

# Multi-site and Multi-modal Imaging Methods for Studying Spinal, Brain, and Vascular Dynamics

By

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**B.S., Medical Physics - 2013 - Riga Technical University**

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Ph.D. Thesis

Submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in

Biomedical Engineering at Brown University

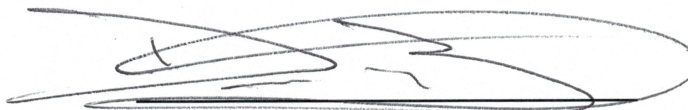
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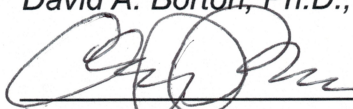
This dissertation by Dmitrijs Celinskis is accepted in its present form by the Center for Biomedical Engineering as satisfying the dissertation requirement for the degree of Doctor of Philosophy in Biomedical Engineering.

Date 10.25.2022



David A. Borton, Ph.D., Advisor

Date 10.24.22



Christopher T. Moore, Ph.D., Advisor

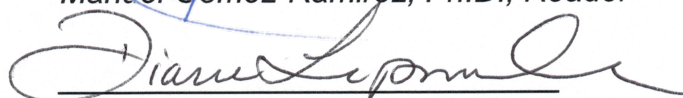
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# Curriculum Vitae

## EDUCATION

### **October 2022**

Ph.D. in BIOMEDICAL ENGINEERING, **Brown University**, Providence, Rhode Island  
Dissertation: "Multi-site and Multi-modal Imaging Methods for Studying Spinal, Brain, and Vascular Dynamics" | Advisors: Prof. Christopher MOORE (neuroscience) and Prof. David BORTON (neuroengineering)

### **May 2015**

M.Sc. in BIOMEDICAL ENGINEERING, **Arizona State University**, Tempe, Arizona  
Thesis: "Investigation of Ultrasonically Powered Implantable Microdevices for Wireless Tissue Impedance Measurements" | Advisor: Prof. Bruce Towe

### **February 2013**

B.Sc. in MEDICAL Physics and PHYSICAL TECHNOLOGIES IN MEDICINE, **Riga Technical University**, Riga, Latvia

Summa cum laude

Thesis: "Photogrammetric System for Mobile Telemedicine Screening Complex" |

Advisor: Prof. Aleksejs KATASHEVS

Engineering Design Project: "Medical Gas Supply Network for Pauls Stradins Clinical University Hospital" | Advisor: Uldis JASPERS



## **Spring 2011**

Exchange studies at **Norwegian University of Science and Technology**, Trondheim, Norway

## **PEER-REVIEWED PUBLICATIONS**

- D. CELINSKIS, N. Friedman, M. Koksharov, J. Murphy, M. Gomez-Ramirez, D. Borton, N. Shaner, U. Hochgeschwender, D. Lipscombe, C. I. Moore, Miniaturized Devices for Bioluminescence Imaging in Freely Behaving Animals, 2020 42nd Annual International Conference of the IEEE Engineering in Medicine Biology Society (EMBC), 2020.
- DMITRIJS CELINSKIS, Mark D. Grabiner, Claire F. Honeycutt, Bilateral early activity in the hip flexors associated with Falls in Stroke Survivors: Preliminary evidence from laboratory-induced falls, In Clinical Neurophysiology, 2017.
- D. CELINSKIS and B. C. Towe, "Characterization of the implantable neurostimulator-based wireless bioimpedance measurement technique," 2016 IEEE EMBS International Student Conference (ISC), Ottawa, ON, 2016, pp. 1-4.
- D. CELINSKIS, "Investigation of Ultrasonically Powered Implantable Microdevices for Wireless Tissue Impedance Measurements," Thesis, 2015, pp. 1-113.
- D. CELINSKIS and B. C. Towe, "Wireless impedance measurements for monitoring peripheral vascular disease," 2014 36th Annual International Conference of the IEEE Engineering in Medicine and Biology Society, Chicago, IL, 2014, pp. 6937-6940.

- D. CELINSKIS, Katashev A. "On Criteria for Wide-Angle Lens Distortion Correction for Photogrammetric Applications," International Symposium on Biomedical Engineering and Medical Physics, 10-12 October, 2012, Riga, Latvia.

## **ABSTRACTS**

- D. CELINSKIS, J. Murphy, N. Shaner, U. Hochgeschwender, D. Lipscombe, C. Saab, C. I. Moore, D. A. Borton, Miniaturized functional microscopy of the brain and spinal cord using bioluminescent and fluorescent indicators, UIUC student conference 2022.
- D. CELINSKIS, J. Murphy, N. Shaner, U. Hochgeschwender, D. Lipscombe, C. Saab, C. I. Moore, D. A. Borton, Imaging the cortex and spinal cord using bioluminescent calcium indicators and miniaturized microscopy, Society for Neuroscience Meeting, 2021.
- N. Shaner, D. CELINSKIS, E. Ikefuama, E. D. Petersen, E. F. Murphy, A. Bjorefeldt, J. Murphy, M. Prakash, A. I. More, L. G. Budet, Bioluminescent Activators and Indicators in Neuroscience, Society for Neuroscience Meeting, 2021.
- D. CELINSKIS, J. Murphy, N. Shaner, U. Hochgeschwender, D. Lipscombe, C. I. Moore, D. A. Borton, Next generation tools for imaging in the cortex and spinal cord, 14th Canadian Neuroscience Meeting, 2021.
- A. Barrios-Anderson, D. CELINSKIS, C. Black, C. Saab, D. A. Borton, Calcium imaging of neurons in the dorsal horn of the lumbar spinal cord in awake behaving mice, 7th BRAIN Initiative® Investigators Meeting, 2021.

- D. CELINSKIS, M. Gomez-Ramirez, D. A. Borton, C. I. Moore, Miniaturized devices for bioluminescence imaging of brain and spinal cord, Society for Neuroscience Meeting, NeuroNex Investigator Meeting, 2019.
- J. W. Murphy, N. G. Friedman, M. Prakash, D. Celinskis, R. St. Laurent, D. Lipscombe, N. C. Shaner, U. Hochgeschwender, C. I. Moore, Cell-Target Specific BioLuminescent-OptoGenetic Synaptic Signaling in vivo: BioLumInescent Trans-Emitters (B-LiTE), Society for Neuroscience Meeting, NeuroNex Investigator Meeting, 2019.
- DMITRIJS CELINSKIS, Arto Nurmikko, Wearable Ultrasound Sensor for Detection of Embolic Events, 40th International Conference of the IEEE Engineering in Medicine and Biology Society, 2018.
- D. CELINSKIS, B. C. Towe, Wireless ultrasonically-powered neurostimulators with bioimpedance measurement capacity, Society for Neuroscience, 95.03 / KKK63, 2016.
- D. CELINSKIS, M. D. Grabiner, C. F. Honeycutt, Role of stretch and startle reflexes in falls following stroke: Insights from treadmill induced balance perturbations, Society for Neuroscience, 610.18/P23, 2015.

## **HONORS & AWARDS**

### **2020 - 2022**

#### **GRADUATE COMMUNITY FELLOWSHIP (Providence, RI, USA)**

- Created a peer-to-peer mentorship program to support new international students at Brown

- Moderated a panel for newly admitted international students on tips and tricks of thriving at Brown University and academia during the pandemic
- Organized virtual and in-person socials for the international students to promote well-being, peer support and cross-disciplinary connections
- Collaborated with the administrative teams from the Graduate School and Global Brown Center
- Managed a budget for the international student community events

### **2018**

**FELLOWSHIP** to attend the ETH-Wyss-Brown "Translational Neuroscience and Neural Engineering" workshop (Newport, RI, USA)

### **2015**

**BEST STUDENT'S PRESENTATION AWARD** at the 2nd IEEE EMBS International Summer School of Neural Engineering (Shanghai, China)

### **2015**

**TRAVEL AWARD** for participation in the 2nd IEEE EMBS International Summer School of Neural Engineering (Shanghai, China)

### **2014**

**GRADUATE SCHOOL TRAVEL AWARD** to support the attendance of IEEE EMBS 2014 conference

### **2013**

**FULBRIGHT SCHOLARSHIP AWARD** in support of Master studies at Arizona State University

### **2013**

**GOLDEN FUND AWARD** acknowledging exceptional academic performance, Riga Technical University, Riga, Latvia

**2011**

**ERASMUS EXCHANGE PROGRAM AWARD** in support of exchange studies at Norwegian University of Science and Technology

**2009 - 2013**

**ACADEMIC ACHIEVEMENT** stipend recipient at Riga Technical University

## **TEACHING**

**MENTOR - Spring 2021**

**ADRIEL BARRIOS-ANDERSON** - medical student supported by the BRAIN initiative fellowship

Brown University, Borton Laboratory, Providence, RI

- Provided training on spinal cord and brain surgical techniques, fluorescent labeling techniques for vasculature and neurons, implant design and fabrication processes, animal handling and behavior, miniature microscopy imaging, image analysis and good experimental documentation practices
- Submitted a conference abstract for the BRAIN initiative meeting titled "Calcium imaging of neurons in the dorsal horn of the lumbar spinal cord in awake behaving mice"

**INSTRUCTOR - Summer 2018**

**ENGINEERING DESIGN STUDIO** (middle school)

Brown University School of Professional Studies, Providence, RI

- Led and organized workshops and guest lectures on the topics ranging from nature inspired design to empathy and project planning
- Managed a team of two teaching assistants
- Continuously adapted course content following the direction of student curiosity
- Advised students and their parents on career trajectories

### **INSTRUCTOR - Summer 2017**

#### **ENGINEERING BIOMEDICAL SYSTEMS (pre-college)**

Brown University School of Professional Studies, Providence, RI

- Performed in-class anatomy, medical device and brain machine interface demonstrations
- Organized field trips to fMRI research facility, cardiovascular tissue engineering lab and neuroengineering lab
- Guided students through the labs on electrophysiological recording, wet-lab techniques and 3D printing
- Organized guest lectures by diverse speakers with a focus on contemporary research and career advice
- Managed a team of two teaching assistants

### **TEACHING ASSISTANT - Spring 2019**

#### **C2S NEUROTECH: FROM CONCEPT TO STARTUP - TRANSLATING NEUROTECHNOLOGY (mixed undergraduate and graduate)**

Brown University, Providence, RI Instructor: Dr. John Donoghue

- Organized in-class demonstrations of anatomical specimens, neural recording, stimulation and robotic devices

- Coordinated industrial, clinical and patient guest lectures
- Judged and assessed student project presentations and white papers
- Facilitated the smooth transition of the course to remote learning due to the onset of the COVID-19 pandemic

### **TEACHING ASSISTANT - Summer 2019**

#### **THE HEART OF THE MATTER - DESIGNING IMPLANTABLE DEVICES FOR THE CARDIOVASCULAR SYSTEM** (pre-college)

Brown University School of Professional Studies, Providence, RI Instructor: Dr. Farah Laiwalla

- Guided students through the cardiovascular dissection labs
- Led the discussion on the implantable devices and vascular disease

### **TEACHING ASSISTANT - Fall 2017/2018**

#### **BIOINSTRUMENTATION DESIGN** (mixed undergraduate and graduate)

Brown University, Providence, RI Instructor: Dr. David Borton

- Guided students through the bioinstrumentation labs teaching the hardware operations, coding and electrophysiology techniques
- Organized in-class bioinstrumentation demonstrations and gave guest lectures
- Corrected the quizzes, evaluated students' experimental results and knowledge via oral examination
- Maintained lab equipment by performing the repairs, purchasing new parts and arranging service work
- Managed the team of six undergraduate teaching assistants

### **TEACHING ASSISTANT - Spring 2017/2018**

## **NEUROENGINEERING** (mixed undergraduate and graduate)

Brown University, Providence, RI

Instructors: Drs. Leigh Hochberg and Arto Nurmikko

- Developed a computational neuroscience assignment sequence implemented in Jupyter Notebooks using NEURON packages
- Wrote homework and exam questions based on lectures and reading assignments
- Led weekly office hours, corrected homework assignments and exams
- Maintained website with announcement, lecture and homework postings
- Led student visits to fMRI research facility, Rhode Island Hospital and Veterans Affairs Medical Center

### **TEACHING ASSISTANT - Summer 2017/2018**

## **ENGINEERING BIOMEDICAL SYSTEMS I** (pre-college)

Brown University School of Professional Studies, Providence, RI

- Facilitated group discussions and guided students through the lab experiments
- Organized medical device demonstrations and lectured on graduate level research in neuroscience and neuroengineering
- Provided introductory training on rapid prototyping techniques

### **TEACHING ASSISTANT - Summer 2017**

## **INTRODUCTION TO ENGINEERING** (pre-college)

Brown University School of Professional Studies, Providence, RI

- Demonstrated the applications of electrophysiology for control of computer hardware



- Facilitated group discussions, and guided students through the design and fabrication process

## **CERTIFICATIONS / WORKSHOPS**

### **July 2020**

#### **NEUROMATCH ACADEMY: AN ONLINE SCHOOL FOR COMPUTATIONAL NEUROSCIENCE**

Organized by a worldwide community of computational enthusiasts

- Learned a wide range of computational techniques such as machine learning, dimensionality reduction, reinforcement learning and deep learning, and their applications in computational neuroscience
- Implemented our own analysis of Neuropixel electrophysiology data as part of the final project team work
- Practiced discussed computational techniques using Python notebooks running on Google Colab

### **Fall 2019, Spring/Fall 2018**

#### **TEACHING CONSULTANT PROGRAM**

Sheridan Center for Teaching and Learning, Brown University, Providence, RI

- Developed and refined the skills in peer observation and feedback, leadership, and discussion facilitation
- Facilitated workshops on inclusive teaching, feedback in STEM courses and good teaching portfolio design practices
- Co-facilitated a semester-long workshop on reflective teaching

### **Spring 2019**

## **DEALING WITH CONFLICT**

University Ombuds Office, Brown University, Providence, RI

- Learned the essentials of conflict dynamics and resolution
- Practiced skills for more constructive and effective conflict resolution and management
- Learned and practiced conversation facilitation and conflict mediation techniques

**Spring 2019**

## **COURSE DESIGN SEMINAR**

Sheridan Center for Teaching and Learning, Brown University, Providence, RI

- Explored integrated course design principles with primary emphasis on backward course design, inclusive teaching and engaged student learning practices
- Designed two course syllabi, scaffolded assignment sequences and assessment rubrics
- Collaborated with the teaching community at Brown on sharing and receiving feedback on designing learning experiences in different disciplines

**Spring 2018**

## **EFFECTIVE PERFORMANCE: COMMUNICATION AND IMPROVISATION TECHNIQUES FOR GRADUATE STUDENTS**

Trinity Repertory Theater and Brown University, Providence, RI

- Worked on discovering a story behind the research
- Explored theater techniques, experimenting with play, spontaneity, and connecting to the audience
- Developed comfort and confidence in making an engaging research statement

## **Fall 2016**

### **REFLECTIVE TEACHING**

Sheridan Center for Teaching and Learning, Brown University, Providence, RI

- Developed and refined fundamental teaching and assessment strategies and communication skills based on how students learn
- Explored the importance of supporting the diverse students in their efforts to learn by creating inclusive and respectful learning environments

## **WORK EXPERIENCE**

### **2017 - 2018**

Volunteer data analyst at **DISCOVERYENGINE**, Providence, RI

### **2014 - 2015**

Research assistant at Human Mobility Lab, **ARIZONA STATE UNIVERSITY**, Tempe, AZ

### **2014 - 2015**

Equipment safety assistant at Biomaterials Lab, **ARIZONA STATE UNIVERSITY**, Tempe, AZ

### **2012 - 2014**

Medical engineer at NMS ELPA, Riga, Latvia

### **2012 - 2013**

Clinical engineer at cardiovascular surgical department, **PAULS STRADINS CLINICAL UNIVERSITY HOSPITAL**, Riga, Latvia

### **2011 - 2012**

Research assistant at bioinstrumentation lab, **RIGA TECHNICAL UNIVERSITY**,  
Riga, Latvia

**Summer 2012**

Summer medical physics intern at linear accelerator department, **DAUGAVPILS  
REGIONAL HOSPITAL**, Daugavpils, Latvia

## **SOCIETY MEMBERSHIPS**

- Canadian Association for Neuroscience (2021 - present)
- Society for Neuroscience (2015 - present)
- American Association for the Advancement of Science (2015 - 2018)
- IEEE Engineering in Medicine and Biology Society (2014 - present)
- IEEE – Institute of Electrical and Electronics Engineers (2014 - present)
- IEEE Ultrasonics, Ferroelectrics, and Frequency Control Society (2016 - present)
- IEEE - Eta Kappa Nu (active since 2014)
- Tau Beta Pi - The Engineering Honor Society (active since 2014)
- Alpha Eta Mu Beta - National Biomedical Engineering Honor Society (active since 2015)

# Preface

Throughout my PhD one of the many things I relearned or reinforced is that nothing is ever simple. A simplest experiment or a simplest non-science task has an infinite number of degrees of freedom that can break apart even with the most elaborate plan in hand. The challenge is to develop the sense of appreciation and awe for this complexity. Anything is possible, things are chaotic and unpredictable, and it is so powerful to be able to mindfully receive any twists of life's threads with kindness, curiosity, open mind and empathy to others and yourself. I hope to become better at this some day, but for now, my PhD at least helped me to become more aware of these factors...

# Acknowledgements

I would like to thank my family, friends, both of my labs, my advisors, my committee, everyone in the Bioluminescence Hub team and everyone whom I encountered throughout my complex journey at Brown. Every even smallest interaction has shaped my journey and helped me to reach the point in life where I am right now. I am grateful to every person.

I would like to thank everyone in the Moore Laboratory for always being uniquely supportive, welcoming and kind team members. I especially would like to acknowledge everyone's extreme level of mindfulness about communicating around the use of shared resources. I would like to thank Chris Deister for help with troubleshooting and understanding the ins and outs of 2-photon hardware, genetic mouse lines and calcium data analysis. I would like to thank Sinda Fekir for introducing me to the practical and theoretical aspects of 2-photon imaging, the analysis of two photon vascular data, tail vein injections in mice and the sensitivities around coordinating the use of shared resources. I would like to thank Nina Friedman for introducing me to the *in vivo* bioluminescent experiments requiring the proper preparation procedures for all the chemicals and animals. I would like to thank Manny Gomez-Ramirez for helping me to explore the development of MRI-compatible microscopy tools for mice and marmosets, introducing me to miniscope hardware, brain surgical procedures and the basics of bioluminescence imaging. I would like to thank Eric Klein for always being available and open-minded about discussing science, sharing his thoughts and pro-actively sharing all of his own discoveries ranging anywhere from finding new behavior sensors and building new hardware to unique surgical and imaging tricks. I would like to thank Ian

More for introducing me to some aspects of the mouse awake whisker behavior, headpost surgeries, in-lab custom calcium image analysis software and for sharing viruses. I would like to thank Jeremy Murphy for being always eager to do his best to help with absolutely anything from unique 3D-printing challenges to teaching me how to do acute electrophysiology surgeries, use emCCD and OpenEphys software and hardware, and brainstorm any thoughts, ideas or challenges surrounding science and academia. I would like to thank Laurie Lynch for being a universally supportive friend, colleague and a lab manager capable and super-eager to help with anything from getting all the needed equipment for the experiments as fast as humanely possible, making sure the entire lab is fully stocked and in compliance with all the regulations, and everything functions properly to taking care of the well-being of our animals and lab members. Last but not least, I would like to thank Chris Moore - a hugely inspiring, supportive and kind lab leader, mentor, academic advisor and a good friend. Chris showed again and again a deep capacity to inspire further work, and find positive and scientifically curious aspects in almost every single data point that I ever shared with him no matter how strongly it would seem to me as noisy or lacking any new information. Chris also was always making efforts to hear, support and be present for everyone around the lab even during the hardest of times, for which I'm very grateful. Many aspects of work in science which I would love to carry with me and share with others throughout the rest of my career are inspired by Chris. Thank you Chris for never giving up on me...

I also would like to thank everyone from the NSF NeuroNex Bioluminescence Hub. It was very enriching, inspiring and stimulating to be a part of a multi-institutional

team with members across different institutions having unique expertise while being driven by the shared goals. Being an active member of Bioluminescence Hub gave me very easy access to different experts which I could comfortably contact any time and be met with

immediacy, thoughtfulness and care. Being a part of NeuroNex also gave me access to other NeuroNex hubs and unique meetings, which I got to utilize to a bit limited degree, but this still was an impactful component of the recipe for the success of my PhD. I would like to thank Justine Allen for being a brilliantly supportive program manager, always being available to help with any ordering, scheduling, outreach, publishing and any other issues requiring thoughtful coordination. I would like to thank Ute Hochgeschwender and her lab at Central Michigan University for developing all the bioluminescent molecules encoding viruses used in my work and, in general, for being great resources when it comes to any questions pertaining to adeno associated viruses. I would like to thank Mikhail Koksharov for helping with testing and learning about different luciferins. I would like to thank Diane Lipscombe for generously sharing two optogenetic mouse lines with me that were crucial for studying sensory information processing. I would like to thank Krystal Literman for helping with coordinating meetings and outreach activities towards sharing hub's work. I would like to thank Eric Petersen for enriching scientific discussions about bioluminescent indicators and imaging. I would like to thank Jason Ritt for valuable discussions around animal behavior and imaging data analysis. I would like to thank Nathan Shaner for his supportiveness, friendliness and almost constantly instantaneous availability for any random questions and help with molecular tools.



I would like to thank everyone in the Borton laboratory for being a wonderful support throughout many engineering challenges faced during my PhD by providing me access to equipment and knowledgeable people, helping me to better my soft and hard skills by regularly offering diverse unexpected tasks and offering opportunities to reinforce the sense and spirit of being a part of broader scientific community rather than walking my

PhD journey completely alone. I would like to thank Anusha Allawala for setting up the initial hardware for the spinal cord work, for showing me the basics of immunohistochemistry and for introducing me to the basic ideas behind the spinal cord imaging for pain. I would like to thank Christopher Black for being the main person throughout my PhD with whom I could always share any challenges that I was facing with spinal cord work. I'm also grateful to Chris for showing me how to cryosection mouse spinal cord tissue and for always being such a cheerful source of support. I would like to thank Adriel Barrios-Anderson for his interest in learning the spinal surgical procedures from me, for helping me to practice my surgical training skills and for successfully performing one simultaneous brain-spinal cord surgery that allowed me to collect meaningful data. I would like to thank Radu Darie who was the first person who I got to collaborate with on a project when I just joined Brown. Radu was always a super encouraging, positive, supportive and open-minded colleague and a friend. I would like to thank Ki-Soo Jeong for regularly participating in our sensory work meetings and sharing his thoughts. I would like to thank Evan Matteson for being the first person who made me feel that I was not alone in Norton lab. I would like to thank Rebecca Poncin for being a super well-organized, responsive and supportive mouse colony manager

throughout most of my PhD, and for being equally wonderful Borton lab manager throughout the last 6 months of my student journey. I would like to thank David Xing for being a great friend and for helping me to feel as a part of the lab. I would like to thank my co-advisor David Borton for always challenging me and always finding surprising new ways to bring me down. It trained me to get better at managing difficult conversations, doing the things I do not believe in, being patient, managing stressors and distractions, and being persistent at trying to explain my side of the matter. Thank you David for your time,

for funding part of my PhD, for your patience and for always being eager to find time to meet. David was my main inspiration for joining and even applying to Brown University to begin with - without him I wouldn't have met so many other wonderful people.

I would like to thank my committee members David Borton, Manuel Gomez-Ramirez, Diane Lipscombe, Christopher Moore and Kimani Toussaint for generously devoting their time to administer the final oral and written examinations of my PhD, and helping me to make the written part of my dissertation work even better.

I also would like to thank a large group of individuals not affiliated with any of the groups I was a part of. I would like to thank Carl Saab for always being ready to discuss any questions pertaining to pain and being a great resource on pain science. I greatly appreciate Carl's openness to any even silliest questions. I would like to thank Daniel Aharoni for his help with troubleshooting the hardware and software of UCLA miniscopes, and for sharing the miniscope hardware prototypes with me freshly minted directly in his lab. I would like to thank Caleb Kemere and his former lab member Jill Juneau for sharing their custom miniature voltage-imaging microscope with me and for

their willingness to support me through the use of their microscope. I would like to thank Thomas Kirkland and Joel Walker from Promega Inc. for sharing with me the experimental samples of their luciferases. I would like to thank Arto Nurmikko for supporting a portion of my PhD journey and for helping me to learn how to navigate some very unique academic challenges. I also would like to thank the members of the Nurmikko lab Naubahar Agha, Farah Laiwalla and Zeyang Yu for being great friends and support. I would like to thank Adriana C. Salazar Coariti for valuable help with the questions surrounding optics, writing and formatting my dissertation, and optimizing the optical hardware for more homogeneous illumination. I would like to thank Christopher Schaffer and Yu-Ting Cheng for hosting me in their lab at Cornell University, and introducing me to their spinal cord surgical and imaging techniques. I would like to thank Sabina Stefan for valuable discussions around optical image analysis, sharing the pursuit of better cohesion amongst the international graduate students at Brown and for being a good friend. I would like to thank the associate dean Maria Suarez for her support during the very last stage of my PhD, especially for supporting my family's visit to Brown. I would like to thank Bruce Towe and Claire Honeycutt, my advisors from my Master studies, for having a continuous presence in my education even during my PhD. I would like to thank Brown University's librarian Andrew Creamer for helping me to explore different electronic notebook options and properly formatting the references of images in my dissertation. I also would like to thank my non-human support team, which included my puppy Nicholas who was tirelessly committed to discovering novel ways of stimulating my life and all the experimental animals who contributed to my work.

Most of all, I would like to thank my Latvian family and my newly built US family. Family has always been the core value for me and being separated from my family as an international student has probably been the biggest challenge of my PhD. I'm grateful for the strength of the family bond that we managed to maintain throughout all the years of separation. I would like to thank my friends Phuong Truong, Jeray Thelwell, Bradley Adams and Cristian Buc Calderon who during my PhD became a part of my extended and highly cherished family similarly to Adriana and Nicholas. I also would like to thank a wonderful group of my friends from NMS Elpa led by Ilmārs Čurkste, Aija Čurkste and Normunds Patmalnieks who always helped me to feel especially welcomed back in Latvia even after prolonged times away from home. I'm also grateful to them for willing to support me through life challenges even while I was across the world. Thank you to my family for being my guiding sunshine through sometimes densely wild and dark forest of life. I am especially grateful to Olegs and Adriana for all the precious direct help, and my grandma and mom for moral support especially towards the end of my doctoral journey. I especially would like to thank my grandma, whose positive fire of life has always been a huge source of inspiration, and Adriana, who was patient and present for me during the times when no one else could. I also would like to thank my mom for growing patience, kindness and understanding throughout the years.

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Engineering, National Science Foundation (NSF NeuroNex 1707352) and National Institute of Health (NIH R01 NS108414).

The above list is not exhaustive as it is hard to give justice to every single person I had a chance to interact with throughout my PhD journey. I am nonetheless grateful to all people (as well as animals and plants ☺) listed and not listed here.

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# CHAPTER 1

## 1. Introduction

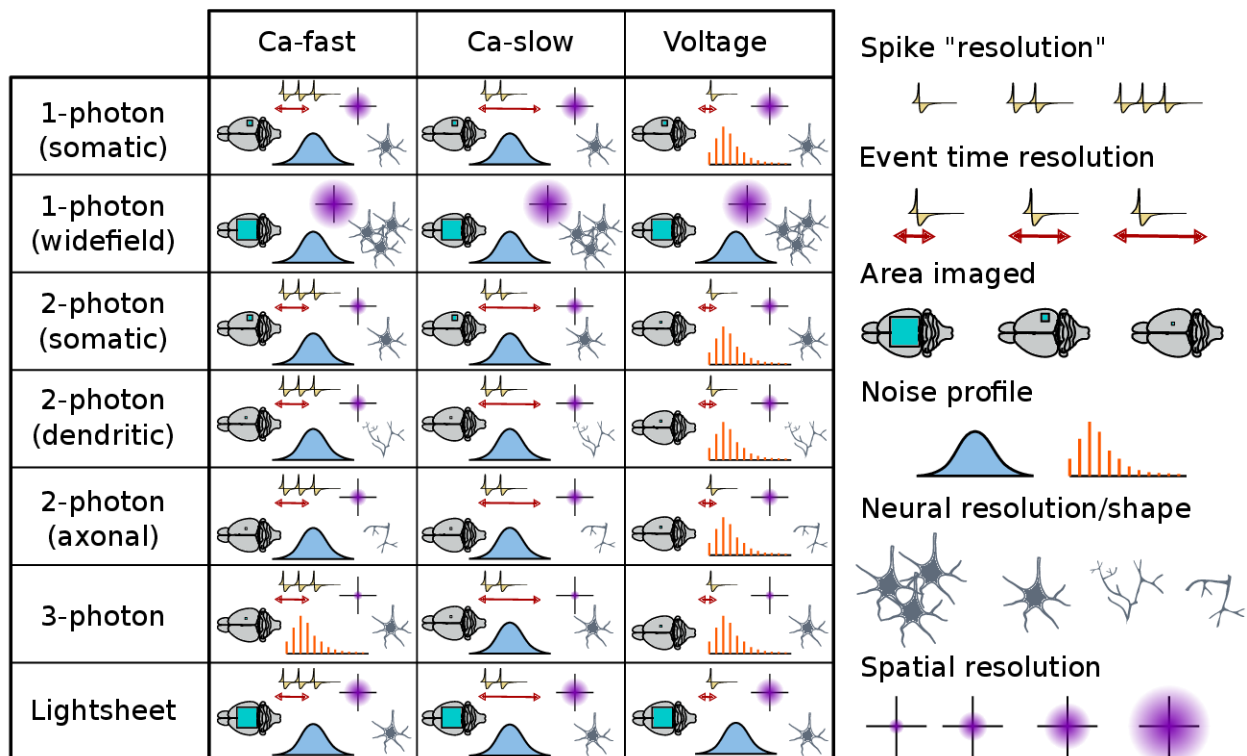
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## 1.1. State of the art in **fluorescent** imaging of the brain and spinal cord in 2022

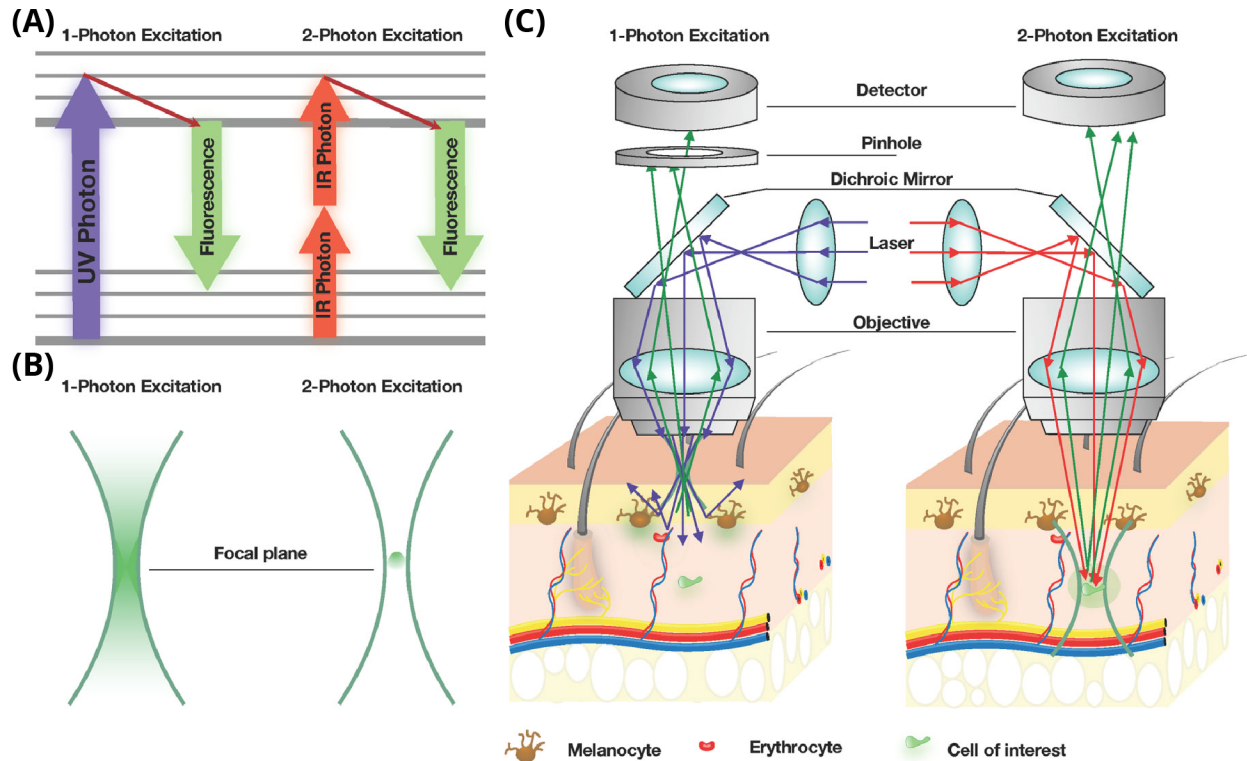
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Chronic 1-photon and 2-photon epifluorescent imaging of calcium ( $\text{Ca}^{2+}$ ) dynamics in neurons of the brain are well-established approaches. The key initial discovery that enabled these technologies occurred in 1997; the development of a green fluorescent protein (GFP) based  $\text{Ca}^{2+}$  indicator [1]. Since then, a wide range of other fluorescence-based indicators have been developed, offering a variety of signal brightness levels, dynamic ranges, spatio-temporal resolutions, fields of view (FOVs), noise levels, spectral characteristics, and 2-photon excitability cross-sections ([Fig. 1](#)) [2].

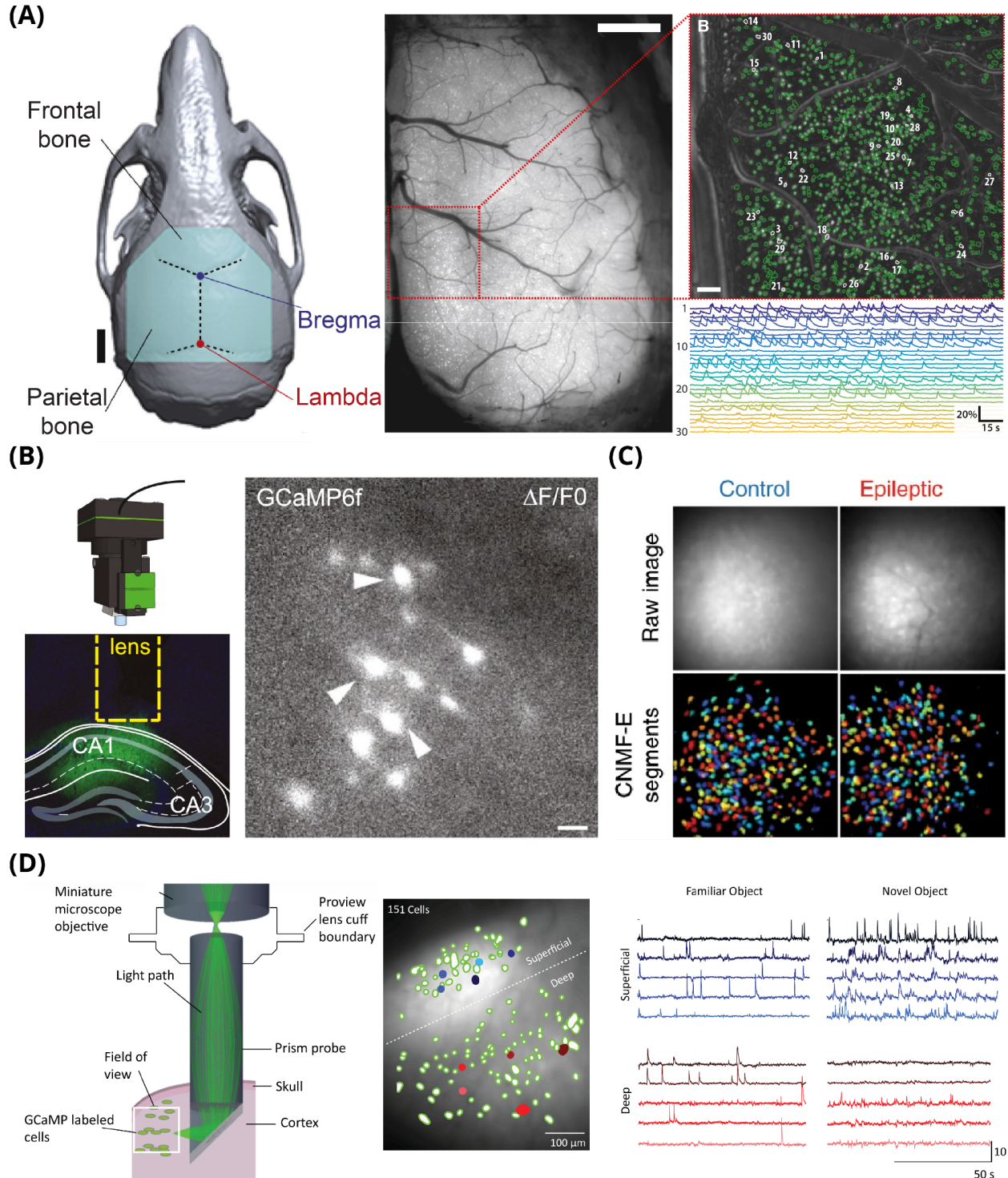


**Figure 1.** Comparison of different microscopy modalities commonly used for functional brain imaging. In addition to the comparative metrics included in this figure, every imaging modality offers a different maximal imaging depth. 1-photon and lightsheet offer the smallest penetration depth, 2-photon offers medium penetration depth and 3-photon offers the deepest imaging. These microscopy modalities also differ in terms of the cost of hardware. Cost-wise, 1-photon microscopes are most accessible and 3-photon microscopes cost >200x more than 1-photon and >2x more than 2-photon. Reprinted, with permission, from [2], © 2022 Benisty et al., licensed under [CC BY-NC-ND 4.0](#).

1-photon microscopy has higher scattering noise, higher photobleaching, higher phototoxicity and shorter imaging depth compared to multi-photon microscopy ([Fig. 2](#)) while offering the most cost-effective option for measuring single-neuron and gross population dynamics ([Fig. 1](#)). With sufficiently-sparse labeling of neurons, 1-photon imaging allows reliable tracking of somatic  $\text{Ca}^{2+}$  ([Fig. 3A](#)) [3]–[5]. 1-photon imaging works especially well for imaging superficial cortical cells, for example, interneurons expressing the neuron-derived neurotrophic factor (NDNF) in layer 1 of the cortex [3]. 1-photon also allows imaging of somatic  $\text{Ca}^{2+}$  dynamics in deeper structures such as the hippocampal CA1 region [6], [7] and cortical layer 5 [8]. These studies used implantable GRadient Index of Refraction (GRIN) lenses and micro-prisms, respectively ([Fig. 3B-D](#)).



**Figure 2.** Comparison of 1-photon and 2-photon microscopy. **(A)** demonstrates the need for a single photon vs. 2 photons to excite the fluorophores in the case of 1-photon vs. 2-photon microscopy, respectively. **(B)** demonstrates a significantly higher focality of 2-photon compared to 1-photon microscopy with optical power being smeared throughout a significantly larger volume in the case of 1-photon imaging. **(C)** depicts the basic optical hardware layouts and beam paths. 1-photon imaging involves a significant amount of forward photon scattering and shallower imaging depth. 2-photon imaging offers less forward scattering and deeper imaging because of the more red-shifted wavelength, and less noise because of the higher focality of the 2-photon effect. 3-photon microscopy is similar to 2-photon, except it requires 3 photons for excitation while offering even deeper and more focal imaging. Reprinted, with permission, from [9], © 2021 Zayats, Uzonova and Murooka, licensed under [CC BY 4.0](https://creativecommons.org/licenses/by/4.0/).



**Figure 3.** Benchtop 1-photon microscopy and wearable miniature microscopes (aka. miniscopes) allow calcium imaging in individual cortical and deeper layer neurons. **(A)** An example of calcium imaging from an estimated one million cortical neurons using 1-photon microscopy through the “Crystal Skull” implant. Reprinted, with permission, from [5], © 2016 Hyun Kim et al., licensed under [CC BY-NC-ND 4.0](https://creativecommons.org/licenses/by-nc-nd/4.0/). **(B)** An example of miniscope imaging of  $\text{Ca}^{2+}$  in individual CA1 hippocampal neurons using a GRIN lens. Reprinted, with permission,

from [7], © 2017 Xia et al., licensed under [CC BY-NC-ND 4.0](#). (C) An example of pre-clinical application of a wire-free miniscope to analyze the spatial coding of epileptic seizures. Adapted by permission from Springer Nature Customer Service Centre GmbH: [6], © 2020 Shuman et al., under exclusive license to Springer Nature America, Inc. (D) An example of miniscope  $\text{Ca}^{2+}$  imaging using an implanted microprism probe to image individual neurons throughout multiple layers of the somatosensory cortex (S1). Reprinted, with permission, from [8], © 2017 Gulati et al., with explicit permission to reuse this image from JoVE Journal.

In the past decade 1-photon microscopy has grown significantly in popularity because of the **miniaturization of the optical hardware** allowing  $\text{Ca}^{2+}$  imaging in freely behaving mice. Imaging using traditional benchtop epifluorescent 1-photon microscopes relies on restraining the animal under the microscope to allow high-quality imaging with the minimal amount of motion. Restrained imaging of rodents is still a powerful technique that allows studies of awake behavior such as processing of sensory signals generated by the deflection of whiskers [10], [11] or responses to the sensory stimulation of the paw [12]. However, by restraining the animal, we modify the animal's natural way of executing tasks important for its survival; in their natural habitat, animals typically are not fixed in a single spot in space. Even though the restraint can simplify the interpretation of data by removing many confounding processes happening simultaneously in free animals, the restraint inevitably modifies the neural dynamics and the organization of the nervous system that we are trying to study [13]. Miniature microscopes (aka. miniscopes) are one of the recent advances in imaging that have removed the need for restraint [14]. Miniscopes can weigh as little as 1.6 g [15], and can be head-mounted with reduced impact on natural behavior. Despite their significantly simplified optics, miniature microscopes allow us to conduct longitudinal studies to track  $\text{Ca}^{2+}$  dynamics in individual neurons, and with the imaging quality comparable to benchtop microscopy ([Fig. 3A](#) for benchtop 1-photon vs. [Fig. 3B-C](#) for the miniscope).

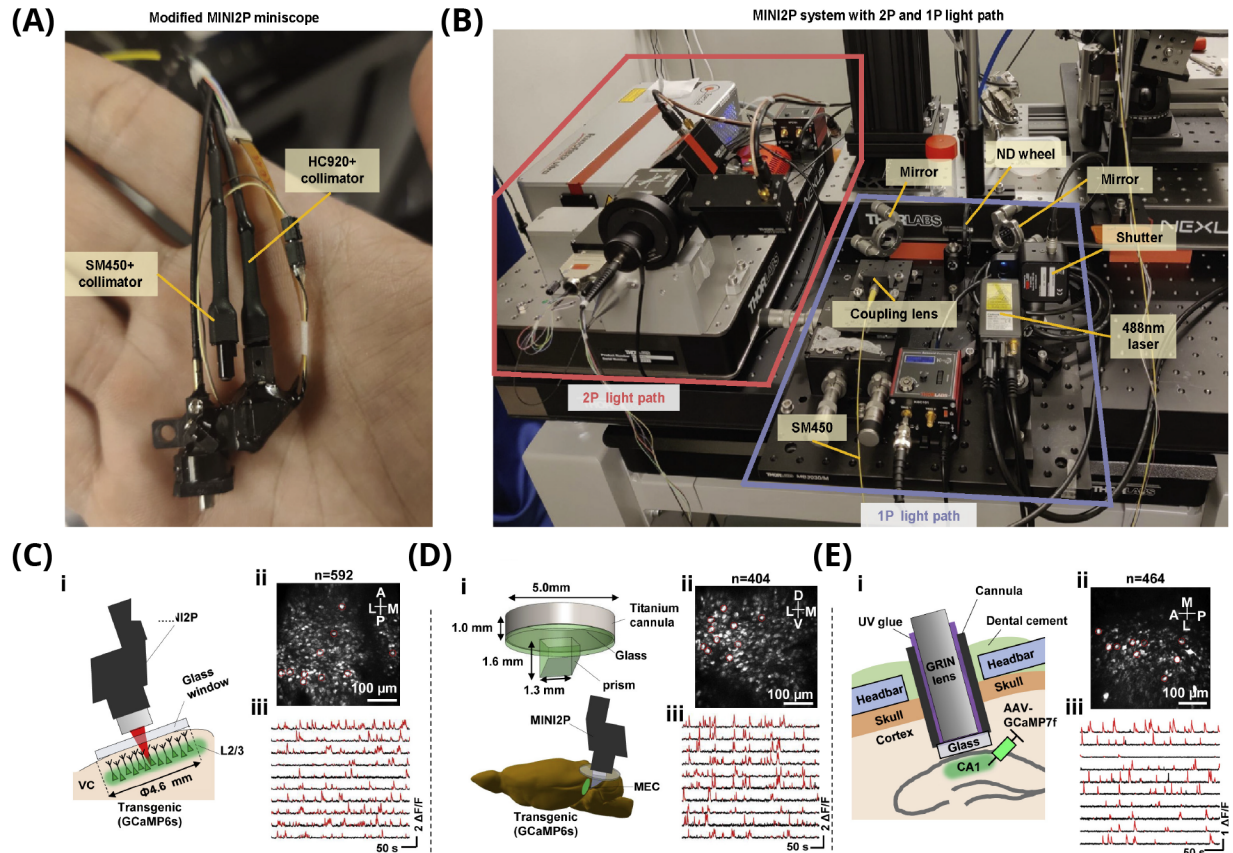


The two key milestones that lead to broad adoption of miniature microscopes by the research community were the development of high-quality commercial miniature microscopes [16] followed by the development of more cost-effective open-source miniscopes [17] ([Fig. 4](#)). The commercial miniscopes offered by Inscopix Inc.® (Mountain View, California, USA) provided hardware solutions ready to be used out of the box, and in-lab assistance from field scientists with the use of their system and implementation of the animal models. This kind of assistance from the field scientists is gradually becoming more and more important for implementing complex behaviors by the non-tech developing labs and in the future this might become one of the important services offered by the national imaging facilities [4]. However, commercial miniscopes are expensive. On the other hand, open-source miniscopes while being more affordable, require in-house expertise in optics, electronics, behavior, neuroscience, surgery and data science. Despite all of these challenges, miniature microscopes have been widely adopted and application examples include > 25 different designs [14], [18]–[20]. Some of these designs were the modifications of the UCLA miniscope (e.g. my publication on bioluminescence miniscope BLmini v1.0 [21]), but in many cases researchers developed designs completely independent of the UCLA miniscope. These designs include 1-photon [15], [22]–[26], 2-photon [27], intrinsic imaging [28] and photoacoustic miniscopes [29]. Some of these designs are open-source and can be adopted by any lab. However, the most widely adopted miniscope was developed by UCLA [17]. This is because of their detailed documentation, streamlined access to the parts and assembled miniscopes, active development of improved hardware versions, very responsive team of developers and vibrant community of users.

| (A)                                                                                                                                                             | (B)                                                                                                                                          | (C)                                                                                                                                                                           | (D)                                                                                                                                                          |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------|
|                                                                                |                                                             |                                                                                             |                                                                           |
| FinchScope                                                                                                                                                      | miniScope                                                                                                                                    | UCLA Miniscope                                                                                                                                                                | CHEndoscope                                                                                                                                                  |
| Dim: 10 x 6 x 21 mm<br>Wired: 1.8 gram<br>Wireless: ~ 4 gram<br>FOV: 880 x 600 $\mu$ m<br>Frame Rate: 30 Hz<br>Focus: turret<br>DAQ: Arduino<br>Software: MacOS | Dim: 12 x 12 x 20 mm<br>Wired: 2.4 gram<br>FOV: 1.1 x 1.1 mm<br>Frame Rate: 10 Hz<br>Focus: turret<br>DAQ: Opal Kelly<br>Software: Win & Mac | Dim: 16.5 x 13 x 22.5 mm<br>Wired: ~ 3 gram<br>Wire-free: 4.5 gram<br>FOV: 700 x 450 $\mu$ m<br>Frame Rate: 60 Hz<br>Focus: linear slider<br>DAQ: custom PCB<br>Software: Win | Dim: 15.9 x 17 x 32.5 mm<br>Wired: 4.5 gram<br>FOV: ~ 500 $\mu$ m across<br>Frame Rate: 20 Hz<br>Focus: turret<br>DAQ: direct to PC<br>Software: Win & Linux |

**Figure 4.** Examples of specifications of the open-source miniature 1-photon microscopes. Reprinted, with permission, from [30], © 2019 Aharoni and Hoogland, licensed under [CC BY 4.0](https://creativecommons.org/licenses/by/4.0/).

In contrast to 1-photon microscopy, 2-photon microscopy offers finer spatial resolution, lower photon scattering noise and deeper imaging at the cost of more complex and more expensive hardware ([Fig. 5B](#)). Despite more complex optics, several groups have successfully implemented and disseminated wearable versions of 2-photon microscopes ([Fig. 5A](#)) [15]. 3-photon microscopy remains accessible to a limited number of labs because of the significantly higher cost compared to 2-photon microscopy. At the same time, active work is underway towards 3-photon imaging in freely behaving animals [31], [32].



**Figure 5.** (A) and (B) show the hardware for the miniature 2-photon microscope (mini2p). (C)-(E) show the examples of lens configurations for mini2p, images of individual neuron somas and calcium dynamics. Reprinted, with permission, from [27], © 2022 Zong et al., licensed under [CC BY 4.0](https://creativecommons.org/licenses/by/4.0/).

Overall, all imaging modalities used for studying the biology underlying animal behavior are converging towards a form factor (i.e. dimensions, weight, tether) compatible with free behavior. The trend away from restrained behavior suggests that the field of neuroscience is reaching a pivotal point. The field has a base of knowledge of the mechanisms underlying artificially constrained behaviors and can now focus on the function of the nervous system during naturalistic behaviors.

### 1.1.2. Spinal cord imaging

Awake imaging in the spinal cord is significantly less developed than imaging in the brain. All of the above examples involved brain imaging. At the beginning of my



thesis work there was not a single study demonstrating the awake imaging in the spinal cord. Furthermore, until recently, there have not been reports of chronic neural  $\text{Ca}^{2+}$  imaging in the spinal cord. Early imaging in the spinal cord focused on vascular, microglial and astrocytic cells [33] and acute imaging of dorsal root ganglia [34], [35]. Most early studies employed acute imaging using  $\text{Ca}^{2+}$  sensitive dyes [36]. The biggest advancement in spinal cord imaging came in 2012 with the development of spinal cord window implants for chronic imaging under anesthesia [33].

The complexity of spinal cord imaging has limited the number of studies published by 2022 that succeeded in imaging individual neuron calcium in the spinal cord to 7 studies ([Table 1](#)). Five of these studies have been published between December 2021 - April 2022. The recency of progress in this field indicates that the tool development for chronic spinal cord imaging of  $\text{Ca}^{2+}$  in individual neurons in awake animals is finally reaching a mature level. Tools are commercially available or there is sufficient know-how for broad adoption of chronic imaging of the spinal cord. The data generated using these methods offers significance for understanding biology and behavior in animal models of health and disease.

| Imaging target                         | Data                                           | Microscope type                              | Application example                                   | Transgenic indicators                               | Injectable labels                                                           | Animal state                               | Level          | Methodological reproducibility | Reference                          |
|----------------------------------------|------------------------------------------------|----------------------------------------------|-------------------------------------------------------|-----------------------------------------------------|-----------------------------------------------------------------------------|--------------------------------------------|----------------|--------------------------------|------------------------------------|
| neurons (somas)<br>astrocytes<br>axons | calcium (astrocytes, neurons), anatomy, motion | custom wearable 1p-miniscope and benchtop 2p | sensory information processing (tactile, nociception) | Thy1-eYFP-H (for axons), SOM-tdTomato (for neurons) | AAV5-GfaABC1D-GCaMP6f (for astrocytes)<br>AAV9-CaMKII-GCaMP6f (for neurons) | awake<br>freely moving<br>under isoflurane | L4-L5 vertebra | low                            | Sekiguchi et al. Nature, 2016 [37] |
| neurons (somas)                        | calcium (neurons)                              | benchtop 2p                                  | sensory information                                   | Thy1-eYFP-H (for                                    | NA                                                                          | awake                                      | L4-L5 vertebra | good                           | Chen et al. Nat                    |

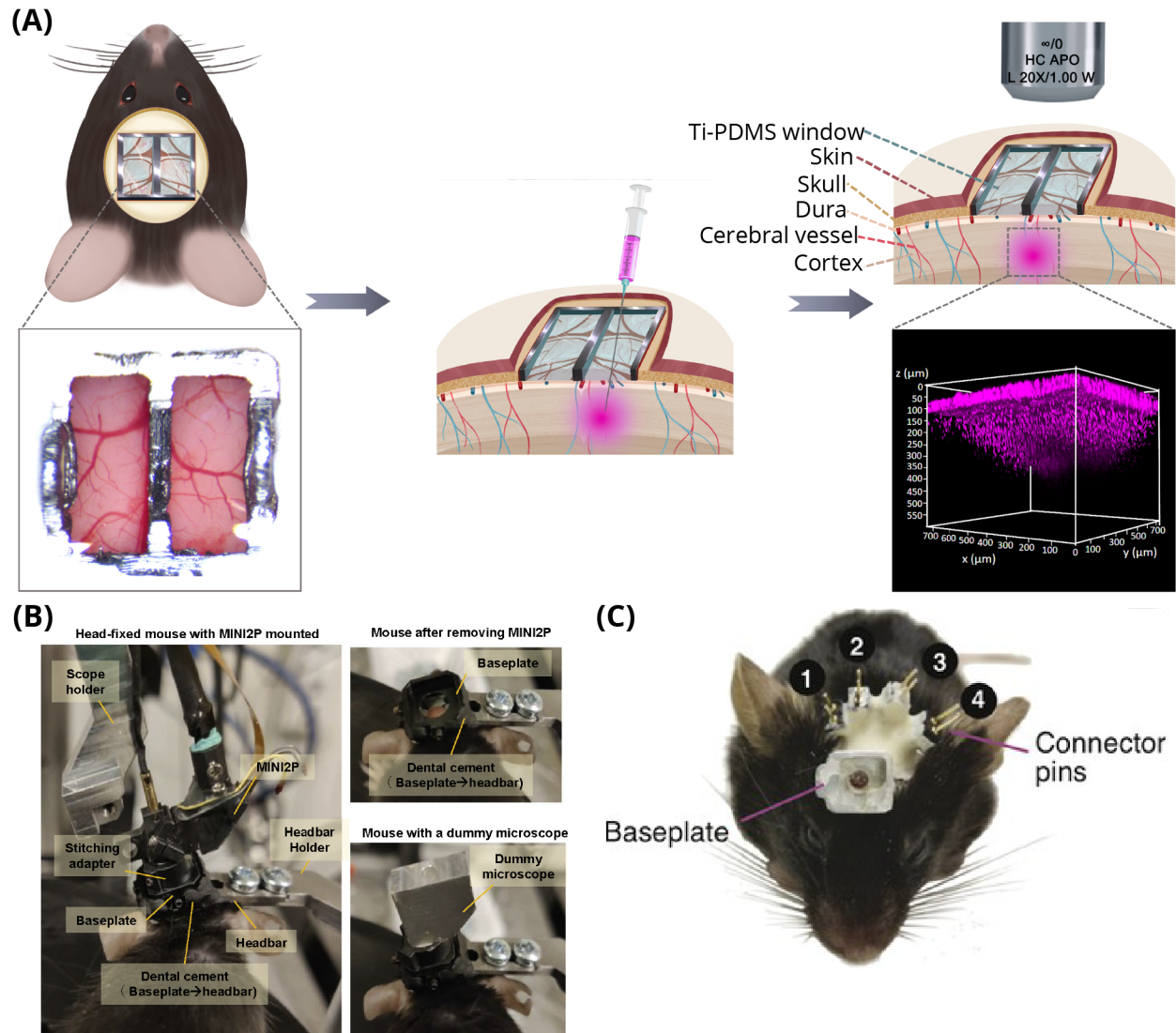
|                                                       |                                                                  |                                                     |                                                                                       |                                                                                                        |                                               |                                            |                                        |      |                                           |
|-------------------------------------------------------|------------------------------------------------------------------|-----------------------------------------------------|---------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------|-----------------------------------------------|--------------------------------------------|----------------------------------------|------|-------------------------------------------|
| axons                                                 |                                                                  |                                                     | processing (inflammatory pain)                                                        | axons and neurons<br>Thy1-GCaMP6s (for neurons)                                                        |                                               | under anesthesia                           |                                        |      | Commun 2019 [38]                          |
| neurons (somas)<br>astrocytes                         | calcium (astrocytes, neurons)                                    | custom wearable 1p-miniscope (multi-color)          | sensory information processing (tactile, nociception)                                 | GFAP-GCaMP6f (for astrocytes)                                                                          | AAV9-CaMKII-H2B-GCaMP7f-TaggRFP (for neurons) | awake<br>freely moving                     | T12-T13 vertebra (L2-L5 spinal levels) | low  | Shekhtmyster et al. bioRxiv 2021 [39]     |
| astrocytes                                            | calcium (astrocytes)                                             | custom wearable 1p-miniscope (wide-field)           | sensory information processing (tactile, nociception)                                 | GFAP-GCaMP6f (for astrocytes)                                                                          | NA                                            | awake<br>freely moving                     | T12-T13 vertebra (L2-L5 spinal levels) | low  | Shekhtmyster et al. bioRxiv 2021 [40]     |
| neurons (somas)<br>axons                              | calcium (neurons)                                                | wearable 2p                                         | sensory information processing (tactile, nociception, temperature, nerve injury)      | Thy1-GFP line M                                                                                        | AAV9-CaMKII-GCaMP6s (for neurons)             | awake<br>freely moving                     | L3-L4 spinal levels                    | low  | Ju et al. bioRxiv 2022 [41]               |
| neurons (somas)                                       | calcium (neurons)                                                | benchtop 2p                                         | sensory information processing (tactile, temperature, nociception)                    | vGluT2-GCaMP6s, vGluT1-GCaMP6s                                                                         | NA                                            | under isoflurane                           | T12-T13 vertebra (L3-L5 spinal levels) | good | Sullivan and Sdrulla. JNeurosci 2022 [42] |
| microglia<br>blood vessels<br>axons<br>collagen (SHG) | morphology across days (microglia)<br>axons                      | benchtop 2p with SHG<br>stimulated Raman scattering | inflammatory response<br>axonal damage                                                | Cx3Cr1-GFP (B6.129P2(Cg)-Cx3cr1tm1Litt/J) (for microglia)<br>Thy1-YFP/Cx3Cr1-GFP (axons and microglia) | Texas Red dextran (70 kDa)                    | under anesthesia                           | T11-L2 vertebra                        | high | Wu et al. Nat Commun 2022 [43]            |
| neurons (somas)<br>neuropil<br>blood vessels          | calcium (neurons)<br>blood flow<br>blood vessel diameter changes | UCLA miniscope, benchtop 1p emCCD, benchtop 2p      | sensory information processing (tactile, nociception)<br>endogenous light sensitivity | Trpv1-ChR2-EYFP (for stimulating Trpv1), Cacna1h-ChR2-EYFP (for stimulating LTMRs)                     | AAV2/9-CaMKIIa-GCaMP6f-P2A-nls-dTomato        | awake<br>freely moving<br>under isoflurane | L1-L3 vertebra                         | high | Present work, 2022                        |

**Table 1.** Summary of the previously published studies using chronic  $\text{Ca}^{2+}$  imaging in spinal dorsal horn and dorsal root ganglia. Methodological reproducibility was assessed based on my subjective assessment of the descriptions of procedures provided in the corresponding articles. LTMR = low threshold mechanoreceptor, L# = lumbar, T# = thoracic.

### 1.1.3. Implant approaches for imaging

A large number of implant designs have been proposed and characterized in recent years that achieve reliable, multi-functional imaging [44]. In addition to examples in [Fig. 3](#) and [Fig. 5](#), other reported designs include brain implants compatible with intracortical injections without the need of removing the implant ([Fig. 6A](#)) allow 2-photon imaging in freely behaving animals ([Fig. 6B](#)), and miniscope implants that combine miniscope imaging with optogenetic stimulation of the imaged or downstream brain areas ([Fig. 6C](#)). The diversity of brain implant designs indicates the need for customizing the implant designs for the specific experimental questions at hand. To this point, new designs continue being released addressing the problems that haven't been tackled before.

Spinal cord implant development has been informed by brain surgical preparations, but brain and spinal cord require different opto-mechanical interfaces. Hence, the starting point of my thesis involved gaining the understanding of all the constraints of state-of-the-art spinal cord implants, and then improving the implant designs and techniques for optically accessing the spinal cord alone. Spinal cord is a more challenging target for chronic optical studies compared to brain and hence required significant methodological developments. This was followed by developing universal implants that allow optical access to the brain and spinal cord in the same animal and finally, developing the bioluminescence imaging tools that hold the potential of improving imaging quality both for the brain and spinal cord.



**Figure 6.** Examples of the cranial window implants used for chronic imaging of fluorescent calcium indicators in the brain using benchtop and wearable microscopes. **(A)** Titanium-silicone cranial window for transcranial viral injections and imaging. Reprinted, with permission, from [45], © 2022 Yang et al., licensed under [CC BY 4.0](https://creativecommons.org/licenses/by/4.0/). **(B)** Baseplate and headbar implant for imaging using wearable 2-photon microscope (mini2p). Reprinted, with permission, from [27], © 2022 Zong et al., licensed under [CC BY 4.0](https://creativecommons.org/licenses/by/4.0/). **(C)** A baseplate implant with 4 optogenetic probes that allows imaging through the gradient index (GRIN) lens using a wearable microscope. Reprinted, with permission, from [15], © 2020 de Groot et al., licensed under [CC BY 4.0](https://creativecommons.org/licenses/by/4.0/).

## 1.2. State of the art in manipulation techniques of neural and vascular dynamics

The strategies developed in my thesis provide technology allowing optical access to several distinct foci within a neural processing stream, from peripheral sites to neocortex. Optical methods for neural control provide many unique benefits [46]. Light can be highly selective and spatially precise in its targeting. With the advent of modern microbial opsins as a strategy compatible with mammalian neurons [47], these methods (generally termed ‘optogenetics’) provided a much-needed selective approach to manipulate genetically-specified neural elements [48], [49]. These methods have not only revolutionized the study of neural systems, they have also been broadly applied, including to vascular targets [50]–[52]. Interestingly, expression of endogenous opsins and, more generally, light-sensitivity in vascular elements provides a powerful avenue for optical control, independent of the need for an additional opsin genetic targeting intervention [53].

In the past 5 years, optical control methods have been effectively reduced to practice using shaped patterns of 2-photon light, allowing not only cell-type specificity, but also the selection of functionally-defined neural ensembles [54]–[57]. An exciting forefront in optogenetics is the multiplexing of this capability with chemogenetic control options in the same molecule, by attaching a bioluminescent moiety to the opsin [58]–[60]. While broadly applied for testing the mechanisms and meaning of neocortical circuit dynamics [61], [62], optogenetic methods have also been adapted to the spinal cord [58], [63]–[76] and dorsal root ganglia [77]. Compared to spinal cord imaging, spinal cord optogenetics is more established because of the less strict surgical and

optical access requirements. Specifically, even though high light scattering by myelin [74], post-surgical spinal cord fibrosis and motion still affect the efficiency and longevity of spinal cord optogenetics, the impact of these factors on optical stimulation is not as strong as on imaging for the similar physical reasons why bioluminescence potentially offers higher signal-to-noise imaging. Optogenetic excitation requires light delivery only in one direction. Hence, photons undergo only forward scattering, eliminating the issue of backward scattering and halving the impact of light on the tissues along the full return path needed for the fluorescence imaging ([Fig. 7](#)) [21]. Importantly, the implant procedure needed for chronic optogenetic stimulation is significantly simpler compared to the spinal cord imaging implants. The main source of simplification for the spinal cord optogenetic surgery comes from the fact that the implant can be made out of a single piece of hardware and the transcutaneous connection can be made few millimeters in diameter (instead of dozens of millimeters required for the spinal cord chronic imaging implants) and tunneled to different parts of the body [63], [68], [69]. The more exotic recent advances in spinal cord optogenetics include the use of bioluminescent optogenetics (BL-OG), which does not require any implants [58]. Another example of an important progress direction for the optogenetic toolbox is the use of near-infrared upconversion nanoparticles to eliminate the need of placing the light source directly at the stimulation target [72].

### 1.3. Motivation for the present work

Every biological process in every organ in our body is interlinked with the biological dynamics evolving in other organs. Understanding of these processes requires the ability to simultaneously record the biological activity across multiple organs

as I am demonstrating in the present thesis. The imaging tools and techniques I developed were inspired by the biological questions about sensory and nociceptive processing. These signals originate from the skin at peripheral sites and propagate from the skin to the spinal cord and then either ascend to the brain (i.e. brain-spine continuum) or form the local reflex loops.

The current state-of-the-art approach for studying animal behavior relies on looking at a single slice of tissue at the time [7], [37], [78]. This can be, for example, a slice of the brain [7], spinal cord [33], [37], dorsal root ganglion [38], skin [79], [80], heart [81] or intestinal tissue [9]. The most sophisticated studies to-date study a single organ while applying the stimuli outside the studied organ, such as the tactile stimulation of the skin while imaging the brain [9], [12] or the spinal cord [9].

Simultaneous optical access to the brain and spinal cord in the same animal would significantly expand our mechanistic understanding of complex bodily circuits in health and disease. Every even most anatomically focal disease inevitably affects the parts of the body outside of its focal origins and hence is a problem requiring the tools for multi-site intervention to allow better understanding and better treatments. One approach is to track the dependency of brain activity on neurovascular activity in the spinal cord in response to a spinal cord injury (SCI). This can be tracked throughout injury, healing and treatment processes. An example from our colleagues includes the application of bioluminescent optogenetics (BL-OG) to drive locomotor recovery following the SCI [58]. In this example, we would like to know what happens to the brain following the SCI and following the treatment while also knowing the status of the spinal cord circuitry. Another powerful application example is to study the mechanisms of

sensory information processing in normal and chronic pain conditions. The neuro-glio-vasculo-immune time course of the chronic pain remains unknown [82] even individually for the brain and spinal cord, and it has not been even attempted to look at the interdependence and co-evolution of the brain-spinal cord states throughout the development of the chronic pain.

At the same time, even the studies investigating the **spinal cord** function in awake behaving animals are scarce and the predominant knowledge in the field stems from imaging, and recording electrical activity from the dorsal-root ganglia (DRGs) and spinal dorsal horn (SDH) under anesthesia [83]. To date, there is evidence for the involvement of the SDH parvalbumin (PV) interneurons in gating the touch-evoked pain [84] via synaptic disinhibition mediated by the retinoic acid receptor (RAR $\alpha$ ) [85] with multiple downstream effects including the inhibition of the excitatory interneurons that express the  $\gamma$  isoform of protein kinase C (PKC $\gamma$ ) [86] or by activating the local astrocytes [87]. Furthermore, the role of inhibitory and excitatory neurons has been elaborated by observing that the vAAT-positive inhibitory neurons are more sensitive to innocuous touch and less sensitive to the thermal stimuli than the vGluT2-positive excitatory neurons with the sensitivity of excitatory neurons being amplified by the capsaicin-induced pain [42]. Besides the neurons, astrocytes [37], [39], [40] and hemodynamics [88] have been demonstrated to be modulated by cutaneous inputs.

Three out of seven chronic spinal cord imaging studies listed in [Table 1](#) have been published by the group that offers very innovative solutions towards spinal cord imaging in awake animals. However, unfortunately these publications describe the results acquired using custom implants and custom optical hardware that are not



described at the level that allows simple adoption of the tools used in those studies. Rather, the methods are described at the level that requires extended testing by any labs willing to adopt these tools.

Fluorescent imaging is a powerful tool that provides deep insights into biological function, however, **bioluminescence** holds the promise of deeper imaging using simpler optical systems at the cost of several engineering considerations. Despite its tremendous value for biological science, fluorescence imaging is constrained by its reliance on the excitation light source and the endogenous fluorescence (aka. autofluorescence) of the tissue all around the body [89], [90]. The excitation light used to excite the fluorophores suffers from scattering, resulting in less “sharp” images and constrained imaging depth ([Fig. 7](#)). Furthermore, continuous application of external light is associated with photobleaching [91], phototoxicity [92] and confounding modulation of cellular capacitance [92], [93] and mitochondrial function [94]. In addition, even though fluorescent miniature microscopes have been able to achieve a remarkably small footprint of 21 mm and 2 grams in weight [15], these dimensions become problematic when trying to fit multiple of such miniscopes throughout the body of a mouse. These dimensions increase even further when we try to increase the number of simultaneously imaged colors or transition towards wire-free miniscopes.

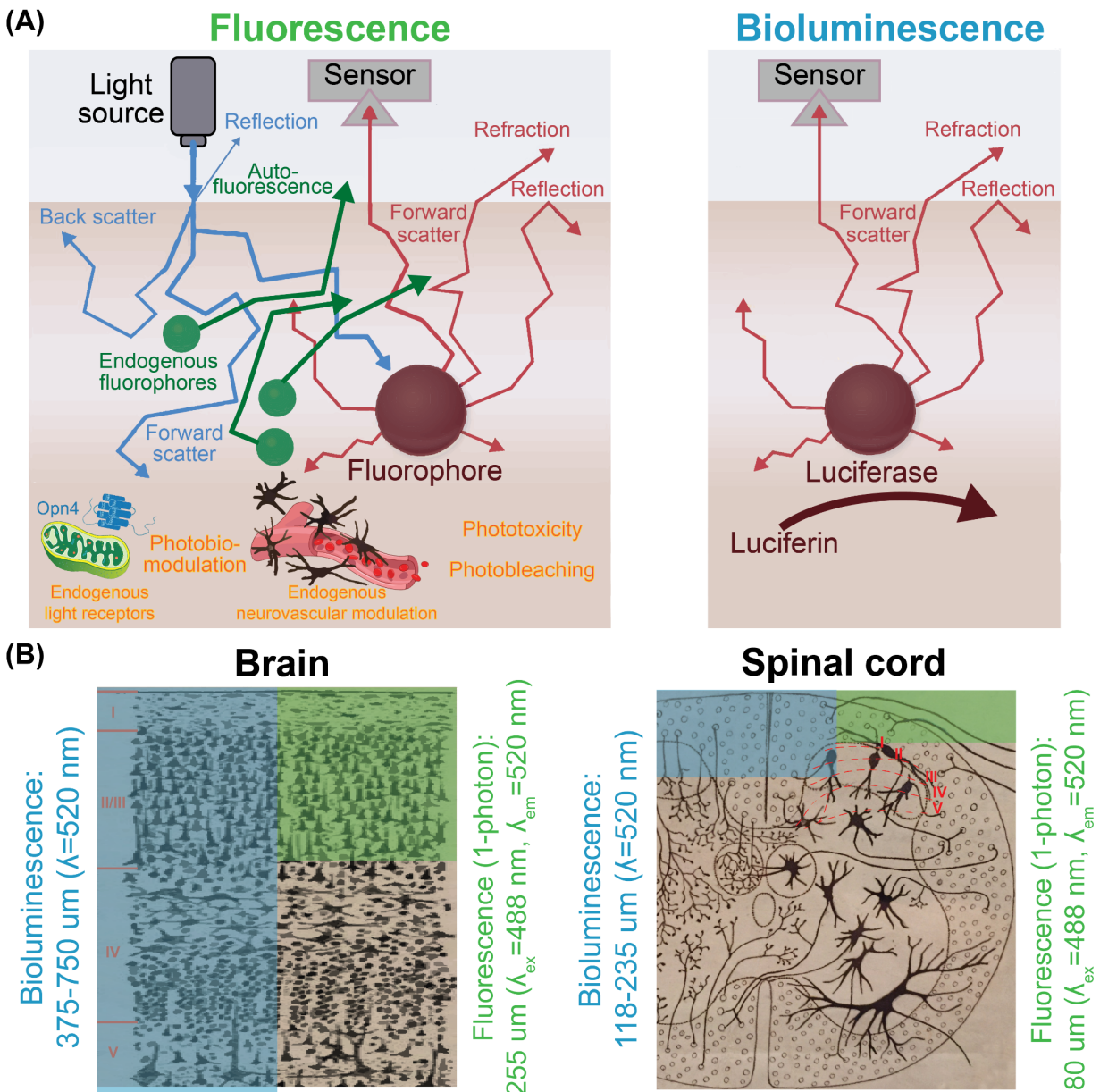
Bioluminescence solves all the above problems at the cost of a few alternative engineering considerations. Bioluminescence does not require an excitation light source, hence it does not suffer from photobleaching, phototoxicity, autofluorescence and it offers a potential 3-fold increase in the imaging depth compared to 1-photon fluorescence imaging ([Fig. 7](#)). However, the actual imaging depth limit for

bioluminescence has not been yet determined experimentally. In [Fig. 7](#), the bioluminescent imaging depth for the brain was estimated using Monte Carlo simulations [95]. For the spinal cord, the bioluminescence imaging depth was estimated by looking at the ratio of 1-photon fluorescent imaging depths in the brain vs. the spinal cord. By assuming that bioluminescence imaging depth limit will be comparably affected by spinal cord myelination scattering as fluorescence imaging depth limit, we can estimate the limit for the spinal cord using bioluminescence depth limit in the brain. Bioluminescence imaging Monte Carlo simulations utilized the detectability of circular 10  $\mu\text{m}$  light sources mimicking neurons above the noise floor in <70% of cases as the imaging depth limit [95]. For fluorescence imaging, the imaging depths for 1-photon imaging were taken from experimental data. The deepest targets that can typically be resolved are the blood vessels rather than individual neuron activity (e.g. blood vessels in the SC can be imaged using 2-photon microscopy as deep as 200-300  $\mu\text{m}$  [33], [96], [97] and using 3-photon as deep as 500  $\mu\text{m}$  [96]). It is also important to emphasize that the depth limit definitions here exclude the depths achievable when using invasive hardware such as GRIN lenses, prisms and implantable fibers, as well as the depths achievable when using longer wavelengths (e.g. near-infrared 1-photon fluorescence imaging has been shown to allow spinal vasculature imaging as deep as 350  $\mu\text{m}$  [98], which exceeds even the 2-photon imaging depth reported up to date). Finally, it can be noted that even though the spinal cord is significantly harder to image as deep as the brain due to light scattering, another part that contributes to the limited imaging depth achieved so far in the spinal cord is the significantly fewer spinal cord studies. The abundance of brain imaging studies drives the momentum of rapid tool development

building up upon previously developed tools. In the case of the spinal cord, the progress heavily relies primarily on adopting brain tools to the spinal cord with much fewer uniquely spinal cord tools ([Fig. 10](#)).

Bioluminescence imaging also offers significant benefits from the hardware perspective. The lack of need for the excitation light also renders a much cheaper and lighter microscope ([Table 2](#)). The important engineering consideration is the fact that in general while offering a higher signal-to-noise ratio compared to fluorescence, the bioluminescent signal is dimmer. Some previous studies estimated bioluminescence to be about 1,000 dimmer than fluorescence with the emission of  $10^3$ - $10^5$  photons/s vs.  $10^8$  photons/s, respectively [99]. However, the exact brightness comparison is hard to estimate as it is dependent on the experimental conditions, and the brightness of the bioluminescent indicators has been steadily increasing over the years [100]. Another part of the equation is that imaging cameras are becoming more and more sensitive, with the avalanche detectors approaching the market [101]. However, the increasing sensitivity of the cameras raises the importance of the light-tightness between the sample and the camera. With the high sensitivity cameras, even dim light sources in vicinity of the sample can significantly affect the performance of the camera, especially when imaging without optical filters. Finally, an important consideration must be given to the administration of the bioluminescent substrate, luciferin. There are many possible routes of administration for luciferin in mice and each route offers different temporal dynamics, signal brightness, impact on the animal's body, compatibility with the animal's behavior and the level of practical implementation complexity. Given the converging progress in the bioavailability and brightness of the molecular and hardware tools,

imaging of bioluminescent dynamics using low-cost miniature cameras seems to have reached the point of feasibility [21] and hence is pursued in this thesis. I address the above outlined challenges of bioluminescent imaging in this thesis to make the benefits of bioluminescent molecular tools more accessible to the scientific community.



**Figure 7.** Bioluminescence offers fewer confounding optical factors due to the lack of excitation light and offers a potential increase in the imaging depth for up to 3 fold for the brain and spinal cord compared to 1-photon fluorescence imaging. **(A)** Illustration of the factors contributing to

noise and confounding effects in the case of fluorescence (left) and bioluminescence (right). **(B)** Illustration showing a potential increase in the imaging depth with bioluminescence compared to 1-photon fluorescence in the brain and spinal cord. Mouse somatosensory cortical layers and spinal dorsal horn laminae marked in red indicate the approximate deepest cortical layers [10], [97], [102] and laminae [97], [103] that can be imaged in an adult mouse via fluorescence and bioluminescence. Imaging depths were determined from [95]. Brain background image in (B) is from [104] and is in the [public domain](#). Spinal cord background image in (B) is from [105], licensed under [Public Domain Mark 1.0](#).

The **vascular endogenous light sensitivity** part of this thesis presented in Chapter 2 was inspired by recent reports on endogenous sensitivity of the blood vessels in the brain [53] and lungs [106], [107]. However, the endogenous light sensitivity properties have not been previously investigated for the spinal cord neurovascular system. As the development of the surgical and imaging tools presented in this thesis required the examples of reliable imaging of the neurovascular changes induced in a temporally precise manner, the question on endogenous light sensitivity was perfectly positioned as an example of the kind of novel unanswered question that can be addressed using the imaging methodology developed in this thesis.

#### 1.4. Multi-site, multi-modal imaging can help us understand nociception, and the link between acute and chronic pain following peripheral nerve injury

Processing of noxious and innocuous sensory information plays an important role in acute and chronic pain of peripheral etiology. Acute and chronic pain of peripheral etiology, for example, chronic postsurgical pain (CPSP), engages the neuro-glio-vasculo-immune circuitry at the periphery (e.g. skin), dorsal root ganglia (DRGs), spinal cord and the brain [108]–[110]. The complex nature of acute and chronic

pain involves multiple parts of the body and, consequently, is a difficult topic to study. As a consequence of significant knowledge gaps, chronic pain affects an estimated 20% of people worldwide and is responsible for 15-20% of physician visits [111]. For the past 2 decades there has not been progress in decreasing the prevalence of conditions like CPSP [112]. At the same time, the opioid epidemic has been on the rise [113], [114] and new treatments either fail or show variable efficacy in heterogeneous populations and pain etiologies [110], [115], [116]. The methodology I developed in this thesis can provide critical knowledge on many open questions pertaining to the processing of noxious and innocuous sensory information in health and disease. In this subchapter, I discuss the examples of specific questions pertaining to nociception, acute pain and chronic pain that can be studied using my methodology.

Recently it has been recognized that the progress in developing effective pain treatments lacks behind in part because of a simple factor of the lack of agreement on the definitions relevant for the study of pain. In addition to that, many animal studies often do not specify whether they focus on acute or chronic pain because it is challenging to differentiate between the stages of pain [117]–[119]. Hence, for the purposes of this study, it is important to begin with some core definitions.

#### 1.4.1. Definitions

The detection process of noxious stimuli (pain sensation is not necessarily implied) by the primary sensory neurons is referred to as **nociception** [108]. The sensory neurons responsible for nociception are called **nociceptors**, and are categorized into the classes C, A $\delta$ , and to a lesser extent, A . Nociceptors have activation thresholds or sensitivities that are different from the thresholds of neurons

that process innocuous signals and are activated by stimuli (mechanical, thermal and chemical) capable of causing tissue damage [108]. Nociceptors innervate not only the skin, but also deep tissues and internal organs [109], the brain being one of the few organs lacking nociceptors. In a healthy state, the processing of nociceptive information by the brain can lead to the experience of acute pain. In a disease state, nociception, acute pain, damage of the nervous system, cancer and many other factors can lead and contribute to chronic pain. According to the International Association for the Study of Pain (IASP), **pain** is defined as “an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage”. IASP does not provide unified definitions of acute and chronic pain, as the nature of pain varies significantly depending on etiology of pain. According to Carr and Goudas 1999, a common definition of **acute pain** is “the normal, predicted physiological response to an adverse chemical, thermal or mechanical stimulus... associated with surgery, trauma and acute illness” [120]. The International Classification of Diseases (ICD) of the World Health Organization (WHO) defines **chronic pain** as “pain that persists past normal healing time and hence lacks the acute warning function of physiological nociception” [111]. Pain is commonly assumed to be chronic when it lasts or recurs for more than 3 to 6 months [111]. Importantly, both acute and chronic pain have affective (sometimes referred to as emotional) and sensory components [109], [113], [120], [121]. The **affective component** influences the experience of pain by contributing psychological and social factors such as attitudes, beliefs, culture, past experiences and personality. Multiple brain areas have been identified to be responsible for processing the affective component such as basolateral amygdala (BLA), prefrontal

cortex (PFC), anterior cingulate cortex (ACC) and periaqueductal gray area (PAG) ([Fig. 9A](#)) [109], [121]. The **sensory discriminative component** of pain contributes the sensory experiences of pain such as localization, perception of spatial spread, intensity and other qualities of pain (e.g. stabbing, pricking, burning, aching). Some of the brain areas implicated in the sensory component of pain include primary (SI) and secondary (SII) somatosensory cortices, thalamus and cerebellum [109]. Neither affective nor sensory components of pain exist outside the brain, and it has been shown that by electrically stimulating thalamus in healthy individuals it is possible to evoke the sensation of pain [122], [123]. These kinds of observations support the growing view of pain as a central disorder [124], [125]. However, the idea that pain can be autonomously sustained by sensitized brain circuits alone without interacting with the spinal cord circuits or the periphery has not been experimentally confirmed yet. This question could not be addressed up to date because it has not been possible to measure what is happening in the periphery and the spinal cord during seemingly autonomously generated pain [124].

#### 1.4.2. Gaps in the knowledge

A rich plethora of studies have been conducted in an attempt to develop mechanistic understanding of different types of pain. These studies examine pain at many different biological scales: behavioral [126], [127], psychological [128]–[130], gross brain [131]–[133] and spinal cord circuit [134]–[137], cellular [86], [109], [138], [139], molecular [138], [140], [141] and epigenetic [142]. This abundance of studies has yielded a large number of therapies. Most of these therapies are still under investigation: exercise [143], [144], mindfulness [145], [146], transdermal drugs [147],



injectable hydrogels [148], acupuncture [149], neurostimulation (e.g. deep brain stimulation [150], [151], spinal cord stimulation [152]–[154], repetitive transcranial magnetic stimulation [155], dorsal root ganglion stimulation [152], [154], [156], vagus nerve stimulation [157]), focused ultrasound stimulation of the brain [158], [159], gene therapy [160], tissue transplants [161]. The hallmarks of many pain therapies are variable [110] or lacking efficacy [162], [163], and side effects [164]. Consequently, despite well defined biopsychosocial side effects (e.g. respiratory depression, tolerance, addiction, loss of independence, need for therapy) and high economical cost [164], opioids remain the main treatment of choice. According to Centers for Disease Control and Prevention (CDC), in 2017 the cost of opioid use disorder and fatal opioid overdose totalled \$1,021 billion [165]. Yet there are simply no better alternatives to opioids. **Opioids** are the essential medications, and the most effective drugs for the relief of severe pain, chronic pain and suffering [164], [166]. Opioids have a rapid onset of pain relief, are recognized as generally safe by the CDC, can be readily produced in natural and synthetic ways, and has the longest use history amongst all medications - the knowledge about opioid psychological effects and usefulness in relieving pain and diarrhea by Sumerians dates back to 4000 BC [164]. The impactful negative effects of opioids stem from the wide distribution of opioid receptors throughout the body. Opioid receptors are present throughout the peripheral and central nervous system, in the immune white blood cells [164], [166] and in neural plexuses of the gastrointestinal tract [164]. The complexity of opioid receptor function and their widespread presence throughout the body leads to many efforts to harvest the analgesic benefits of opioid receptor modulation without the side effects [164]. Furthermore, a predominant number

of studies that aim to develop new therapies focus on chronic rather than acute pain. Even though acute pain is part of a healthy response, in the cases where acute pain needs to be mitigated, patients often have to rely on treatments developed for chronic pain [117]. This potentially can limit the efficacy of acute pain therapies because of the lack of knowledge of the mechanistic effects of chronic pain treatments on acute pain.

The above issues in efficacy of pain treatments follow from many fundamental gaps in knowledge about the mechanisms of pain, especially when it comes to the link between acute and chronic pain, as well as the temporal dynamics of processes happening across distant parts of the body. The specific knowledge gaps that need to be addressed are as follows:

- there is a lack of acute pain specific treatments because the majority of studies focus on chronic rather than acute pain [117],
- the exact duration of acute pain phase and biomarkers of pain are not known neither in humans nor in animals [167]–[169],
- it is still not known whether acute and chronic pain represent a continuum of a single disease or two concurrently developing diseases or some more complex combination of states [117], [170],
- the exact time courses of sensory information journeys from the periphery through DRG and spinal cord towards the brain is not known,
- many specific genetic identity cells have been implicated in nociception and pain ([Fig. 9A](#)), but we do not know enough about their temporal dynamics and circuit interactions throughout the development of pain,

- it is not known how multiple types of pain (e.g. migraine and peripheral nerve injury) or multiple types of diseases (e.g. pain and depression, pain and bone injuries) interact with each other at the circuit level [171]–[174],
- it is not known whether pain can be autonomously sustained by the brain without the interactions with the spinal cord and the periphery [124],
- it is not known how some of the experimental pain therapies (such as spinal cord stimulation (SCS)) work or how combined therapy (e.g. combining transcranial alternating current stimulation + photobiomodulation (PBM) + exercise) affect different parts of the nervous system,
- it is not known how the efficacy of treatment depends on the time passed since the onset of chronic pain (e.g. initiate the SCS right at the onset of chronic pain or years later) [175], [176],
- it is not known whether pain has just 2 clinically relevant time points (i.e. onset of pain and transition time point from acute to chronic pain) or many more relevant time points throughout pain development...

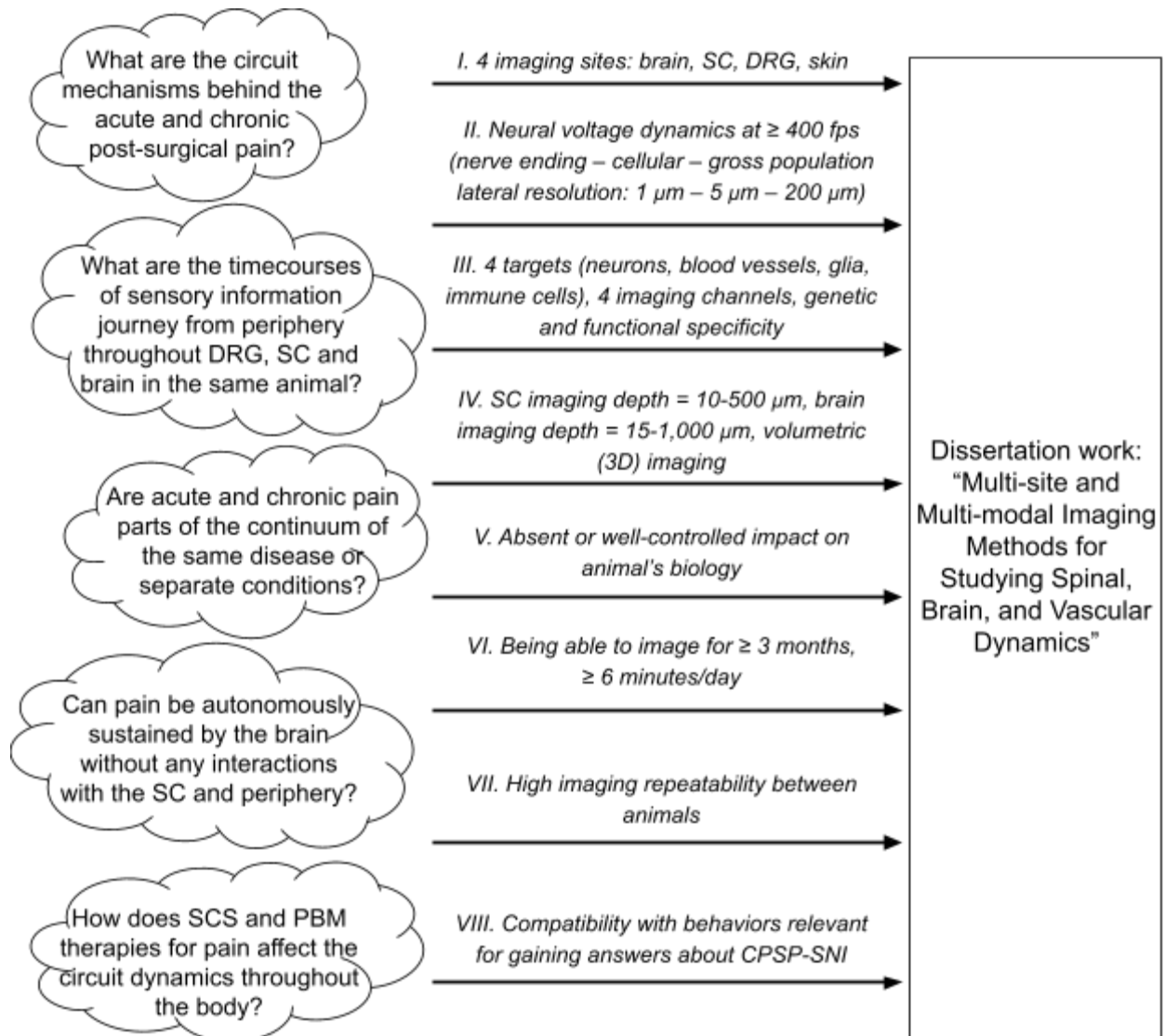
We can gain deeper insights into many of these questions only by looking at multiple parts of the body in the same animal and by collecting the dynamic data along the continuum of disease rather than collecting the data from discrete time points. Furthermore, even with the current state-of-the-art technologies, to understand what is happening in the brain and spinal cord during similar conditions, we would need a double number of animals to study each organ independently. As we increase the number of imaging targets and sites, the number of needed animals will keep increasing. This means extra variability in the data, extra confounds, higher costs,

longer lab-to-knowledge timelines, lower repeatability and, most importantly, higher numbers of animals exposed to pain. Being able to collect the data in the brain and spinal cord in the same animal alleviates all of these multiplicative issues.

#### 1.4.3. Engineering specifications for imaging methodology needed to understand circuit mechanisms behind the acute and chronic postsurgical pain

The tools developed in this dissertation are important for understanding a variety of diseases and pain conditions of different origins. Here I consider a particular example of CPSP [112]. CPSP can be caused by a variety of triggers, but at least one-third of cases have neuropathic components such as direct nerve injury [112]. CPSP is a common condition. It has been reported that 22.5% of patients attending the pain clinics attribute their pain to past surgeries. Moderate CPSP occurs in 11.8% of surgical patients and severe CPSP in 2.2% of surgical patients in Europe [112].

CPSP after peripheral nerve injury is a disease that can particularly benefit from multi-site imaging tools developed in this dissertation because its spatio-temporal onset can be well defined. In an animal model, CPSP can be induced by a sciatic nerve injury (SNI) [177]–[179]. This offers a precisely controlled onset in time and a clearly defined target for imaging in the periphery. Precise temporal and spatial localization of CPSP-SNI makes it possible to track dynamically the state of neuro-glio-vasculo-immune circuits throughout the development of disease pre- and post-injury. However, in order to conduct this form of dynamic circuit mapping throughout the body and throughout the full development process of CPSP-SNI, the imaging method needs to meet the following specifications ([Fig. 8](#)):



**Figure 8.** The input (goal) specifications of the imaging method were defined at the beginning of this dissertation work based on the knowledge gaps surrounding acute and chronic pain. SC = spinal cord, DRG = dorsal root ganglion, SCS = spinal cord stimulation, PBM = photobiomodulation, CPSP-SNI = chronic post-surgical pain caused by sciatic nerve injury.

- I. 4 imaging sites: brain, spinal cord (SC), dorsal root ganglia (DRG) and skin are well known parts of the sensory signaling pathways important for pain of peripheral etiology [110]. By imaging the injury site concurrently with DRG, SC and brain, we can understand the interactions of the dynamic processes happening throughout the circuits distributed throughout the body ([Fig. 8. I](#)).

- II. Neural voltage dynamics at  $\geq 400$  fps with spatial resolution of 1-200  $\mu\text{m}$ : to understand the function of neural circuits of acute and chronic pain, we need to be able to image neural voltage dynamics. The timescale of action potentials requires imaging at the framerates of  $\geq 400$  fps and we need to have an appropriate spatial resolution to study the mechanistic roles of different neural components. 1  $\mu\text{m}$  resolution for imaging nerve endings is based on the imaging resolution used in [79] to image calcium responses to capsaicin of *Trpv1* nerve endings in the skin, 5  $\mu\text{m}$  resolution is based on the size of spinal dorsal horn neurons being on the order of 10  $\mu\text{m}$  [180], and 200  $\mu\text{m}$  resolution is based on functional magnetic resonance imaging (fMRI) studies in mice focusing on gross brain area dynamics during the processing of noxious and innocuous sensory signals [181]–[184] ([Fig. 8, II](#)).
- III. 4 imaging targets and channels with genetic and functional specificity: to understand pain, it is not enough to image only neural dynamics. Beyond neurons, it is important to image blood vessels, glial cells and immune system (4 targets) [185] and, ideally, simultaneously in the same animal (4 imaging channels) with genetic and functional specificity. It is important to be able to image not only cells, but also the signaling mechanisms, such as imaging the neurotransmitters [78], [186]. The need for 4 imaging channels refers not only to the ability to image simultaneously all 4 targets, but also imaging 4 different cell types within the same target (e.g. 4 different populations of neurons) or any other combinations. One example of multi-channel imaging is the use of multiple fluorescent [91], [187], [188] or bioluminescent [189]–[191] colors. Genetic

specificity refers to the ability to target cells of specific genetic identities. Functional specificity refers to the ability to target cells based on specific functional phenotypes (e.g. fast spiking vs. slow spiking neurons) ([Fig. 8. III](#)).

- IV. 10-500  $\mu\text{m}$  imaging depth in the spinal cord and 15-1,000  $\mu\text{m}$  imaging depth in the cortex with ability to image in 3D: to understand pain, we need to understand the dynamics across V dorsal horn laminae in the spinal cord and 6 cortical layers of the somatosensory cortex in 3D. The minimal imaging depth requirement in the spinal cord of 10  $\mu\text{m}$  corresponds to the average size of spinal dorsal neurons and 500  $\mu\text{m}$  corresponds to the depth of lamina V - the deepest dorsal horn lamina known to be involved in sensory information processing [83], [97], [192]. The minimal imaging depth requirement in the brain of 15  $\mu\text{m}$  corresponds to the average soma diameter in the primary visual and frontal cortices [193], and 1,000  $\mu\text{m}$  in the brain corresponds to the thickness of the somatosensory cortex in adult mice [194], [195]. Even though the somatosensory cortex is one of the key areas for processing the innocuous and noxious sensory stimuli, “pain matrix” spans many brain areas [109] and ideal imaging depth would span the entire mouse brain (~4 mm [196]) to avoid the use of more invasive optical imaging approaches such as implantable lenses, prisms and fibers ([Fig. 8. IV](#)).
- V. Absent or well-controlled impact on animal’s biology: an ideal imaging method should allow to collect the data without biological effects confounding the study (e.g. it can be challenging to derive meaningful conclusions about vascular function when an imaging method requires the application of isoflurane

anesthesia because of its known effects on vascular function [53]). However, an imaging method that impacts animal's biology in a controlled manner is a powerful tool for generating new insights (e.g. restraint impacts animal's natural behavior, but allows to control many experimental factors such as consistent application of optogenetic stimulation to study nociception [126]). Part of the compatibility with natural behaviors includes the lack of adverse effects on animal's health and consequently, the lack of uncontrolled (or minimal well known) effects on the investigated neuro-glio-vasculo-immune processes ([Fig. 8. V](#)).

- VI. *Being able to image for  $\geq 3$  months,  $\geq 6$  minutes/day*: we want to be able to image for as long as necessary to understand the development of disease. Many insights into nociception can be gained from acute studies whereas chronic pain requires the ability to track the disease for days or months [121]. The goal of being able to image for  $\geq 3$  months is based on the definition of chronic pain for humans.  $\geq 6$  minutes/day goal is based on our in-lab experience of needing  $\geq 30$  trials of sensory stimulation to start gaining insights into how a given sensory system functions (e.g. stimulation of vibrissa processed by somatosensory cortex). Based on the typical timing of vibrissal stimuli employed in our lab,  $\geq 6$  minutes are needed to collect 30 trials. The specific pain behaviors developed in our lab require the ability to perform animal's behavior for  $\geq 1$  hour [126]. Hence, in order to gain insights into sensory information processing dynamics, we need to be able to image for at least 6 minutes/day or for more sophisticated behaviors, for  $\geq 1$  hour/day ([Fig. 8. VI](#)).



- VII. High imaging repeatability between animals: the imaging method should allow to obtain comparable results across different animals within the tolerances of natural biological variability (e.g. it should be possible to observe comparable features of responses across different animals when applying the same noxious hindpaw stimulation and imaging in the same brain-spinal cord areas under comparable experimental conditions) ([Fig. 8, VII](#)).
- VIII. Compatibility with behaviors relevant for gaining answers about CPSP-SNI: the imaging method should be compatible with behavioral essays needed to fully understand the disease mechanisms. In the case of pain, this often includes von Frey reflex testing [121], thermal stimulation of the hindpaw [79], place preference test [197], videographic tracking of animal motion [127], [198] and grimace [199], [200] for pain scoring, and optogenetic stimulation behavior for self-report of nociception [126]. These behaviors can require anesthetized, restrained and freely moving animal preparations ([Fig. 8, VIII](#)).

It should be noted however that the above specifications are not aimed at defining the ultimate imaging system capable of addressing every imaginable question pertaining to CPSP-SNI. Rather, the imaging method that meets the above specifications would significantly advance the basic and translational systems neuroscience knowledge of circuit dynamics underlying CPSP-SNI (as well as rich variety of other questions pertaining to pain of other etiologies, wound healing, spinal cord injury, photobiomodulation therapy, spinal cord stimulation therapy, etc.). In defining the above specifications, I particularly considered the feasibility given current state-of-the-art. Without considering the feasibility, the ideal method would be the one

that allows us to image the dynamics of everything from individual molecules to full body-spanning circuits at sub-millisecond temporal resolution in naturally behaving animals living in the wild. Even though the focus of this thesis work was entirely on optical microscopy, the physical nature of the imaging method was intentionally not implied when defining the specifications in [Fig. 8](#) because many other imaging modalities such as photoacoustic imaging and functional ultrasound imaging offer rich promise in the domain of circuit systems neuroscience [88], [201].

#### 1.4.4. Experimental approaches demonstrated in this thesis towards studying the processing of noxious and innocuous sensory signals

The scope of this dissertation has been on developing multi-site, multi-modal imaging methods for studying spinal cord, brain, and vascular dynamics towards meeting the specifications outlined in [Fig. 8](#). Given this scope, I demonstrate how my methodology can be applied to study the noxious and innocuous sensory information processing in healthy animals. The development of this methodology brings us closer towards being able to study acute and chronic pain using the same exact methodological building blocks demonstrated in this dissertation.

To demonstrate the ability to study the processing of **noxious sensory signals** using methods developed in my thesis, I employed mechanical pinching and the optogenetic stimulation of hindpaw using *Trpv1*<sup>Chr2EYFP+/-</sup> transgenic animals ([Fig. 9B](#)). Peripheral nerve injury (e.g. surgical sciatic nerve injury) is known to involve activation of transient receptor potential cation channel subfamily V member 1 (*Trpv1*) receptors [107]. In the absence of injury, activation of *Trpv1* by strong (i.e. noxious) heat or capsaicin is part of the healthy nociceptive processing. Transgenic *Trpv1*<sup>Chr2EYFP+/-</sup>

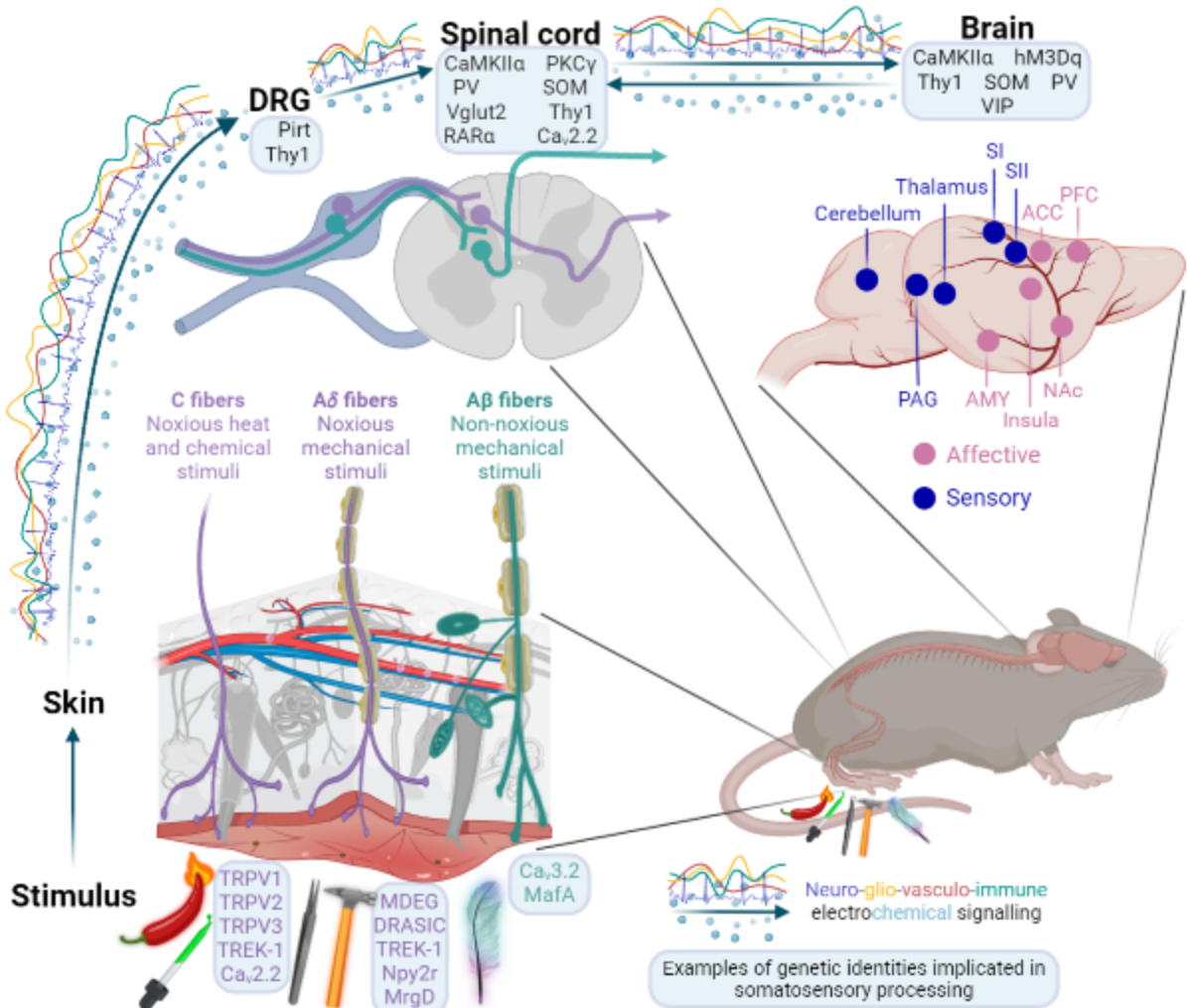
animals allow to open *Trpv1* ion channels by shining blue light (the peak absorption of channelrhodopsin-2 (*ChR2*) is around 470 nm [202]) at the tissues naturally containing *Trpv1* proteins. In our case, we are specifically interested in *Trpv1* ion channels expressed in A and C fiber peptidergic neurons, their axons in the sciatic nerve and nerve endings in the skin responsible for detection of noxious heat [203]. To demonstrate the ability to study the processing of **innocuous sensory signals**, I employed mechanical touch and the optogenetic stimulation of hindpaw using ***Cacna1h*<sup>Chr2EYFP+/-</sup>** transgenic animals ([Fig. 9C](#)). Transgenic *Cacna1h*<sup>Chr2EYFP+/-</sup> animals allow to control *Cav3.2* ion channels by shining blue light at the tissues naturally containing *Cav3.2* proteins and being genetically modified to also express the light-sensitive opsin *ChR2*. *Cav3.2* ion channels have been implicated in acute itch [204] and in the hindpaw skin these channels are primarily responsible for C- and Aδ-fiber low-threshold mechanoreception (LTMR) [205]. LTMRs have also been shown to drive pain in the case of allodynia [206], [207] and suppress nociception when the inputs of innocuous LTMR coincide in time with the noxious signals [208] as has been hypothesized by the Gate Theory of Pain [209]. In essence, optogenetic *Trpv1*<sup>Chr2EYFP+/-</sup> and *Cacna1h*<sup>Chr2EYFP+/-</sup> animals allow us to probe with nanosecond temporal precision of light delivery the neural pathways known to be implicated both in healthy nociception and chronic pain.

The somatosensory circuits responsible for processing the noxious and innocuous signals are summarized in [Fig. 9A](#). In the case of noxious signals, the illustrated pathways can lead to the experience of acute pain. The same pathways are also implicated in chronic pain, however in the case of chronic pain the stimulus of the

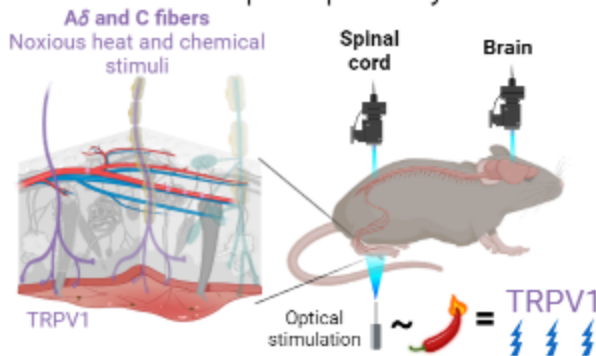
skin is no longer necessary to experience pain. Consequently, [Fig. 9B-C](#) highlights that the transgenic animal models used in this thesis allow to directly study parts of the somatosensory pathways implicated in nociception, acute pain and chronic pain. [Fig. 9A](#) also highlights a number of molecular targets that have been reported to be relevant for processing of somatosensory signals (Trpv1 [210], Pirt [211], Thy1 [38], CaMKII $\alpha$  [121], [212], [213], PKC $\gamma$  [86], PV [86], [214], SOM [215], Vglut2 [216], RAR $\alpha$  [85], VIP [217]). The listing of these molecular targets in [Fig. 9A](#) is by no means comprehensive and rather provides examples of relevant molecular identities.

Beyond the transgenic optogenetic lines, I employed wild type animals to test the optical sensitivity of blood vessels in the spinal cord. Being able to modulate the function of blood vessels directly in the central nervous system is another approach in modulating the biological function (e.g. the processing of sensory information, the biodistribution of pharmaceuticals) in a temporally and spatially precise manner. Finally, part of my dissertation work involved development of bioluminescent imaging methodology. Bioluminescence imaging is an essential part of work towards achieving the required methodological specifications defined in [Fig. 8](#).

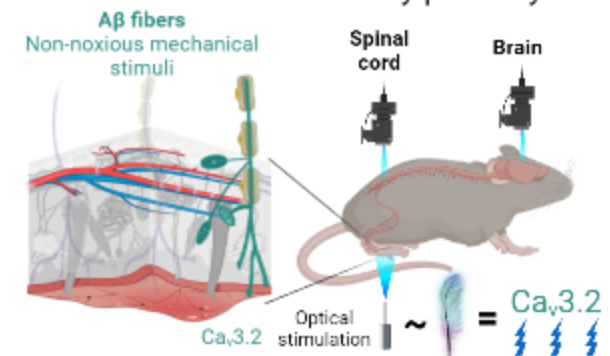
**(A) Somatosensory pathways of acute pain and non-noxious sensation**



**(B) Skin optogenetic stimulation in TRPV1 x ChR2 animals engages high-threshold nociception pathways**



**(C) Skin optogenetic stimulation in Cacna1h x ChR2 animals engages non-noxious mechanical sensory pathways**



**Figure 9.** The experimental models demonstrated in this thesis (*Trpv1* x *ChR2*, *Cacna1h* x *ChR2*, mechanical stimulation, endogenous light modulation) allow the study of pathways involved in the processing of noxious and innocuous signals. **(A)** Somatosensory pathways in

healthy states are responsible for the processing of innocuous and noxious signals. Noxious signals can lead to the experience of acute pain. Listed brain areas were informed by [109], [113], [218]. Listed skin receptors were informed by [79]. **(B-C)** The pathways activated experimentally in this thesis using *Trpv1 x ChR2* and *Cacna1h x ChR2* allow partial engagement of the pathways shown in (A). Brain pain network from [109], [218]. S1 = primary somatosensory cortex, SII = secondary somatosensory cortex, ACC = anterior cingulate cortex, PFC = prefrontal cortex, PAG = periaqueductal gray area, AMY = amygdala, NAc = nucleus accumbens, DRG = dorsal root ganglia. Created with [BioRender.com](https://www.biorender.com).

## **CHAPTER 2**

### **2. Multi-site and Multi-modal Optical Imaging**

### **Methods for Studying Spinal Cord, Brain, and Vascular Dynamics**

#### **2.1. Preface**

This chapter summarizes my efforts to improve existing optical methods for brain, spinal cord, and vascular imaging and modulation. My work in this chapter was inspired by observing significant gaps in chronic spinal cord imaging and modulation methodologies. In parallel with developing the optical tools for the spinal cord, I was working on imaging the sensory brain responses. As I was trying to achieve reliable response imaging independently in the brain and spinal cord, it became obvious that many questions pertaining to the processing of noxious and innocuous signals require synchronous study of the brain and spinal cord. Following this realization, I became fascinated about the idea of functional neuro-glio-vasculo-immune imaging with single cell resolution throughout the entire body. Present chapter is my first step towards this distant dream.

#### **2.2. Contributors to this chapter**

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## 2.3 Abstract

Despite advances in targeted imaging described in Chapter 1, these have almost uniformly been realized through imaging in a single brain area at once. However, the biological dynamics underlying behavior, and their failure in disease, require coordination and often transformation in multiple body regions. Specifically, methods for simultaneous, chronic imaging from the Brain and Spinal Cord (BASC) is a key next step to enable advances in understanding topics such as the coordinated multi-site activity required for motor action and tactile sensation, the functional representation of pain, recovery from injury, and comparative progression of diseases such as ALS and spinal cord injury. **In this Chapter, I describe a new, systematic approach I developed for chronic BASC imaging in transgenic and wild-type mice.** I describe strategies for allowing multi-site microscopic access, surgical strategies, implant designs, and long-term adaptation of mice to these methods. I then provide multiple

examples of neural and vascular imaging in both foci up to 230 days post-surgery. The examples of responses to the optogenetic activation of thermo-chemical receptors (*Trpv1*-expressing) and low-threshold mechanoreceptors (*Cacna1h*-expressing), as well as the endogenous light responses of blood vessels in the spinal cord have never been demonstrated before. I conclude with a discussion of key next steps in refining BASC.

## 2.4 Introduction

Transformational advances have been made in the last 15 years using genetically-targeted expression in specific cell types [219], [220]. Expressing calcium indicators in a single neuron subtype allows measurement of their relative rates and correlations, perception, action and cognition, and in disease models. Until recently, genetically-targeted imaging in the brain was almost exclusively realized in relatively focused regions of interest. Such single foci typically span a few hundred micrometers. Increasingly, larger window sizes have permitted ‘mesoscopic’ access to a broader field of view [221], including the entire dorsal neocortical brain surface [5] or large regions in deeper structures such as the dorsal striatum [78].

While these approaches permit broader access to a single brain area, they typically do not allow imaging of multiple distinct brain areas, for example, beyond just the neocortex. However, even the simplest behaviors require more than a single brain area, and more than just the brain alone [109], [222], [223]. As one example, processes such as explicit memory of a single item are widely considered to require an engram, in which specific ensembles of cells that encode the relevant information are chained to other downstream specific ensembles, and their combined, interdependent activity

results in the recall of information [224]. Understanding this phenomenon will necessarily require studying how transmission depends on the conjoint activity of multiple regions, or how the co-emerging dynamics occurring in coordinated regions (but, that would be invisible by analysis of any one region alone) are key.

Another fundamental process important to our biological existence is the processing of noxious and innocuous environmental stimuli. The process of perceiving skin deformation (touch) engages the receptors in the skin [225], [226], the primary afferents [227], the neurons in the dorsal root ganglia [35], the interneurons and astrocytes in the dorsal spinal cord [36], [225], [226], [228], [229], the local reflex loops throughout the spinal cord [139] and many brain areas such as brainstem [230], thalamus [223] and the somatosensory cortex [230]. Traditionally when studying sensory and nociceptive information processing most groups focus on the brain alone [12], [212], [215], [231]. One of the main areas of great interest is **primary somatosensory cortex (S1)** studied both in humans [232] and awake behaving rodents [12], [215], [231]. Although the studies of the brain alone have been instrumental in furthering our understanding of the sensory information processing, they neglect the downstream long-term and short-term neuro-glio-vasculo-immune dynamics in the spinal cord, dorsal root ganglia and the skin. Other groups study the activity of the spinal cord [37], [39], [40], [42], [84]–[88], dorsal root ganglia [38] and the skin alone [79]. Spinal cord studies have demonstrated the importance of not only neurons [37], [39], [42], [84], [86], [87], but also astrocytes [37], [39], [40] and hemodynamics [88] during the processing of cutaneous inputs. However, the brain, spinal cord, dorsal root ganglia and the skin cooperate together to orchestrate the perception of sensory

information. Hence, to fully understand the biology behind animal behaviors and the mechanisms of such diseases as chronic pain, amyotrophic lateral sclerosis and spinal cord injury, we need to be able to study the multiple parts of the body engaged in the behavior or affected by the disease.

Despite the clear need for a multi-site approach in many contexts, multi-site imaging studies have yet to be widely adopted, likely due to the many technological challenges. Such studies require long-term healthy survival after multiple invasive surgeries, maintenance of optical access in multiple positions (ideally chronically), successful behavioral adoption by the animal of multiple distinct implants, and resolution of steric hindrance between different imaging microscopes/devices.

Building on decades of use of chronic electrophysiological chambers [233], [234] and development of methods for chronic intrinsic imaging [235], [236], imaging of genetically-targeted cell types in neocortex has grown relatively linearly over the past few decades ([Fig. 10A](#)). While many challenges remain, this mode of functional brain microscopy is a mature technology, and has been applied for months [237]–[239] or even years [240]–[242] in the same preparation.

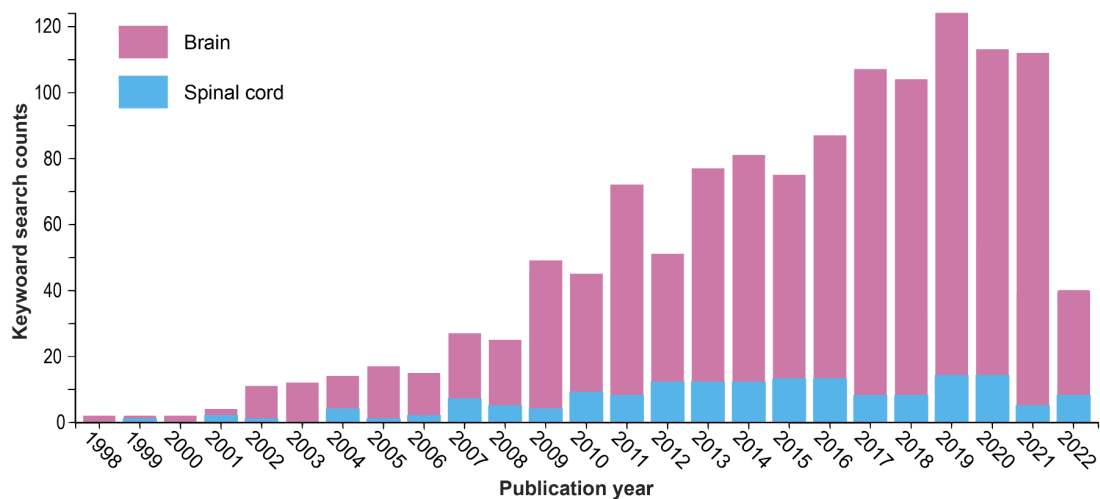
In contrast, chronic spinal cord microscopy has received significantly less attention ([Fig. 10A](#)). In the recent years the counts of publications on brain microscopy per single year started to approach the sum of all articles published on chronic spinal cord microscopy over the last 25 years (i.e. 124 articles on brain microscopy in 2019 vs. 165 articles on spinal cord microscopy over the past 25 years). While there has been exciting recent progress ([Fig. 10B](#)), the widespread adoption of spinal cord imaging remains slow because of the multiple challenges imposed by the physio-anatomy of the

spinal cord. Spinal cord is more challenging to image than the brain from the optical perspective because of the higher photon scattering noise and shallower imaging depth caused by imaging through the dense myelinated fiber tracts on the dorsal surface [74]. Further, and likely the most important factor retarding the adoption of these methods despite their clear need, is the more complex surgical preparation compared to the neocortex. The factors that make the spinal cord surgical procedures particularly challenging include:

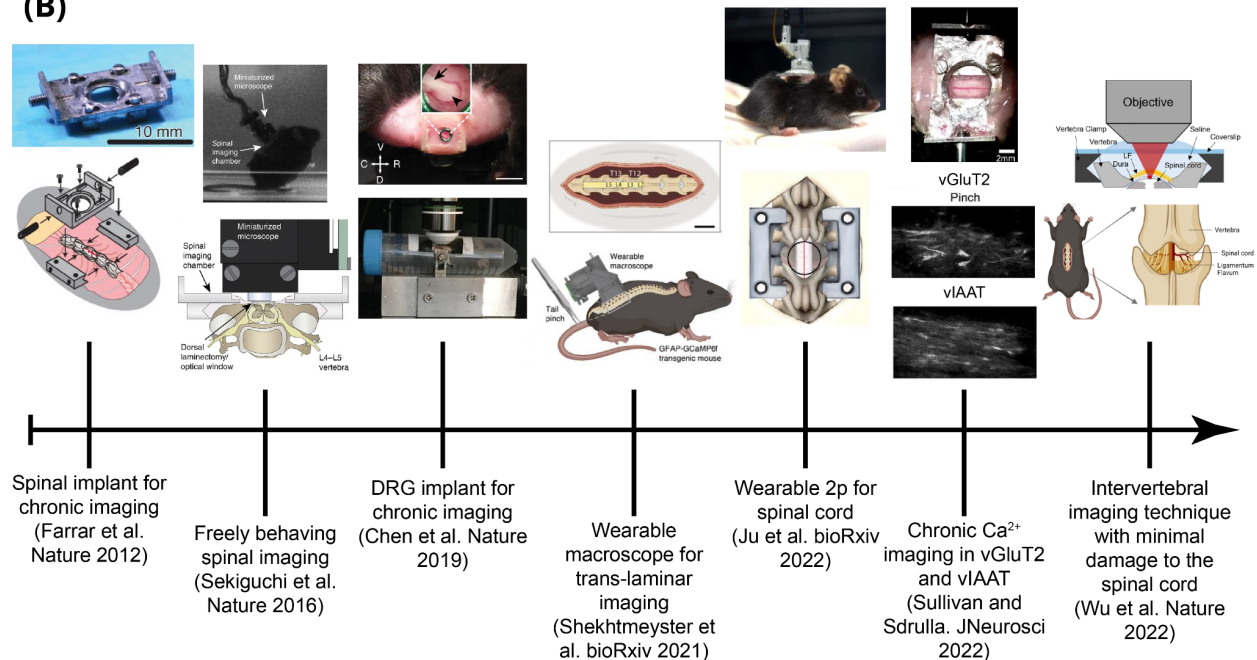
- i. **The vertebral column is a mobile organ** undergoing constant bending and twisting in 3D space, which makes the tissue around the spinal cord implants more susceptible to injury and reduces the longevity of spinal cord windows;
- ii. In mice, the vertebral bone is approximately 65  $\mu\text{m}$  thick [243], is trabecular in structure, and the vertebral column is around 3 mm in diameter. These aspects of anatomy offer much **less stable and more flexible structure for securing** the implant hardware in place compared to the brain where the skull provides a massive, stable surface for securing the implant;
- iii. High mobility and lower structural strength of the vertebral bones make it challenging to design a spinal cord implant composed of **a single part** (plus a glass or polymer window) akin to the brain implants [244]. The most widely adopted implant developed by Farrar et al. [33] is composed of **7 parts**. The large number of components complicates the surgical procedure, introduces additional points of failure and ultimately, reduces the quality and longevity of the overall surgical preparation.

The complexity of the surgical procedure leads to the high adoption barrier for any spinal cord procedures. Spinal cord surgeries require more practice compared to the brain surgeries and are significantly harder to learn from reading the publications alone. Hence, every spinal cord methodological report including present work requires extreme attention to smallest details and good visualizations of the procedures as the procedural repeatability is especially challenging with the spinal cord.

(A)



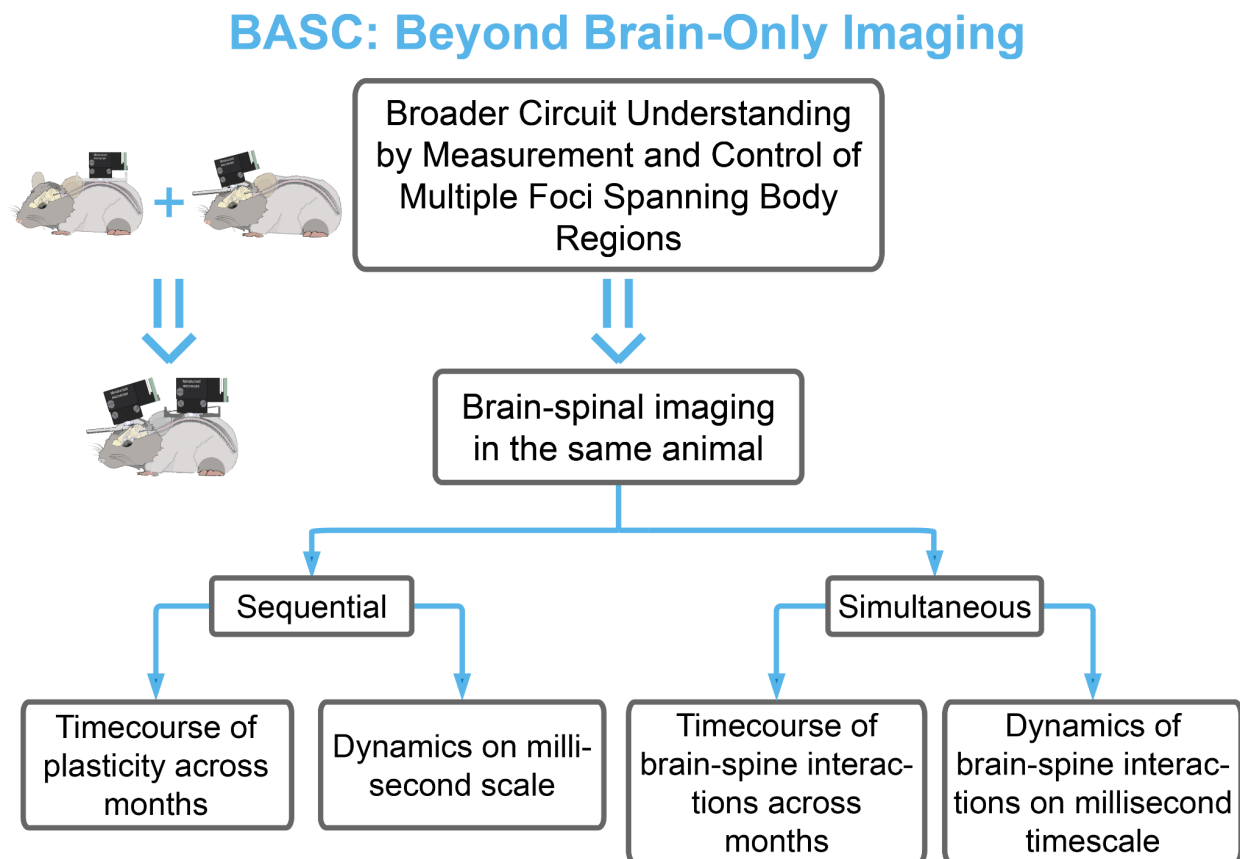
(B)



**Figure 10.** The spinal cord and brain-spinal microscopy lacks the well-established tools available to brain scientists with the majority of methodological progress for spinal cord imaging happening over the last several years. **(A)** Bar plot summarizing the publication counts throughout the last 25 years for the brain and spinal cord microscopy studies. The publication counts were obtained from the Web of Science using the following keywords: (i) Brain, mice, imaging, microscopy; (ii) Spinal, cord, mice, imaging, microscopy. The search was focused only on the field of Neuroscience. **(B)** Last decade milestone overview in chronic imaging of the spinal cord and dorsal root ganglia. Image sources from left to right: adapted by permission from Springer Nature Customer Service Centre GmbH, [33], © 2012 Farrar et al., under exclusive license to Springer Nature America, Inc.; from [37], © 2016 Sekiguchi et al., licensed under [CC BY 4.0](#); from [38], © 2019 Chen et al., licensed under [CC BY 4.0](#); from [39], © 2021 Shekhtmeyster et al., licensed under [CC BY-NC-ND 4.0](#); from [41], © 2022 Ju et al., licensed under [CC BY-NC-ND 4.0](#); from [42], © 2022 Sullivan and Sdrulla, licensed under [CC BY 4.0](#); from [43], © 2022 Wu et al., licensed under [CC BY 4.0](#). No changes were made to any images except for rearrangement and removal of panel letter labels.

The present study describes methods for multi-site BASC imaging. As shown in [Fig. 11](#), answers to key questions would benefit from, or require, this approach. However, BASC imaging has not been established. The feasibility of simultaneous BASC imaging was demonstrated in a review article by Nelson et al. 2019 showing an example of the 2-photon imaging of the brain and 1-photon imaging of the spinal cord using a custom miniscope [97]. While a valuable proof-of-concept, this review article did not include any methodological details on how to replicate the demonstrated example of BASC imaging. Another more distant example of prior art includes the imaging of multiple brain areas such as cerebellum and cerebral cortex in unrestrained mice using custom miniscopes (NINscope) [15]. Albeit focusing on a single organ, this study recognized the importance of studying the dynamics across multiple foci of a complex system rather than a single point. Besides the basic science value of multi-site imaging, the preclinical value has been also demonstrated by Figley et al. 2013 [245] by imaging the tumor micrometastases from the brain to the spinal cord using bioluminescence outside the body followed by the implantation of a spinal cord window for more precise

anatomical localization of tumor cells. The next impactfully informative step would be to image chronically the time course of micrometastases in parallel with progressive changes in brain and spinal cord activity. This is one of the many potential applications of the methodology outlined in this report with some other examples including amyotrophic lateral sclerosis, spinal cord infarction, wound healing, cancer, metabolic syndrome, influence of environmental factors (e.g. ionizing radiation, altitude, low gravity) and photobiomodulation.



#### **Benefits of BASC:**

- (i) reduces inter-subject variability by measuring the brain and spine in the same mouse;
- (ii) reduces animal use by broadening the methods applicable in a single mouse;
- (iii) allows inter-related measurements of conjoint brain-spine dynamics

**Figure 11.** The multi-site imaging technique enables sequential and simultaneous brain-spinal cord studies allowing to broaden circuit understanding by measurement and control of foci



spanning brain and spinal cord within the same animal. BASC (i) improves the quality of research studies focusing on the brain and spinal cord at the same time, (ii) leads to more cost-efficient and humane experiments, and (iii) allows new kinds of studies on brain-spinal cord interactions that have not been possible before.

The absence of direct progress in this domain likely reflects the fact that the chronic spinal cord optical access model alone still has not been widely adopted. Also, and perhaps more importantly, the multi-site BASC imaging requires resolution of the challenges associated with both the spinal cord and brain optical access separately, and unique additive challenges created by combining both organ preparations. Surgical preparation of both the spinal cord and the brain within the same animal is one of the crucial considerations that is addressed by the present work. Prior to this study, it was unknown how well the same mouse can handle both a brain and spinal cord window surgical procedure and maintain long-term good health following double implantation.

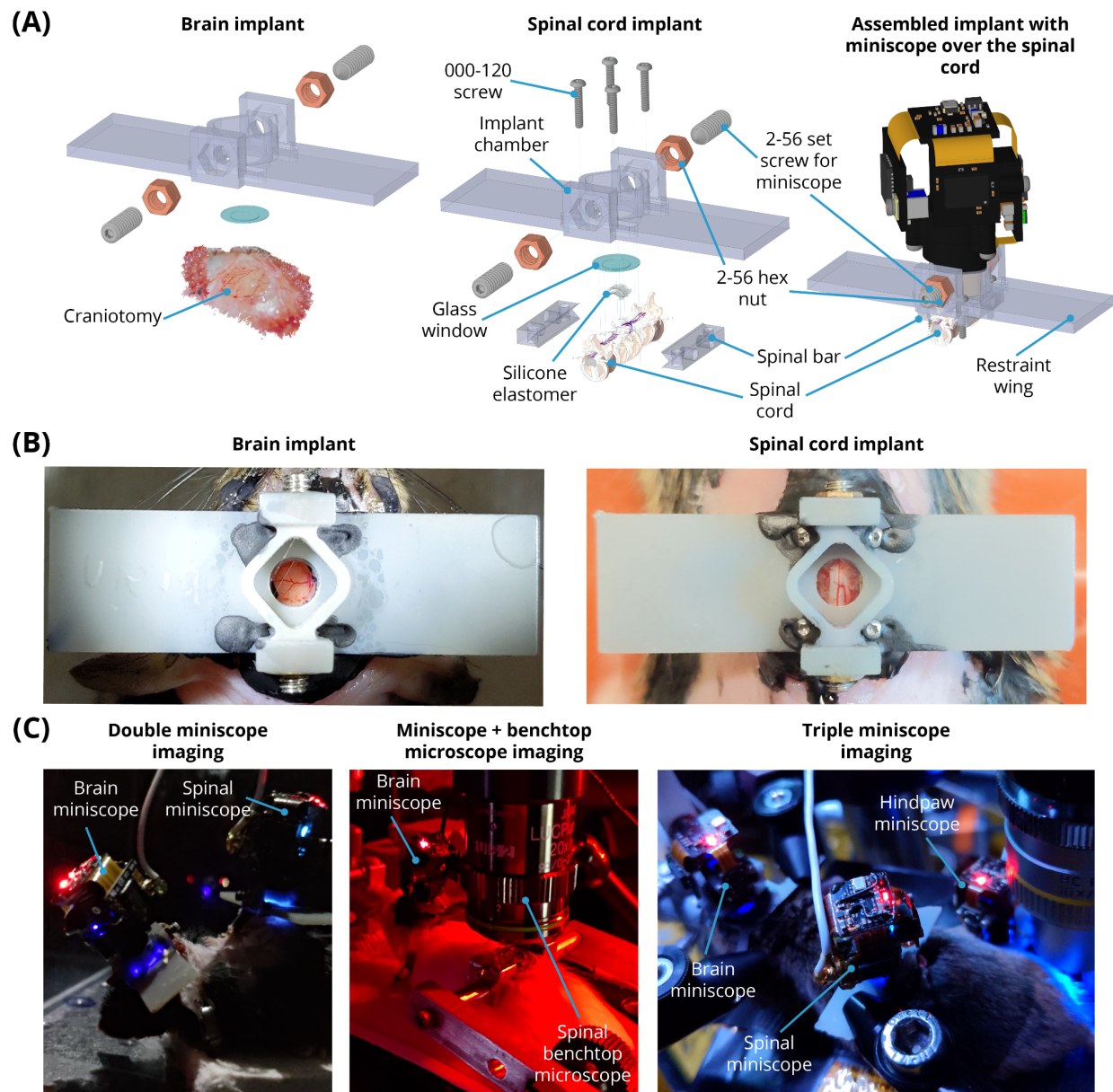
In this report we describe the surgical, imaging and stimulation methodology and tools needed to overcome the hurdles of imaging the brain and spinal cord in sequence in the same animal across months and simultaneously in a single experiment. The examples of the heat receptor (*Trpv1*-expressing) and mechanoreceptor (*Cacna1h*-expressing) activation driven responses, as well as multi-site perfusion examples demonstrate the approach of how described methodology can be applied to study the sensory information processes and drug bioavailability. We preview the future frontiers of integrated full-body studies by demonstrating the feasibility of imaging the perfusion simultaneously throughout three parts of the body with the individual blood vessel anatomical resolution.

## 2.5 Results

### 2.5.1 Low-cost 3D-printed cranial and vertebral implants enable chronic imaging using 1- and 2-photon microscopy and freely behaving animal imaging using miniscopes

A key goal of my technological Thesis is to allow multiple kinds of data acquisition in the same mouse. **To this end, I developed approaches for simultaneous window implantation for imaging and optical control in the brain and spinal cord. Further, my implants can accommodate both restrained behavior imaging using fixed ‘benchtop’ 1- and 2-photon microscopy ([Fig. 12C](#), middle and right) and free behavior with miniscopes ([Fig. 12C](#), left). In addition, this implant can be used both in the brain and spinal cord making it universal for studying different parts of the central nervous system (CNS) ([Fig. 12B](#)).**

As this Thesis goes to press (October 2022), the cost of outsourced high resolution stereolithographic 3D-printing from Accura 60 resin with quick clean finish is ~\$76/implant ([www.3dsystems.com](http://www.3dsystems.com)). Further, 3D-printing cost becomes negligible when using in-house 3D-printing. Machined spinal cord implants cost on the order of \$349/implant for the individual implants or \$171/implant when ordering in bulk ([Neurotar Inc.](#)). Further, our implant chambers are the first that allow imaging both in freely behaving animals with miniscopes and under restraint. Commonly available miniscope baseplates cost  $\geq$ \$32/implant ([miniscopeparts.com](http://miniscopeparts.com)), but do not allow animal restraint and cannot be used for chronic spinal cord imaging.



**Figure 12.** 3D-printed cranial and vertebral implants allow multi-site imaging of restrained and freely behaving animals using benchtop microscopy and miniscopes. **(A)** 3D-design rendering of the brain-spinal cord implant showing the exploded views of the implants for the brain and spinal cord, as well as an assembled spinal cord implant with the mounted UCLA miniscope v4.4. **(B)** A brain implant positioned over the primary somatosensory cortex (left) and a spinal cord implant clamped over Th13-L2 vertebrae (right). **(C)** Application of two implants in the same awake animal for chronic imaging using two miniscopes (left), application of the miniscope for the brain and benchtop microscope for the spinal cord imaging of an anesthetized animal (middle), and application of 2 implants and 3 miniscopes for the concurrent perfusion imaging of the brain, spinal cord and the hindpaw under anesthesia.

Our implants allow long-term functional imaging with comparable impact on animal's health as state-of-the-art implants. Qualitatively we have not observed any differences in animal behavior or gross tissue impact compared to our use of established spinal cord implants machined from stainless steel originally developed by Farrar et al. 2012 [33] and the stainless steel headposts used typically in 2-photon imaging [59]. We were able to image calcium responses of individual neurons in the spinal cord for up to 37 days ([Fig. 16A](#)) and dynamic 'field' 1-photon responses for up to 154 days ([Fig. 16B](#)). The dominant form of spinal cord implant failure was overgrowth of the window ([Fig. 13](#)). **For ease of reference, we refer to our Novel 3D Printed Common format implant as the NPC.**



**Figure 13.** Example anatomical changes of the spinal cord observed through the spinal cord window across day 1, day 3 and day 39 after the spinal cord window implantation.

### 2.5.2 Animals tolerate multi-site surgeries well

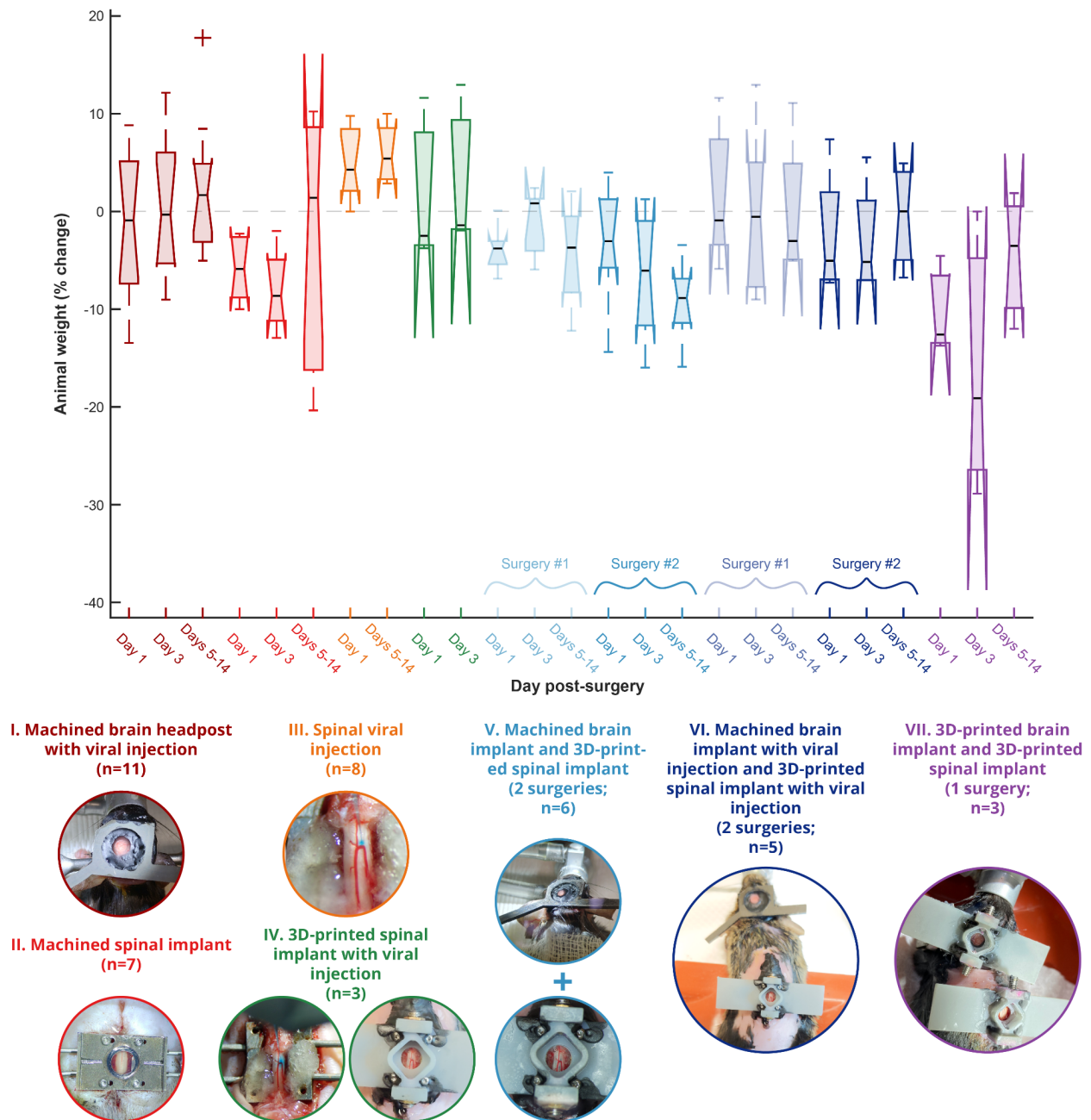
All implant iterations and surgical combinations tested during this work showed comparable impact on animal's health based on qualitative tracking of animal weights and qualitative observations of behavior. The leading implants and surgical combinations tested during this work included 7 different preparations and are summarized in [Fig. 14](#). Cumulatively this analysis included 41 animals. The multiple

one-way ANOVA comparisons using Bonferroni method included the comparison of 300 surgical preparation pairs. For all comparison groups it was hypothesized that different surgical preparations will not result in significant animal weight changes. This analysis identified no significant differences between any of the compared groups when using a significant level adjusted for multiple comparisons (i.e.  $p = 0.05/300 = 0.00017$ ). The surgical preparation groups and the p-values involving corresponding groups were as follows:

- I. Implantation of a machined brain headpost with cortical viral injections (the multiple comparison involving this group included 69 comparisons; 65 comparisons resulted in  $p=1$ ; comparison of group I day 1 and group VII day 3 has  $p=0.642$ , comparison of group I day 3 and group VII day 3 has  $p=0.121$ , comparison of group I days 5-14 and group V days 5-14 has  $p=0.901$ , comparison of group I days 5-14 and group VII days 5-14 has  $p=0.034$ );
- II. Implantation of machined spinal cord implant (design described in [33]; the multiple comparison involving this group included 69 comparisons; 68 comparisons resulted in  $p=1$ ; comparison of group II day 3 and group III day 1 has  $p=0.457$ );
- III. Spinal cord viral injection without implants (the multiple comparison involving this group included 47 comparisons; 40 comparisons resulted in  $p=1$ ; comparison of group III day 1 and group II day 3 has  $p=0.457$ ; comparison of group III day 1 and group V days 5-14 has  $p=0.168$ ; comparison of group III day 1 and group VII day 1 has  $p=0.507$ );

- comparison of group III day 1 and group VII days 3 has  $p=0.007$ ;  
 comparison of group III days 5-14 and group V days 5-14 has  $p=0.487$ ;  
 comparison of group III days 5-14 and group VII days 1 has  $p=0.890$ ;  
 comparison of group III days 5-14 and group VII days 3 has  $p=0.023$ );
- IV. Spinal cord 3D-printed implant with viral injection (the multiple comparison involving this group included 47 comparisons; 45 comparisons resulted in  $p=1$ ; comparison of group IV day 1 and group VII day 3 has  $p=0.695$ ; comparison of group IV day 3 and group VII day 3 has  $p=0.315$ );
- V. Implantation of machined brain implant (surgery #1) followed by implantation of 3D-printed spinal cord implant (surgery #2) (the multiple comparison involving this group included 129 comparisons; 126 comparisons resulted in  $p=1$ ; comparison of group V surgery #2 days 5-14 and group I days 5-14 has  $p=0.900$ ; comparison of group V surgery #2 days 5-14 and group III day 1 has  $p=0.168$ ; comparison of group V surgery #2 days 5-14 and group III days 5-14 has  $p=0.487$ );
- VI. Implantation of machined brain implant with viral injection (surgery #1) followed by implantation of 3D-printed spinal cord implant with viral injection (surgery #2) (the multiple comparison involving this group included 129 comparisons; 127 comparisons resulted in  $p=1$ ; comparison of group VI surgery #1 days 1 and group VII days 3 has  $p=0.231$ ; comparison of group VI surgery #1 days 3 and group VII days 3 has  $p=0.791$ );

VII. Implantation of brain implant and 3D-printed spinal cord implant within a single surgical procedure (the multiple comparison involving this group included 69 comparisons; 58 comparisons resulted in  $p=1$ ; all the  $p$ -values smaller than 1 were listed as part of the comparisons involving all the previous groups).



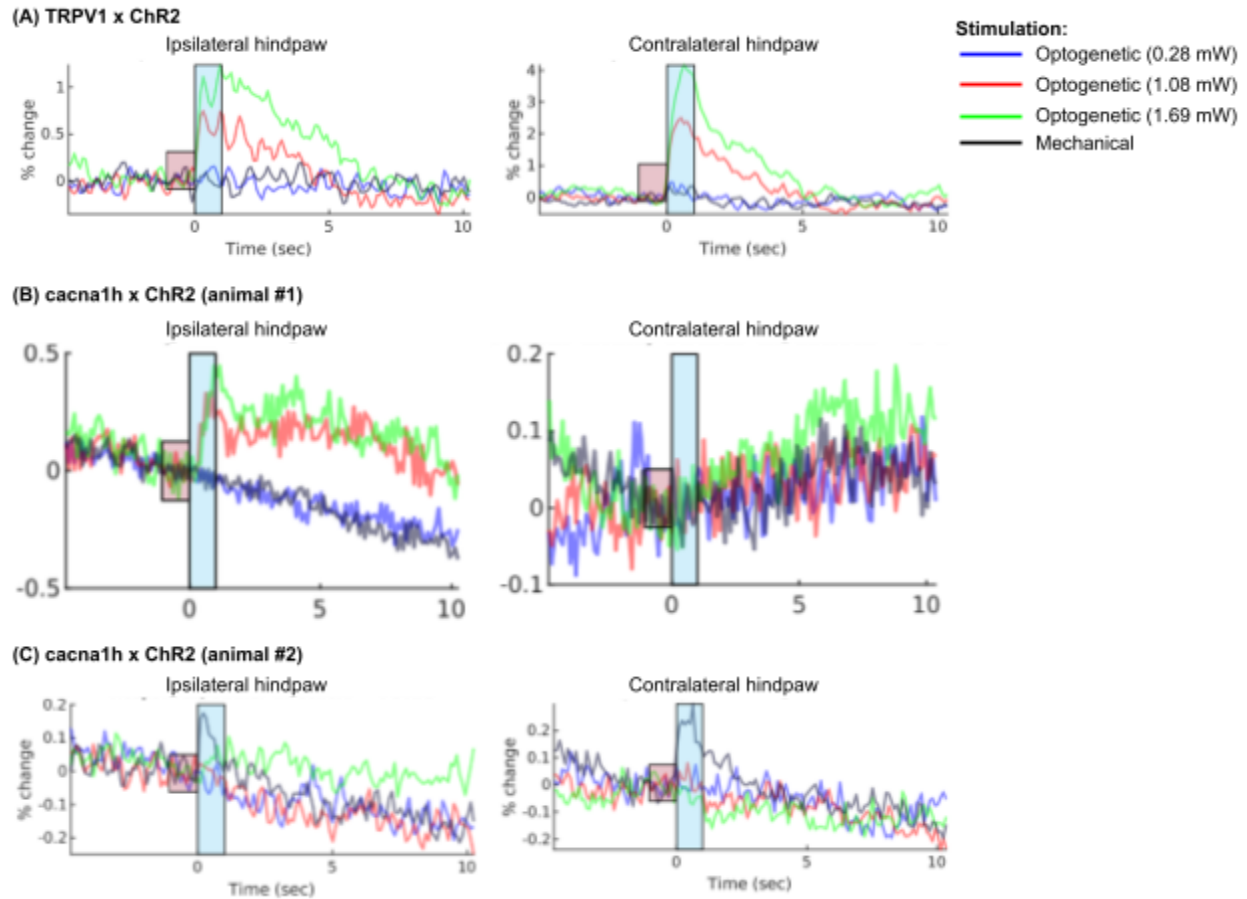
**Figure 14.** Animals tolerate comparably well the standard stainless-steel cranial and spinal implants, our ‘NPC’ 3D-printed implant surgeries, and multiple surgeries combining these strategies. The top box plot summarizes the animal weight changes on post-surgical day 1, day 3 and the average weights throughout days 5-14 for different surgical preparations. The photo-based legend at the bottom explains the depicted surgical preparations by color-coding each surgical group. Multiple one-way ANOVA comparisons using Bonferroni method found no significant differences between any surgical groups. n = number of animals in each surgical group.

### 2.5.3 Response evoked by mechanical stimulation and optogenetic activation of thermo-chemical nociception receptors (*Trpv1*-expressing) and low-threshold mechanoreceptors (*Cacna1h*-expressing) can be reliably imaged in neocortex

Mechanical stimulation and optogenetically evoked brain responses can be chronically imaged in the brain of *Trpv1*<sup>Chr2EYFP+/-</sup> and *Cacna1h*<sup>Chr2EYFP+/-</sup> transgenic animals as far as 203 days following brain viral injections with headpost implantation. [Fig. 15](#) demonstrates cortical responses for left and right hindpaw stimulation with 4 different types of stimulation in 3 animals. Contralateral 1.69 mW stimulation of hindpaw in *Trpv1*<sup>Chr2EYFP+/-</sup> ([Fig. 15A](#)) yielded strongest responses >4% change above the baseline, albeit *Trpv1*<sup>Chr2EYFP+/-</sup> animal showed >1% responses to ipsilateral stimulation as well. The responses to ipsilateral 1.69 mW stimulation in *Trpv1*<sup>Chr2EYFP+/-</sup> were sustained longer by ~1 s compared to the contralateral stimulation, and in both cases the responses were sustained for ≥5 s. The responses to optogenetic activation of C- and Aδ-LTMRs [205] in *Cacna1h*<sup>Chr2EYFP+/-</sup> ([Fig. 15B](#)) were observed reliably in response to the ipsilateral stimulation in one of the two animals. The response had bi-phasic nature with more immediate response around 0.5% change followed by an immediate suppression after the end of stimulation with subsequent sustained activation for the



remainder of the trial. In both cases of *Trpv1*<sup>Chr2EYFP+/-</sup> and *Cacna1h*<sup>Chr2EYFP+/-</sup>, the animals responded reliably to the strongest (1.69 mW) and medium (1.08 mW) stimulation, and only *Trpv1*<sup>Chr2EYFP+/-</sup> showed responses on the order of 0.2% to the lowest (0.28 mW) amplitude contralateral stimulation. The second *Cacna1h* x ChR2 animal (Fig. 15C) showed reliable responses to mechanical stimulation on the order of 0.2% change when stimulating either side, albeit the responses to the contralateral stimulation were stronger in magnitude and were sustained outside of the stimulation interval in contrast to the ipsilateral responses.



**Figure 15.** Optogenetic and mechanical stimulation evoked brain responses in *Trpv1*<sup>Chr2EYFP+/-</sup> and *Cacna1h*<sup>Chr2EYFP+/-</sup> can be reliably imaged under anesthesia for over 6 months using benchtop 1-photon electron multiplying CCD (emCCD) camera. **(A)** Examples of the left and right hindpaw plantar stimulation responses 39 days after the brain surgery in *Trpv1*<sup>Chr2EYFP+/-</sup>

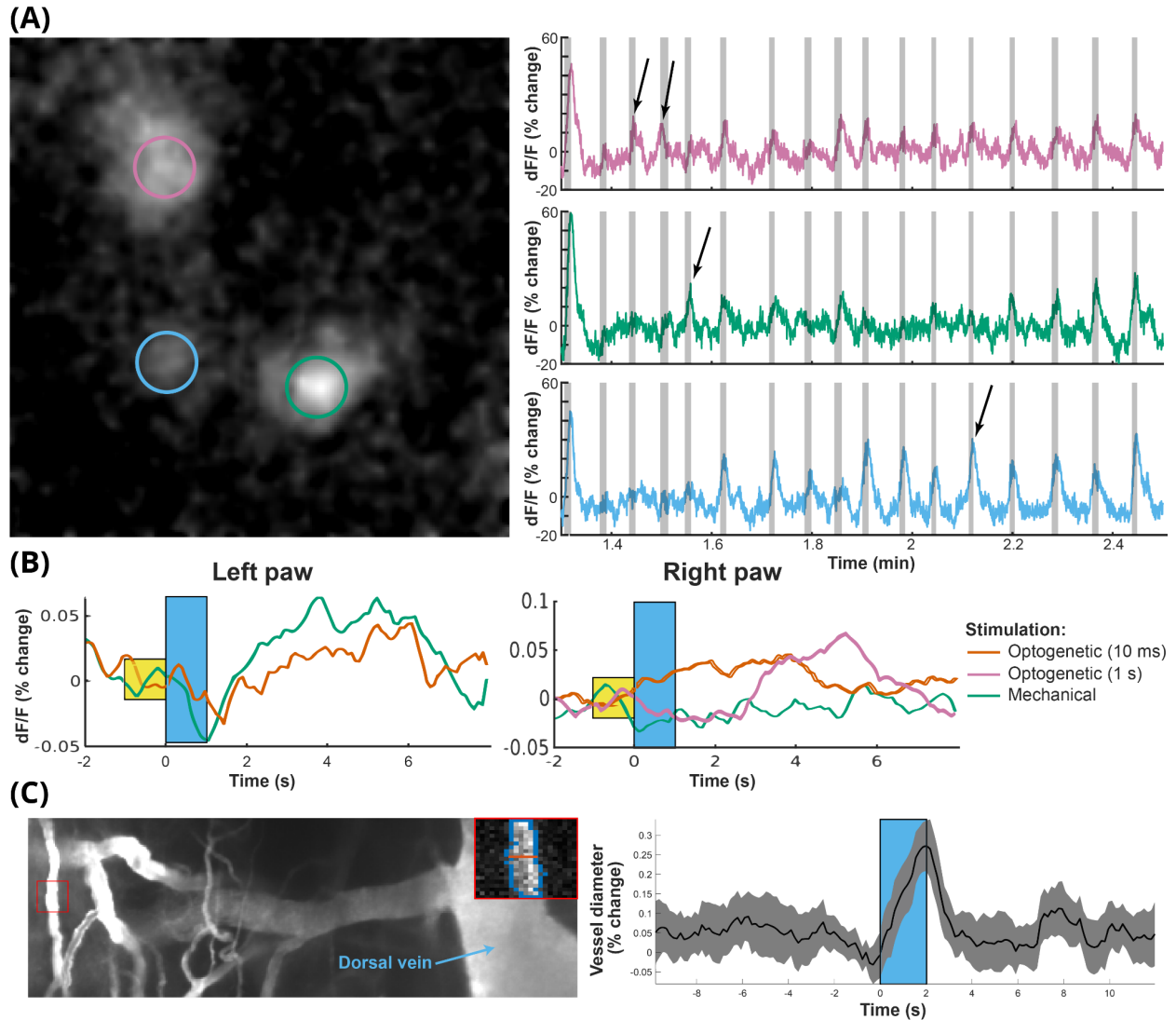
animal (n=1; left hindpaw: 96 trials of 0.28 mW opto, 93 trials of 1.08 mW opto, 81 trials of 1.69 mW opto, 84 trials of mechanical stimulation; right hindpaw: 67 trials of 0.28 mW opto, 77 trials of 1.08 mW opto, 80 trials of 1.69 mW opto, 78 trials of mechanical stimulation). **(B)** Examples of the left and right hindpaw plantar stimulation responses 199 days after the brain surgery in *Cacna1h*<sup>Chr2EYFP+/-</sup> animal (n=1; left hindpaw: 58 trials of 0.28 mW opto, 20 trials of 1.08 mW opto, 15 trials of 1.69 mW opto, 53 trials of mechanical stimulation; right hindpaw: 16 trials of 0.28 mW opto, 17 trials of 1.08 mW opto, 22 trials of 1.69 mW opto, 27 trials of mechanical stimulation). **(C)** Examples of the left and right hindpaw plantar stimulation responses 203 days after the brain surgery in *Cacna1h*<sup>Chr2EYFP+/-</sup> animal (n=1; left hindpaw: 74 trials of 0.28 mW opto, 82 trials of 1.08 mW opto, 79 trials of 1.69 mW opto, 73 trials of mechanical stimulation; right hindpaw: 59 trials of 0.28 mW opto, 81 trials of 1.08 mW opto, 75 trials of 1.69 mW opto, 67 trials of mechanical stimulation). The inlets in every panel show the examples of individual trial scatter plots. All 3 animals had cranial windows around the S1hl area in the left hemisphere (i.e. right paw = contralateral, left paw = ipsilateral), and the data for both right and left hindpaw plantar stimulation were plotted for the same region-of-interest in each animal.

#### 2.5.4 Responses to the optogenetic activation of low-threshold mechanoreceptors (*Cacna1h*-expressing), mechanical stimulation, pinching and blue light evoked vessel dilations can be reliably imaged in the spinal cord

We validated our novel spinal cord implant and surgical procedure by demonstrating the ability to chronically image the pinching, touching and optogenetically-driven calcium responses, and endogenous light sensitivity driven blood vessel dilations. The individual cell responses to hindpaw pinching could be reliably imaged 37 days post-implantation using UCLA 1-photon miniscope ([Fig. 14A](#)). The spatial spinal cord map in [Fig. 14A](#) highlights 3 neurons which responded differently to different pinches. The block arrows in the plot indicate the examples of responses which were present in one of the cells and were absent in other cells. Beyond pinching, we were able to measure field responses to repetitive mechanical poking and optogenetic stimulation of the plantar hindpaw in a transgenic *Cacna1h*<sup>Chr2EYFP+/-</sup> mouse using

emCCD 1-photon camera 154 days after the spinal cord surgery ([Fig. 16B](#)). The optogenetic stimulation using a single 10 ms long pulse of blue (470 nm) light reliably evoked responses within the same ROI in S1-hindlimb (S1hl) of the left hemisphere when stimulating either the left or right plantar hindpaw. On the contrary, mechanical poking stimulation evoked responses comparable to the *Cacna1h* optogenetic responses when stimulating on the ipsilateral side (i.e. left paw), but evoked only mild putative depolarization when stimulating on the contralateral side.

The multi-modal nature of our preparation also allowed us to image the blue light (470 nm) induced vessel dilations imaged in wild-type animal using 2-photon microscopy 24 days after the spinal cord surgery ([Fig. 16C](#)). Within the 2-photon vascular map shown in [Fig. 16C](#) all the segments of the blood vessel that could be segmented reliably showed responses to the blue light. We show an example of the vessel diameter change tracked from the ROI highlighted in red in the vascular map ([Fig. 16C](#)).

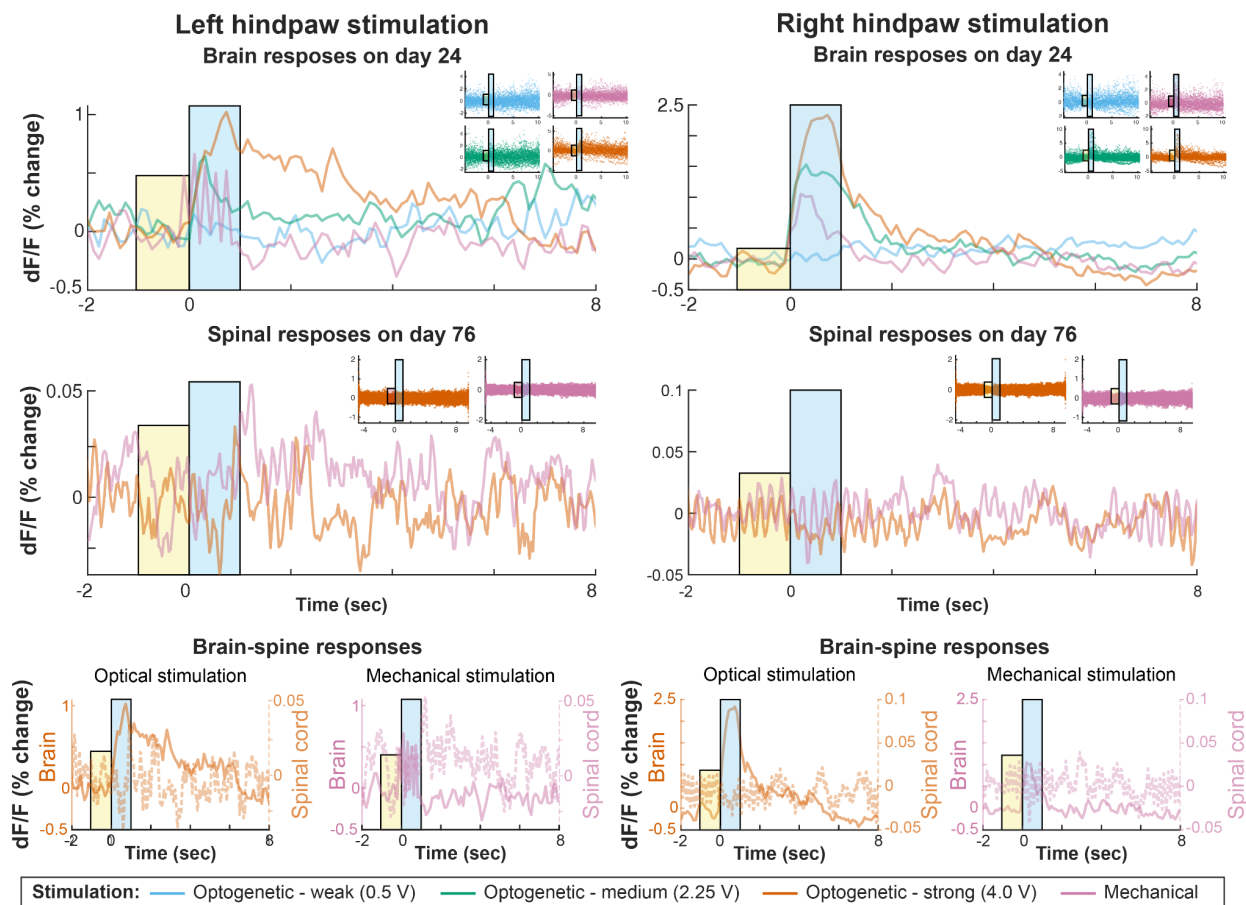


**Figure 16.** 3D-printed spinal cord implant enables the chronic imaging of pinch, optogenetic, mechanical stimulation and endogenous light sensitivity responses using open-source miniscopes, benchtop 1-photon and 2-photon microscopy. **(A)** contains the examples of 1-photon calcium responses from 3 individual neurons to pinching. Responses were imaged under isoflurane 37 days post-implantation using miniscope v4.0. Gray shaded areas indicate pinching time intervals. **(B)** shows gross spinal cord calcium responses to the optogenetic (1 s and 10 ms) stimulation of low-threshold mechanoreceptors and mechanical stimulation of the right and left plantar hindpaws in a *Cacna1h*<sup>Chr2EYFP+/-</sup> transgenic animal. Blue shaded areas indicate stimulation intervals and yellow shaded areas show baseline intervals for each individual trial. Responses were imaged under isoflurane 154 days post-implantation using an emCCD camera. **(C)** shows an example of the endogenous light sensitivity of the spinal cord blood vessels to 470 nm light. The red box on the image to the right shows the location of the responsive blood vessel. The inset on the top right of the image shows a single frame with segmented blood vessel. Responses were imaged under isoflurane 24 days post-implantation

using a 2-photon microscope. Application of the pulses of blue light on average (31 stimulation trials) causes a significant increase in blood vessel diameter. Each panel shows independent individual animals (i.e. 3 animals total). Panel (A) shows individual non-average responses. Panels (B) and (C) show averaged responses. The numbers of trials for different stimulation conditions ranged between 30-60 trials.  $dF/F$  shows the % change from baseline.

### 2.5.5 Separate brain and spinal cord surgeries in the same animal are well-tolerated, and allow sequential and simultaneous long-term imaging of neural dynamics and vascular perfusion in awake and anesthetized animals

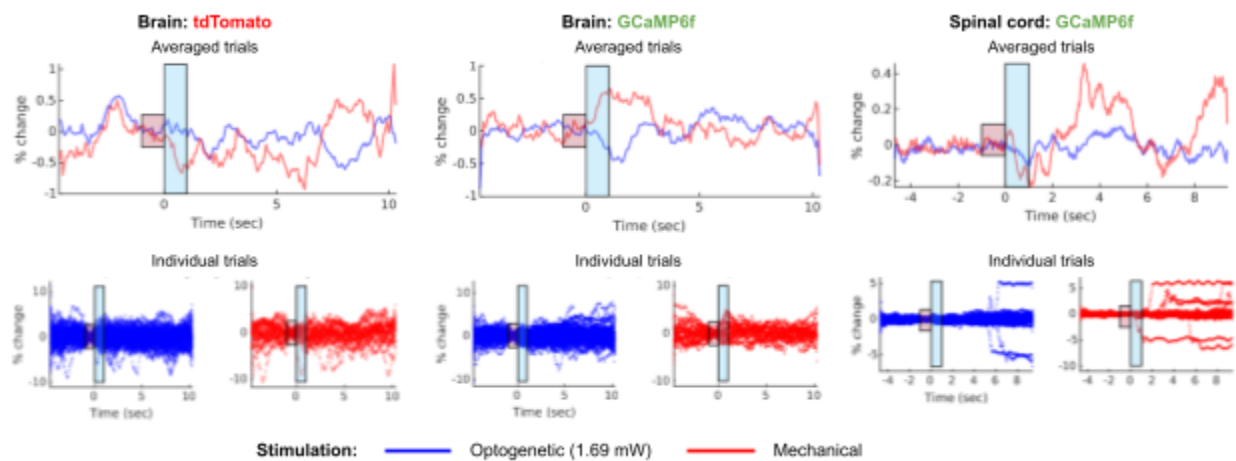
Our custom 3D-printed common-format implants allow imaging of calcium dynamics in the brain and spinal cord sequentially under anesthesia ([Fig. 17](#)) and simultaneously in awake animals ([Fig. 18](#)) to study sensory information processing throughout the central nervous system. Nociceptive response imaging in *Trpv1 x ChR2* animal was first performed in the brain 24 days after the brain surgery and was followed up with the spinal cord surgery that allowed sequentially to image the spinal cord responses 76 days after the spinal cord surgery ([Fig. 17](#)). Plantar optical stimulation of the right hindpaw reliably evoked cortical responses to all stimuli except for the weakest stimulation (i.e. 0.28 mW). Ipsilateral plantar stimulation of the left hindpaw also evoked brain responses, but of smaller (1% change vs. 2.5% change for the strongest optical stimulus) and more sustained magnitude compared to the contralateral stimulation. On the contrary, the spinal cord showed putative responses to mechanical plantar stimulation of the left hindpaw and no obvious responses to the right hindpaw plantar stimulation.



**Figure 17.** Custom 3D-printed implants in combination with multi-site surgeries allow sequential long-term imaging of the brain using an emCCD and spinal cord using miniscope in *Trpv1*<sup>Chr2EYFP+/-</sup> animal under anesthesia. Top row shows brain responses on day 24 after brain surgery for left and right hindpaw stimulation, middle row shows spinal cord responses in the same animal on day 76 after spinal cord surgery in the same animal, and bottom row shows the same plots separated by types of stimuli with brain-spinal cord data overlaid together for each type of stimulation. The inlets in the top right corners show the scatter plots of all the individual trials. All the plots are trial averages for a single animal. dF/F shows the % change from baseline.

We also demonstrated the chronicity, multi-modality and compatibility of our developed technique with awake behavior by co-registering the activity in the brain and spinal cord 230 days after the brain surgery and 48 days after the spinal cord surgery in a *Cacna1h*<sup>Chr2EYFP+/-</sup> animal (Fig. 18). Brain 2-photon imaging included co-registration of the red and green fluorescent channels. Red channel in this case included the labeling

of blood vessels and *CaMKIIa* nuclei, and hence was calcium independent. Green channel for the brain and the spinal cord show the responses from *GCaMP6f*. The optogenetic stimulation evoked hyperpolarization both in the brain and spinal cord. Mechanical stimulation on the contrary evoked depolarization at both foci, but in the spinal cord it first evoked hyperpolarization similar to the optogenetic stimulation followed by depolarization. Within the time interval of spinal cord depolarization, brain activity also exhibited a small (0.25% change for the brain and 0.4% change for the spinal cord) depolarization response.



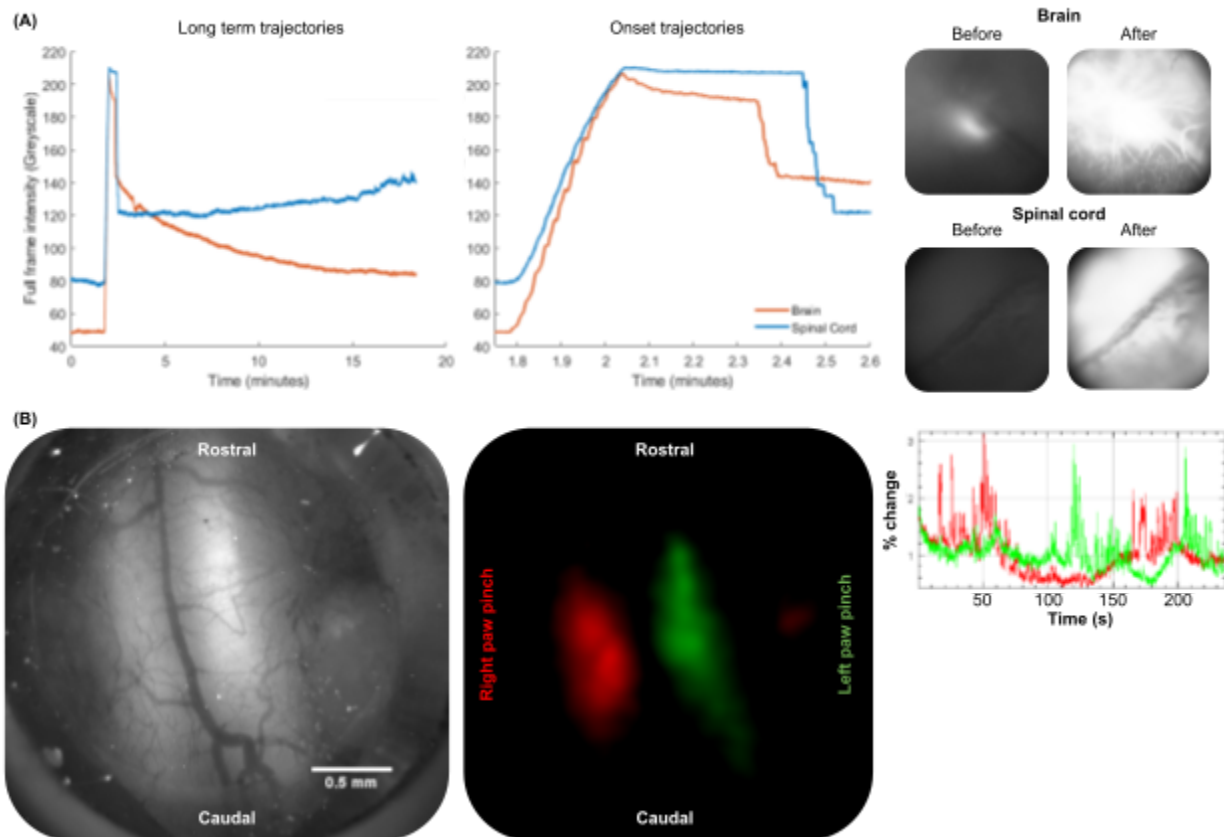
**Figure 18.** Simultaneous brain-spinal cord responses were imaged in awake head-restrained *Cacna1h*<sup>Chr2EYFP+/-</sup> animals using benchtop 2-photon microscopy of the brain and 1-photon miniscope microscopy of the spinal cord. Imaging was performed 230 days after the brain surgery and 48 days after the spinal cord surgery while stimulating the right plantar hindpaw contralateral to the craniotomy. The plots show the trial-averaged and individual trial data. Y-axes show the % change from baseline.

## 2.5.6 Single brain-spinal cord implant surgery allows chronic imaging of pinch responses in transgenic animals

Single brain-spinal cord surgery allows chronic imaging of the brain-spinal cord perfusion up to 9 days post-surgery ([Fig. 17A](#)) and imaging of the transgenic calcium



indicator responses for up to 15 days post-surgery ([Fig. 19B](#)). [Fig. 19A](#) shows a faster rise time for fluorescence following the tail vein injection of FITC dextran. The decrease in signal intensity after 2.3 minutes was associated with the manual decrease of excitation intensity due to the saturation of miniscopes. Field calcium responses of transgenically labeled somatostatin (*SOM*) interneurons showed clear spinal cord somatotopic dependence on the side of stimulation.



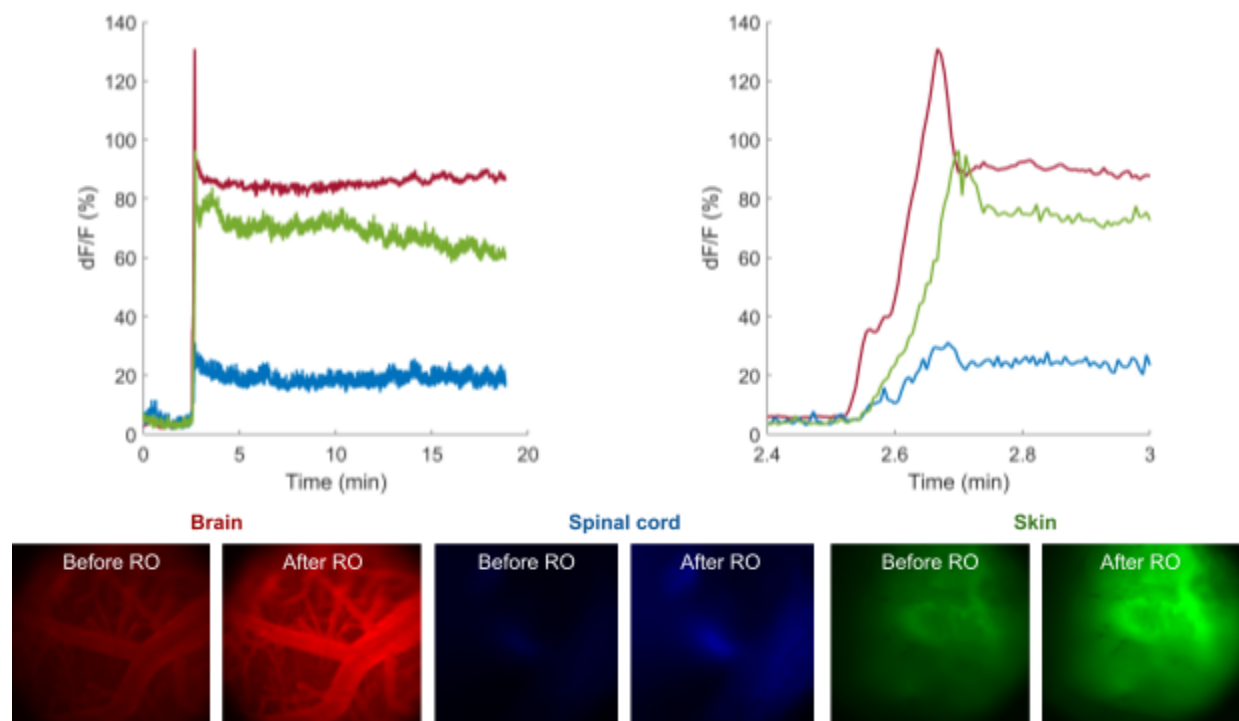
**Figure 19.** Two common format 3D-printed implants can be implanted across the brain-spinal cord within a single surgery to enable chronic perfusion and calcium imaging of sensory responses in a transgenic animal under anesthesia. **(A)** We can simultaneously measure the perfusion time courses for the brain and spinal cord following a tail vein administration of fluorescent dextran 9 days after the surgery. Two plots show the same dataset for different X-axis spans. The spatial maps to the right show the spatial changes in brightness before and after the injection imaged using two miniscopes. **(B)** 15 days after brain-spinal cord co-implantation we can somatotopically map the pinching calcium responses in the spinal cord via transgenic *GCaMP6s*. The left image shows the average *GFP* stack imaged using a benchtop CCD camera. The middle image shows the spatial spinal cord map indicating the



average active areas when pinching the right hindpaw (red) and left hindpaw (green). All the results in this figure are from n=1 *SOM x GCaMP6s x tdTom* transgenic mouse.

### 2.5.7 Miniscopes offer plenty of room for further scaling up: simultaneous imaging of perfusion in the brain, spinal cord and hindlimb

Retro orbital injection of dextran allows simultaneous perfusion imaging in the brain, spinal cord and the skin of a hindpaw using 3 miniscopes. All 3 imaging sites showed a quick immediate peak response followed by a rapid decline towards the new baseline. Similarly to the case in [Fig. 20A](#), cortical signal intensity showed the fastest rise time with the spinal cord showing the slowest rise time dynamics. The same animal was injected with dextran 4 times (data not shown) and in all cases a clear rapid change in brightness was observed similarly to the results in [Fig. 19A](#).



**Figure 20.** Miniscopes in combination with custom 3D-printed implants and multi-site surgery enable 3-site simultaneous imaging of perfusion across the brain, spinal cord and the skin of a hindpaw. In this example the dextran was administered via retro orbital (RO) injection.

## 2.6 Discussion

The hardware and surgical advances presented here were used for observing field calcium dynamics and vascular perfusion of fluorescent molecules from up to 3 sites within the body for over 8 months after the first surgery ([Fig. 12](#)). The novel design we developed provides a well-tolerated, affordable and common-format approach for imaging brain and spinal cord, and imaging in fixed and free-behaving mice. As such, this approach can enable unique observations under conditions where each site is imaged individually but in the same mouse and on the same day, such as the time course of plastic changes in neural ensembles following a peripheral manipulation. The approach can also enable unique correlational studies with simultaneous imaging across sites, for example, determining which conjoint aspects of spinal cord and brain activity indicate nocifensive responses or detection of nociceptive stimuli [126].

For imaging individual cells across multiple sites, the main limiting factor was the longevity of the spinal cord window due to overgrowth ([Fig. 13](#)). Our maximal duration for obtaining quality signals was 37 days after the spinal cord surgery. This finding aligns well with the previous observation using the stainless steel implants that allowed resolving spinal cord axons for more than 35 days in only ~50% of the implanted mice due to fibrosis [33]. Good surgical technique remains the best tool to increase the longevity of the spinal cord windows. The key surgical factor to prioritize is to minimize the injury and bleeding throughout the surgery. The rest of the important factors all ultimately lead to the reduced insult to the spinal cord. These factors include: (i) good seal of all the bone edges throughout the laminectomy using a tissue glue (e.g. VetBond), (ii) minimizing the use of the elastomer (e.g. KwikSil; in the implant design

presented in this work this was achieved by positioning the glass window under the implant chamber akin to the traditional brain implant designs), (iii) increasing the pressure-seal between the glass window and the spinal cord (akin to the traditional brain implant designs); (iv) using the pneumatic drill rather than a mechanical drill or the scissors. Alternatively, the surgical invasiveness can be reduced by imaging between the vertebrae allowing to prolong the chronicity of spinal cord imaging for up to 167 days as has been reported right before this thesis went into press [43]. Presumably, the duration of single-cell resolved imaging might be increased further by treating an implant with gradually releasing antiinflammatory agents to suppress fibrosis [246]. Besides the surgical preparation, when it comes to awake behavior, an equally important factor in maintaining spinal cord window quality is proper habituation of the animal. As discussed in the introduction, high mobility of the spinal cord makes it more prone to injury. Even in the case of miniscope imaging in freely behaving animals, short-term (~2-3 minutes) restraint is necessary to attach the miniscope. In our experience even 2-3 minutes of restraint is enough to significantly disturb the quality of the spinal cord window in a poorly habituated mouse. Hence, extra care should be taken to habituate the animal to restrain and handling. An alternative is to anesthetize the animal to allow simplified attachment of the miniscope. In our experience, attaching the miniscope under anesthesia works well for imaging in unrestrained animals, but cannot be easily applied to restrain the animals because animal awakening can be associated with sudden and forceful movements that can damage the implant. However, the caveat of using the anesthesia is that even in short durations and in the absence of accidents, every cycle of anesthetic such as isoflurane has a negative impact on

animal's health and will ultimately reduce the feasible duration of the study [247]. The overarching timeline describing different major procedures and time intervals for each procedure as was implemented for the final awake behavior experiments in this study are summarized in [Fig. 22](#).

Imaging the perfusion in the skin in concert with the BASC imaging reported here was an accessible milestone and did not require additional surgeries. However, it would be of great value to be able to image other parts of the body together with the BASC such as the gastrointestinal system [9] and dorsal root ganglia [38]. In the case of the peripheral sensory stimulation of the skin, the stimuli modulate the functional activity of the skin nerve endings [79], dorsal root ganglia [38], spinal dorsal horn [37], [42] and the primary somatosensory cortex [12], [248]. From the microscopy hardware perspective all four of the above sites can be readily imaged simultaneously using the set of imaging tools described in this study. We are optimistic this goal can be achieved with the development of the right animal model and use of breeding strategies to drive genetic indicators in all four imaging sites, and with quantitative progress optimizing the speed and quality of the surgical preparation. This form of imaging throughout the body would allow us to expand even further our understanding of how interrelated are the millisecond and second time-scale dynamics throughout different parts of the body? What happens to other parts when we modify the dynamics in one of the parts (e.g. time course of the effects of changes in microbiota on the activity in the brain and spinal cord)? Are there any new therapies that can benefit from closed-loop monitoring of one part of the body and modulating the other part of the body (e.g. applying spinal cord stimulation for pain only when BASC reaches a specific state)? Furthermore, one of the

recent studies offered a glimpse into a time course of the activation of the skin nerve endings in response to capsaicin [79]. However, the open question remains about the changes in the activity happening in the dorsal root ganglia, spinal cord and the brain in parallel with the dynamic changes happening in the hindpaw. This question can now be addressed by building up atop of the methodology developed here.

Similarly to the spinal cord implant design described in [245], because of the use of plastic for the fabrication of the implant chamber, it is more compatible with photoacoustic, ultrasound and MRI imaging, as well as ultrasound stimulation and radiation therapy. Non-optical methods have not been tested as part of this development process and additional modifications might be needed to avoid the artifacts from the screws and the nuts. However, for the non-miniscope imaging the set-screws are not necessary and the plastics tested as part of our work can be threaded without the use of glued nuts. The important factor to keep in mind when threading the plastics reported here is that the threads are more fragile than those in the nuts. In our hands the tapped threads would serve reliably on average for 2-3 screw-unscrew cycles. 000-120 screws can potentially be replaced with the glue, but this would require additional testing and has not been validated as part of our work.

This report serves as a guide for the scientific community for making a leap away from treating an animal's body but a tiny focal cluster of tissue as a black box towards the next big scientific frontier of multi-site multi-organ imaging and manipulation throughout an animal's body.

## 2.7 Methods

### 2.7.1 Animal subjects

All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Brown University. Mouse strains used in this study were purchased from the Jackson Laboratory and included: *C57BL/6* wild-type mice, *SOMcre x tdTomato x Ai148* (*TIGRE-Ins-TRE2-LSL-GCaMP6f*; JAX# 030328), *Trpv1*<sup>Chr2EYFP+/-</sup> (*Trpv1-Cre*<sup>+/+</sup> is JAX#: 017769 and *lox-STOP-lox-ChR2-EYFP* is JAX#: 012569), *Cacna1h*<sup>Chr2EYFP+/-</sup> (*Cacna1h*<sup>Chr2EYFP+/-</sup> line were developed in the Lipscombe laboratory by mating *Cacna1h-Cre*<sup>+/+</sup> with *ChR2-EYFP*<sup>+/+</sup>; *Cacna1h-Cre*<sup>+/+</sup> is also known as *CaV3.2-Cre*<sup>+/+</sup>; the *Cacna1h-Cre*<sup>+/+</sup> mice used for the this thesis were inbred 13 times), *Tie2 x GFP* (*Tg(TIE2GFP)287Sato/J*; JAX#: 003658). Mice were group-housed until the implant surgery and individually housed after the surgery. Mice were provided with bedding and nesting materials, and maintained on a 12 hour light-dark cycle in a temperature and humidity controlled environment.

### 2.7.2 Implant fabrication

All the implants tested for this work were 3D-printed via stereolithography (SLA). The 3D-printing photopolymer resins tested in this work included: (i) Accura Xtreme White 200, (ii) Accura SL 7820 in black color, (iii) Accura Xtreme in gray color, (iv) Accura 60, (v) Accura ClearVue, (vi) Dental SG (Formlabs, FLDGOR01), (vii) Tough 1500\_v1 (Formlabs, FLTO1501). We found both Dental SG and Tough 1500\_v1 to be well suited for fabricating the spinal bars and implant chambers ([Fig. 12](#)). Dental SG offers better biocompatibility than Tough 1500\_v1. However, based on the qualitative

observations, we did not observe any differences between two the implants from these resins in terms of post-surgical animal behavior and tissue quality around the implant. The important considerations to keep in mind is the relatively high translucency and brittleness of the implants made from Dental SG resin. Transparency can be important in the optogenetic studies where it might be undesirable to confound the experimental results because of the endogenous effects resulting from the light exposure outside of the implanted window. Brittleness in our hands was a concern only when trying to thread the implant chamber directly. In contrast with the Tough 1500\_v1, direct threading of Dental SG implants did not allow >1 screw-unscrew operation cycle. However, the issue of translucency can be addressed by painting the implant and the issue of brittleness can be resolved by gluing the commercially available nuts into the implant. All the results presented in this report were collected using the implants fabricated from Tough 1500\_v1 resin ([Fig. 12 B and C](#)).

The spinal cord bars and spinal cord chamber were fabricated from Tough 1500\_v1 resin using an in-house 3D-printer (Form 3, early 2019 edition, FormLabs Inc.). The fabrication steps were as follows:

1. 3D-print the parts,
2. wash the parts in isopropyl alcohol (by hand or using Form Wash washer from FormLabs Inc.),
3. perform only for the implant chamber: apply the UV-curing glue (electronic adhesive 123SBLUE, Norland Products Inc.) and insert the nuts (92736A101, McMaster-CARR Inc.),
4. cure (using Form Cure, FormLabs Inc.),

5. perform only for the implant chamber: after curing we usually apply additional small droplets of 5-minute epoxy (QUIK-CURE, bSi Inc.) at the interface between the nuts and the implant,
6. remove the supports,
7. sand off any residual marks left while removing the supports,
8. thread and test the fitting of all the cylindrical holes:
  - a. spinal cord bars:
    - i. hand-drill the cavities for the bar holders and make sure they fit well on the surgical bar holders;
    - ii. manually tap the vertical holes and test the fitting of the 000-120 screws (90910A603, McMaster-CARR Inc.);
  - b. implant chamber:
    - i. test the fitting of the 2-56  $\frac{1}{4}$ " set screws (90778A022, McMaster-CARR Inc.) into the glued nuts;
    - ii. test the smoothness of the passage of 000-120 screws through the lateral vertical cavities used for threading the screws through the chamber into the spinal cord bars;
    - iii. test the fit of the miniscope into the implant chamber;
9. autoclave before the surgery.



## 2.7.3 Surgical procedures

### 2.7.3.1 Surgeries for brain AAV injections and window headpost implantation

We performed brain injections of adeno associated virus (AAV) and window headpost implantations as parts of a single procedure. Depending on the specifics of AAV injections, this surgical procedure takes on average 3-4 hours plus 1-2 hours of preparation time. It is common for us to still be able to detect brain responses as late as 6 months (data not shown) after the surgical procedure described below.

All surgeries followed the aseptic techniques. We anesthetize the animal using 1.5-2% isoflurane delivered in 100% oxygen gas and eliminated using vacuum suction. We protect the animal's eyes using sterile ocular ointment and the animal's body temperature is maintained stable using a heating blanket. We prepare the surgical site on the skin in a dedicated area away from the surgical sterile field. We inject the animal with intraperitoneal (IP) dexamethasone (0.2 ug/g), subcutaneous (SQ) meloxicam SR (4 ug/g) and local SQ lidocaine (3 ug/g). We then cut off the longer hair from the top of the animal's head and apply a depilation cream (Nair hair-remover lotion, Church & Dwight Inc.) around the planned incision site. We massage the depilator cream into the hair and wash it off together with the detached hair using alcohol after 30 seconds. We then clean and sterilize the skin with a two-stage scrub of betadine and 70% ethanol, repeated three times. After cleaning and sanitizing the skin, we move the animal to the nearby surgical sterile site without interruption to the animal's plane of anesthesia. We then stabilize the animal's head using earbars. We use a 10 mm ring to pressure-mark the edges of the intended cut in the skin. We cut a circular piece of skin using scissors by following the previously marked trace. We then remove all the connective tissue

remaining on the skull and cut the muscle pockets on both lateral sides of the skull. This step is most prone to result in multiple muscle bleeders, especially during the early stages of learning this procedure. During this step and throughout the rest of the surgery we use surgical absorbent gelfoam (Surgifoam 1972, Ferrosan Medical Devices) to control and stop any bleeders. We prepare gelfoam at the beginning of the surgery by applying saline to it to make the gelfoam soft while not free-floating in excessive amount of saline. Throughout the entire surgery we use only the gelfoam softened by saline. Next, using the surgical blade, we make densely distributed surface scrapes in the skull at the top and on the sides of the muscle pockets, and then etch the same skull surfaces using the dentin activator (Universal Dentin Activator Liquid S393, C&B-Metabond®, Parkell) for 10 seconds followed by a wash off with saline. Scraping and etching are important steps to ensure reliable long-term bonding of the dental cement to the skull and attachment of the implant. We then mark bregma and mark the location of somatosensory cortex hind-limb area (S1HL, AP = 0.5 mm and ML = 1.5 mm) relative to bregma. We use the ear punch (3mm disposable biopsy punch, 19D23, INTEGRA Inc.) by gently scraping the surface of the skull to mark out a 3 mm ring for drilling a craniotomy centered on S1HL. We use a Neoburr drill bit (FG ¼, Microcopy) for the initial drilling of the skull through 50-75% of the skull's thickness. We drill through the rest of the skull in gradual pecking motion using a beveled cylinder drill bit (1812.8F, Microcopy). After completing every full drilling circle we apply saline to prevent overheating and flush away the bone chips. Once the craniotomy piece of the skull starts moving with brain pulsations, we remove this bone piece using forceps. We then clean the exposed brain from any blood, CSF and bone chips using a piece of gelfoam.

From this point in the surgery, we always make sure the brain never dries out and is moist with saline. We maintain a good level of moisture both by regularly pouring saline over the craniotomy and keeping the craniotomy covered with gelfoam for longer periods of time. We then take a sterile insulin syringe (1144303, 0.5mL 28G, BD Insulin Syringe, Belton, Dickinson and Co.) and bend the tip of 31G needle by gently tapping the needle against a flat hard surface. This serves as a hook tool for removing the dura. We use such a syringe with the hook to make 2-3 cuts in dura for the upcoming viral injections. We let the brain stabilize after the durotomies while preparing for the viral injections.

We pull glass pipettes (5-000-1010, DRUMMOND WIRETROL, Drummond Scientific Co.) using a pipette puller (Model P-97, Sutter Instrument Co.). We cut the tips using Vannas scissors under the microscope to the tip diameter of 25-50  $\mu$ m. We back fill the pipette with the mineral oil (O121-1, 148223, Fisher Chemical Inc.) and front fill the pipette with the virus using the injector (53311, Quintessential Stereotaxic Injector, Stoelting Co.). We injected *Cacna1h*<sup>Chr2EYFP+/-</sup> animals with 500-750 nL of *AAV2/9-CaMKIIa-GCaMP6f-P2A-nls-dTomato* (Canadian Neurophotonics Platform Viral Vector Core Facility, RRID:SCR\_016477) to the depths of 150-250  $\mu$ m. We injected *Trpv1*<sup>Chr2EYFP+/-</sup> animals to the depths of 150-250  $\mu$ m with 500-1,500 nL of cocktail mixtures of *AAV2/9-CaMKIIa-GCaMP6f-P2A-nls-dTomato* and *SYN-jRGECO* with *GCaMP:jRGECO* proportion of 1:1. We mixed two AAVs and the green food dye (Fast Green FCF Solution, 26053-02, Electronic Microscopy Sciences Inc.) by releasing and withdrawing the mixture 2-3 times with a micropipette. We add green food dye to aid visualization of the flow of AAV during the injection. We insert the pipette into the brain

using a stereotactic manual micromanipulator. We wait for 3 min after inserting the pipette to the targeted depth to allow tissue to stabilize and we inject the AAV at the rate of 60 nL/min. Following the injection, we wait for 5 minutes to allow the tissue to stabilize before withdrawing the pipette.

Once all 2-3 injections are complete, we cover the brain with the glass window. We make our glass windows by gluing together 3 mm (CS-3R, 64-0720, Warner Instruments Inc.) and 5 mm (CS-5R, 64-0700, Warner Instruments Inc.) circular coverslips using the optical adhesive (Optical adhesive 71, Norland Products Inc.). The 3 mm coverslip fits relatively snugly into the craniotomy, forming a tight seal against the drilled skull. This is important to minimize any risk of bone bleeders during the healing and to delay the overgrowth of craniotomy with dura and bone. We press the glass window into the brain using a basic custom pressure applicator with a diameter of 2 mm mounted on a stereotactic manipulator. Once the glass window is tightly pressed into craniotomy, we secure the window in place by applying a 3-component dental cement (C&B-Metabond®, Parkell) around the circumference of the window. Before applying the dental cement to the skull, we add a black paint to the 3-component dental cement mixture to reduce autofluorescence of the dental cement. Once we apply the dental cement around the circumference and the dental cement begins to harden, we withdraw the pressure applicator and start applying the dental cement throughout the rest of the skull. We also fill up with dental cement the muscle pockets that we make at the beginning of the surgery. Next, we cover the bottom of the implant with uncured dental cement mixture and place the implant on the skull centered over the cranial window. We then apply the dental cement mixture from all 4 sides of the implant to increase the

security of its attachment to the skull. We also pour some dental cement mixture through the 4 holes atop of the implant that are used for attaching the screws during the spinal cord surgery below. Finally, we apply tissue adhesive (Gluture, Zoetis Inc.) throughout the perimeter of the dental cement to double secure the attachment of the dental cement mixture to the skull and the skin.

#### 2.7.3.2 Surgeries for spinal cord AAV injections and window implantation

We perform spinal cord AAV injections and window implantations as part of a single surgical procedure. In our hands, the full procedure on average required 3-5 hours plus 1-2 hours of preparation time depending on the specific parameters of the AAV injections and specifics of any given animal. This procedure was developed by the right-handed surgeon and might require small modifications to achieve the optimal timing for the left-handed surgeons.

All surgeries follow the aseptic techniques. We anesthetize the animal using 1.5-2% isoflurane delivered in 100% USP-grade oxygen and exhausts collected using vacuum. We maintain the animal's body temperature stable throughout the surgery using a heating blanket and protect the animal's eyes using sterile ocular ointment. We prepare the animal in a non-sterile area before moving it to the sterile stereotactic frame. For the chronic spinal cord surgeries, we use the same stereotactic setup and injection tools as for the brain surgery, except we add a stack of breadboards (Thorlabs Inc.) with XYZ manipulators (60 mm x 60 mm footprint available from Amazon) needed for spinal cord manipulation ([Fig. 25. S2-1A](#)). The spinal cord clamp holder posts used for all our surgeries were 220 mm in length. The original design by Farrar et al. 2012 included the spinal cord clamp holder posts 50 mm in length. We significantly increased

the length of the implant holder posts to accommodate the dimensions of our stereotactic frame used for brain surgeries (Kopf Inc.). There were no particular benefits for using longer clamp holder posts. However, in our hands the use of XYZ manipulators rather than hand-manipulation like in Farrar et al. 2012 yielded finer control over the implant position and simplified the transfer of knowledge between the students by simplifying this complex procedure.

After moving the animal from the induction chamber to the prepping station, we inject the animal with IP dexamethasone (0.2 ug/g), SQ buprenorphine SR (0.75 ug/g), SQ glycopyrrolate (0.5 ug/g) and local SQ lidocaine (3 ug/g). We then generously shave the animal's back using an electrical buzzer and apply a depilation cream (Nair hair-remover lotion, Church & Dwight Inc.) around the intended incision site. The area for depilation can be determined by finding the apex of the vertebral column's curvature and the location of the last rib. The apex of the vertebral column curvature corresponds to the Th13 vertebra [249]. We massage the depilator cream thoroughly into the animal's back using a cotton swab to avoid any unexposed hair and we remove the detached hair using a razor blade after 30-60 seconds. We thoroughly wash off any remaining depilator cream and detached hair using alcohol pads. We then clean and sterilize the exposed skin with a two-stage scrub of betadine and 70% ethanol, repeated three times. After this, we move the animal to the surgical stereotax for the sterile parts of the procedure.

We begin by aligning the animal's back with the XYZ manipulators to make sure the spinal cord clamps mounted on the custom clamp holder posts can reach the XYZ position of the spinal cord. We then prepare the surgical gelfoam by soaking it in sterile

saline. We proceed by identifying the location for the skin incision by palpating the ribs around the apex of the vertebral curvature. Typically palpation involves active sweeping of the back with the fingers, as well as keeping the fingers in place around the suspected last rib to feel the motion of the last rib while breathing. The last rib emanates from the rostral end of the Th13 vertebra [249]. For the imaging of the spinal cord at the vertebral level L1, we usually aim to have the skin incision that starts about 1-3 mm rostrally relative to the entry point of the last rib, covers the width of the implant (10 mm) and extends 1-3 mm caudally (i.e. 12-16 mm total). As was described by Farrar et al. 2012, it is possible to obtain a good quality spinal cord window even with a significantly larger skin incision. However, it is important to minimize any tissue damage and trying to tailor the incision size towards the implant's size does not have a significant impact on the duration or complexity of the surgery. For the imaging of the spinal cord segments under the L2 vertebra, the skin incision should be moved caudally by 2.5-3.0 mm. Following the skin incision, we repeat the palpation of the last rib this time using the forceps (Fine Instruments Inc.) to locate the rostral end of Th13 more precisely. Once we locate the root of the last rib, we incise the soft tissue overlying the vertebra. The length of the cut is equivalent to the width of the implant. We begin by making a straight cut on the right side as close to the midline as possible while not cutting through the spinous processes. We then turn the surgical blade by more than 70 deg to orient it close to the horizontal plane and cut laterally towards the right to dissect away the overlying soft tissue. After reaching the transverse processes, we reorient the blade vertically and dissect away the tissue off the vertebral bodies not deeper than the thickness of the spinal cord bars (1.5 mm) to offer enough space for fitting the spinal

cord bars under the spinal cord processes flush against the vertebral body. Similar to Farrar et al. 2012, the implant bars merge together 3 vertebrae. Hence, we clean up the lateral surfaces of 3 vertebral bodies, as well as cut off the tendons attached to all 3 vertebral bodies to minimize the motion during post-surgical imaging. After cleaning the lateral sides of vertebral bodies, we clamp the vertebral column from both sides with the spinal cord bars. We align the center of the bars with the approximate center of planned laminectomy over the middle vertebra. We apply a generous amount of pressure to keep the spinal cord in place during the surgery and to promote strong chronic attachment via pressure after the surgery. Next we partially trim away the spinous and transverse processes to maximize the flatness of the implant chamber against the vertebral bodies. The processes are not removed entirely, rather they are flattened by cutting away the superficial parts of the processes while keeping as big parts of the processes as possible to minimize tissue damage and promote strong attachment of the spinal cord bars. In our practice, it is best to cut the processes as early as possible because this step is prone to bone bleeding if done improperly and by cutting the processes early, the tissue has a longer time for coagulation before further cuts must be made. Next, we clean up the intervertebral spaces from all the connective tissue to have a clear tactile and visual feedback on the extents of the middle vertebra. Once we know the extents of the vertebra, we perform a laminectomy using a drill actuated via compressed nitrogen (both diamond burr and ball drill work well for this step). We sequentially drill longitudinally as far laterally as possible first on one side, then on the other and repeat switching sides after every pass, removing thin layers of bone on every pass until the bone becomes thin enough to be pulled up without having to apply a



significant amount of force. Between every pass of the drill we apply the sterile saline to protect the spinal cord from drying.

After the laminectomy, we perform the viral injections following the same steps as for the brain injections. The main difference between the brain and spinal cord viral injections in our experience is that the spinal cord meninges are much harder to pierce through compared to the brain meninges. Hence, we performed durotomies for every spinal cord injection. The examples of pinching responses in [Fig. 16](#) involved injections of an *AAV2/9-CaMKIIa-GCaMP6f-P2A-nls-dTomato* (Canadian Neurophotonics Platform Viral Vector Core Facility, RRID:SCR\_016477) virus at 60 nL/min. We administered the AAV via two injections on the right side of the spinal cord at 150  $\mu$ m and 250  $\mu$ m depths and one injection on the left side at 200  $\mu$ m.

#### 2.7.3.3 Multi-site surgeries as a single or two separate procedures

When performing 2 independent surgeries within the same animal, each procedure is performed exactly as outlined above. The main important factor to consider is that the animal requires at least 3 days to recover after a brain or spinal cord surgery. In our particular experience, we never performed the second surgery sooner than 7 days after the first surgery just out of an abundance of caution. Hence, two surgeries cannot be performed immediately one after another. Furthermore, we typically performed brain surgery first followed by the spinal cord surgery. This choice was based on our experience with maintaining the good quality and responsiveness of surgerized neocortex across months without significant challenges with good consistency between the animals. Hence, the risk of the cranial window to decrease in quality while waiting

for the spinal cord window (e.g. waiting for healing or viral expression) is lower than the risk or the amount of unavoidable quality change for the spinal cord window.

In the case of both brain and spinal cord surgeries performed as part of a single procedure, we follow the same surgical steps as outlined above except in all surgical cases the animals did not receive any viral injections in either of the sites. This had to do with the significantly increasing surgical duration and the impact on the animal's health when trying to incorporate AAV injections into the surgery. The duration of a combined surgery in our implementation is at least 6 hours. We typically would start with a spinal cord implant first and then would proceed with the brain implant. This choice is based on the fact that the spinal cord surgery is more prone to failure and if the spinal cord surgery is done following the brain surgery, the resources invested into brain surgery would be wasted.

As part of the development of surgical methodology it was important to ensure that the surgical developments presented in this thesis are transferable to other scientists. The transferability of principles described here was validated by training a medical student on brain-spine surgeries. The transfer of knowledge could be efficiently performed following the knowledge and materials developed throughout this thesis and trained student was able to successfully surgerize the animal that was used to generate the data presented in [Fig. 19](#).

## 2.7.5 Fluorescence imaging

### 2.7.5.1 1-photon imaging using electron multiplying gain CCD (emCCD) camera

The results shown in [Fig. 15](#), [Fig. 16B](#), [Fig. 17](#) and [Fig. 19](#) were imaged using an emCCD camera. For imaging calcium using a green fluorescent protein based

genetically encoded calcium indicator (*GCaMP*) and the blood vessels labeled with the fluorescein isothiocyanate (*FITC*) dextran ([Fig. 19A](#)) the emCCD camera was equipped with the *GFP* filter cube (DFM1 with *GFP* my excitation and emission filters and dichroic, ThorLabs) and an excitation LED (M470L3, ThorLabs Inc.).

#### 2.7.5.2 Imaging using 2-photon

All the 2-photon imaging was done using a custom microscope assembled by Bruker Inc. Vascular imaging data shown in [Fig. 16](#) were collected using a 960 nm excitation laser wavelength with galvo scanning and 16x water immersion objective (0.80W, DIC N2, Nikon LWD). Brain calcium imaging data shown in [Fig. 18](#) was collected using a 980 nm excitation laser wavelength with galvo scanning and 10x objective (HC PL APO 0.40NA, 506511, Leica Inc.).

#### 2.7.5.3 Imaging using miniscopes

All the miniscope imaging was performed using UCLA miniscopes v4.0, v4.40 and v4.41. Miniscope v4.0 was generously provided by UCLA, v4.41 was purchased from OpenEphys and v4.40 was purchased from OpenEphys. v4.40 miniscope from LabMaker offered the smallest amount of bleed-through excitation light noise and best imaging quality. Miniscopes v4.40 and v4.41 had the lens configuration #1 (combination of lenses #45-089, #45-089 and #45-691 from Edmund Optics with working distance 675  $\mu\text{m}$  +/- 250  $\mu\text{m}$  and FOV of 1 mm x 1 mm) [250] and was used for brain and skin imaging. Miniscope v4.0 had the lens configuration #3 (combination of lenses #45-090, #45-090 and #45-691 from Edmund Optics with working distance 2010  $\mu\text{m}$  +/- 400  $\mu\text{m}$  and FOV of 1.3 mm x 1.4 mm) [250] and was used for spinal cord imaging. We tested

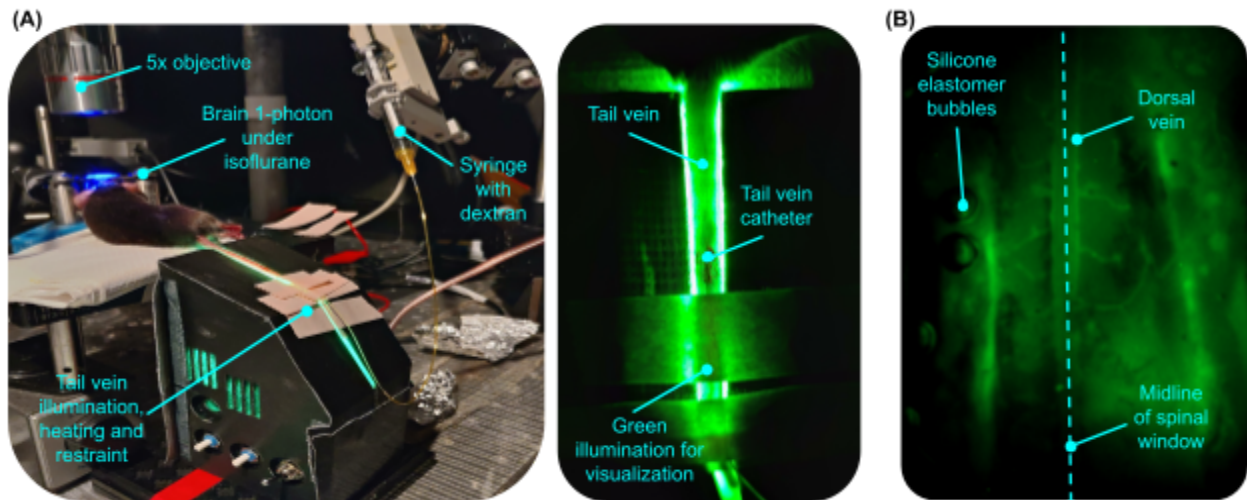
the lens configurations #1 and #2 (working distance for configuration #2 is 975  $\mu\text{m}$   $\pm$  340  $\mu\text{m}$ ) [250] for spinal cord imaging in multiple animals, but the working distance was too short for imaging using the window design and viral injection depth combinations used in this work. In 90% of the cases all lens configurations allowed us to at least reliably image the cortical blood vessels.

For the imaging under anesthesia, the miniscope was attached to the implant chamber using 1 implant screw and for the awake imaging using 2 screws. We also imaged anesthetized animals implanted with the stainless-steel headposts and cranial windows not designed for the attachment of the miniscope. During these imaging sessions the miniscope was mounted in a 3D-printed holder and positioned over the cortex using micromanipulators.

#### 2.7.5.4 Vascular imaging using dextrans

Blood vessel imaging reported in [Fig. 16C](#), [Fig. 19A](#) and [Fig. 20](#) relied on labeling the blood vessels using injectable fluorescent dextran dyes. Dextran was delivered into the animal's body either via retro-orbital injection (used for collecting the data in [Fig. 20](#)) or tail vein injection (used for collecting the data in [Fig. 16C](#) and [Fig. 19A](#)). Dextran was injected by hand using a syringe for collecting the data shown in [Fig. 20](#) and [Fig. 16C](#). To collect the data shown in [Fig. 19A](#), the dextran was injected using a tail vein catheter (MTV-01, SAI Infusion Technologies Inc.). [Fig. 21A](#) depicts the procedure of injecting a dextran using a tail vein catheter. The main benefit of using a catheter is a more precisely controlled timing of the injection via an electronically controlled injector and the ability to perform the injection remotely to avoid the contamination of the imaging with the ambient light needed when injecting manually via a syringe. The dextrans used in

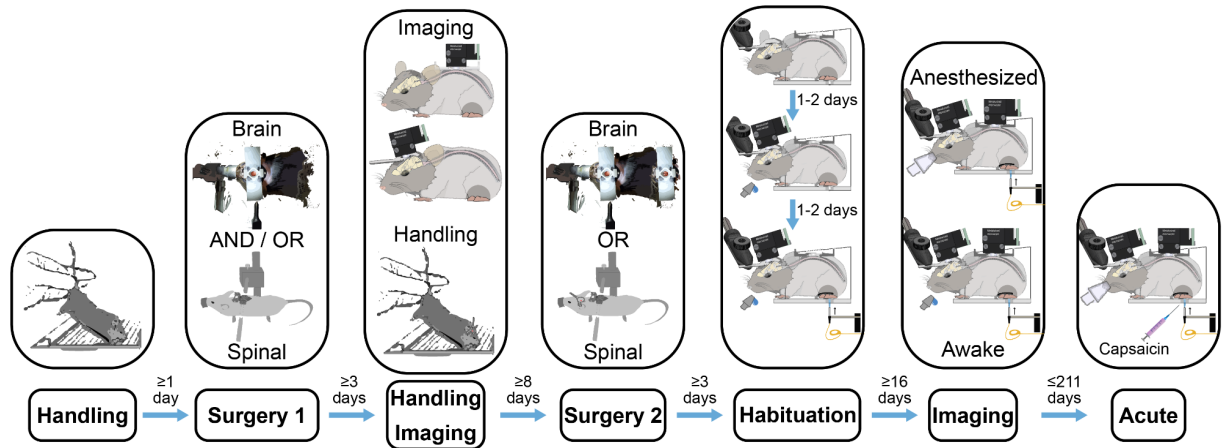
this study were as follows: (i) FITC 70 kDa ([Fig. 19A](#), [Fig. 20](#); D1823, ThermoFisher Scientific Inc.), (ii) FITC 10 kDa ([Fig. 16C](#); D1821, ThermoFisher Scientific Inc.). A typical injection would include 30  $\mu$ L of dextran solution mixed with 70  $\mu$ L of sterile saline.



**Figure 21.** Vascular visualization was achieved via tail vein and retro-orbital injections of fluorescent dextrans. (A) Tail vein dextran injection using a catheter. (B) An example of the spinal cord vasculature imaged using a benchtop 1-photon microscope with 4x objective 50 hours after the surgery. To allow vessel visualization, the animal was injected with the 10 kDa fluorescein isothiocyanate (FITC) dextran via a tail vein.

## 2.7.6 Behavior

The awake imaging results reported in [Fig. 18](#) required the animal to follow the 7-step behavior procedure summarized in [Fig. 22](#). The steps were as follows:



**Figure 22.** Procedural timeline for the animals engaged in multi-site awake imaging in restrained animals using benchtop and wearable microscopes. Achieving awake imaging of responses to repeated stimulation requires at least 7 steps. The habituation during step 5 was split into 3 parts. For the first few days the animal was introduced to being head-restrained for 5-10 minutes. For the next 1-2 days, the animal was introduced to the sucrose reward, imaging of the brain and the restraint was prolonged further to 15-30 minutes. During the final part of habituation, the animal is exposed to the full behavior by increasing the duration of restraint to 1 hour, restraining the hindpaw and applying the stimulation. The hindpaw is gently restrained using tape over the LED mounted on a piezoelectric bender. The illustrated final acute step shows an example of optomechanical stimulation coupled with capsaicin injections to drive sensory neuromodulation.

## I. Handling

A. Handling helps to improve an animal's collaborativeness throughout the experiments. Given how sensitive the spinal cord preparations can be depending on the study and surgeon's technique, as well as the complexity of multisite surgical procedures, it can be beneficial to assess an animal's collaborativeness ahead of the first surgery. Animals that within 3-5 days do not become significantly better at collaborating with the researcher and do not increase in comfort of being handled, it can be beneficial for animal's health and the experiment to assign a given animal

to the acute track or invest additional time to increase the chances for a given animal's successful participation.

B. Handling helps to maximize the animal's comfort with the experiment, minimize the risk of tissue damage, increase the experimental duration and overall, improve the quality of data.

C. Increasing the animal's comfort with being handled by the researcher is especially critical for maintaining the longevity of the spinal cord implants. Even miniscope imaging requires restraining or anesthetizing the animal for 2-5 minutes to attach the miniscope. Hence, by the first time of needing to attach a miniscope, the animal needs to be comfortable with being held in place by the researcher.

## II. Surgery 1

A. This can be either a brain or spinal cord surgery.

B. This could also be a single brain-spine surgery. However, as part of this study, the animals following a single brain-spine surgery received stimulation only under anesthesia and were imaged awake only when looking at the changes in brain-spinal activity during the transition from anesthetized to awake state. Hence, single multisite surgery animals did not follow the behavior protocol for awake study.

## III. Handling and Imaging

A. Between the surgeries it is important to continue handling the animal to increase their comfort with the researcher despite the invasive procedures. A good assessment metric for determining the endpoint for

handling can lie in achieving collaborative behavior qualitatively comparable to the behavior after the initial handling prior to the first surgery.

- B. At this stage it can be useful to image through the implanted window to verify the ability to see the target signal. However, if not sufficient time has passed since the first surgery (e.g. at least 2 weeks for the AAV2/9-CaMKIIa-GCaMP6f viral expression used in this study), the lack of signal or responsiveness to sensory stimuli might not be informative about the prognosis for the signal strength.

#### IV. Surgery 2

- A. This can be either a brain or spinal cord surgery.

#### V. Habituation

- A. This step consists of 3 sub-steps:
  - i. Habituation to the head restraint for 1-2 days, gradually increasing the duration between 5-10 minutes.
  - ii. During the next 1-2 days, add *ad libitum* sucrose (30%) and brain imaging to gently promote the animal's continuous acclimation to the parts of the experimental setup. As part of this step, continue increasing the duration of restraint towards 15-30 minutes. The microscope in this case contributes only the excitation light and it might be beneficial to avoid attempting to image during the habituation to avoid photobleaching. However, we find it useful to start imaging the brain at this earlier stage because the cost of



imaging is low (data storage and experimental time), but the efforts to image at this stage help to learn the particular brain landmarks and probe the responsiveness of different areas.

- iii. Add hindpaw restraint and optomechanical stimulation as the final parts of the experiment. Continue increasing the duration up to 60 minutes. Continue habituating until the animal develops comfort with the needed number of trials or the animal stops improving in its performance. The performance in this case is assessed by the amount of animal's motion and signs of not wanting to continue engaging with the experimental setup. For the purposes of sensory optogenetic and mechanical stimulation experiments ([Fig. 18](#)), the minimal number of trials was 60 (up to 12 minutes) to allow at least 30 trials of optical and 30 trials of mechanical stimulation.
- B. As part of this study, we strongly considered the idea of habituating the animals to head-restraint during between the 1st and 2nd surgery. The benefit would be that the animal learns the behavior before any serious interventions and in theory it should be easier for the animal to learn the task after the 1st surgery than after both surgeries and re-learn the task after the 2nd surgery rather than learning it from zero after the 2nd surgery. Albeit, this approach seems promising, it has not been tested as part of this study.

## VI. Imaging

- A. Imaging involves the use of the same full behavior as the last habituation step. The only difference is the addition of the microscope to image either from both sites or continue image from a single site depending on the experimental goals.

## VII. Acute experiment

- A. As the final step, the animals typically undergo the acute experiments. In this case, every animal underwent the saline and capsaicin injections into the hindpaw to compare the impacts of the strong nociceptive stimulus on the animal's brain-spine responses to stimulation.

Ultimately this procedure is animal-specific and all the durations provided in this procedure might have to be adjusted for a particular animal's phenotype. In addition, given the high value of double implant animals, in the rare cases of brain implant detachment during the habituation, it is possible to reattach the implant. However, it is important to start the surgery immediately to moisturize the brain as soon as possible. The quality of the craniotomy will inevitably decrease in the case of re-implants, hence it is justified to perform this step only in the cases of animals showing exceptionally strong responses. We have not tested spinal cord re-implantation because of the lack of cases of implant detachment. However, given the propensity of the spinal cord towards bleeding and the involvement of silicone elastomer when creating a spinal cord window, the impact of spinal cord re-implantation can be predicted to be inevitably significantly more severe than in the case of brain and will likely be associated with the spinal cord injury.

Animals that were not involved in any awake behavior as part of this study (e.g. the animals participating in the spinal cord endogenous light sensitivity study) did not undergo handling, habituation and awake imaging.

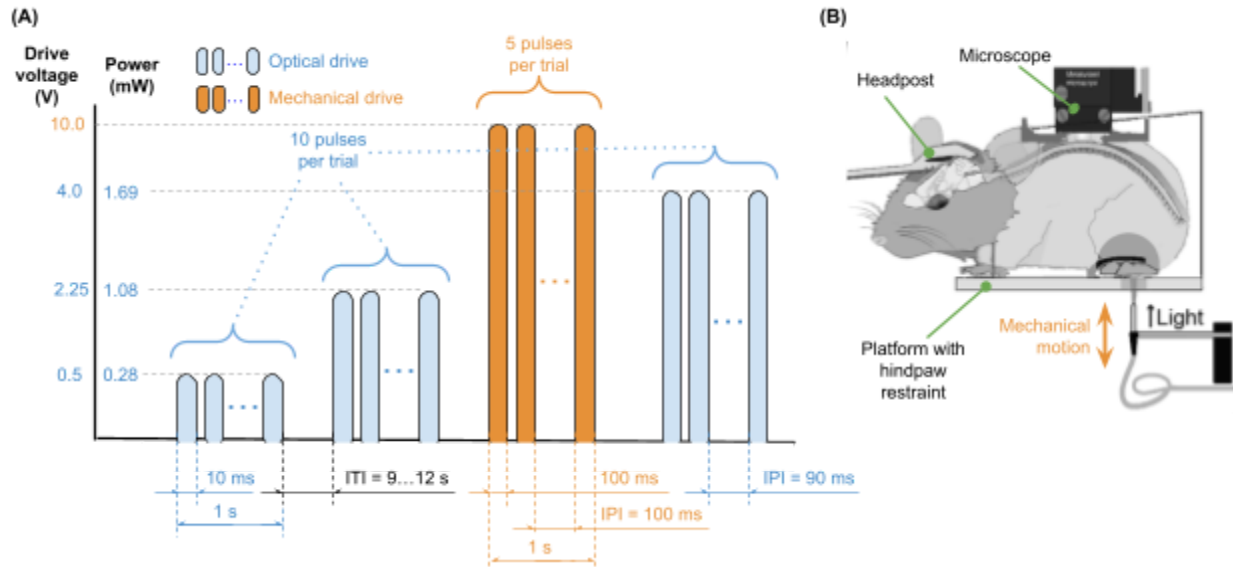
## 2.7.7 Stimulation

### 2.7.7.1 Pinching

Pinching responses were evoked by gently squeezing the hindpaw with the hand-held forceps. The effort was made to apply the pressure consistently at the same location. However, the amount of pressure exerted by every pinch varied pinch-to-pinch because of natural variability present when it is not possible to control precisely the amount and location of applied pressure.

### 2.7.7.2 Optogenetic and mechanical stimulation of the hindpaw

All plantar hindpaw optogenetic stimulation experiments were performed using 470 nm light-emitting diode (LED) (M470F3, ThorLabs Inc.) controlled using an LED driver (LEDD1B, ThorLabs Inc.). Light was delivered to the plantar hindpaw using an optical fiber. The stimulated hindpaw was covered with the black aluminum foil to minimize light propagation beyond the stimulation target. The cannula of the optical fiber was glued using epoxy to the piezo-electric plate bender (CMBP09, Noliac Inc.) to allow interleaved mechanical and optical stimulation trials similarly to [126]. The mechanical stimulation was done by poking the animal's plantar hindpaw with the optical fiber's cannula by actuating the piezo-electric plate bender via an amplifier. The stimulation waveform parameters are shown in [Fig. 23](#).



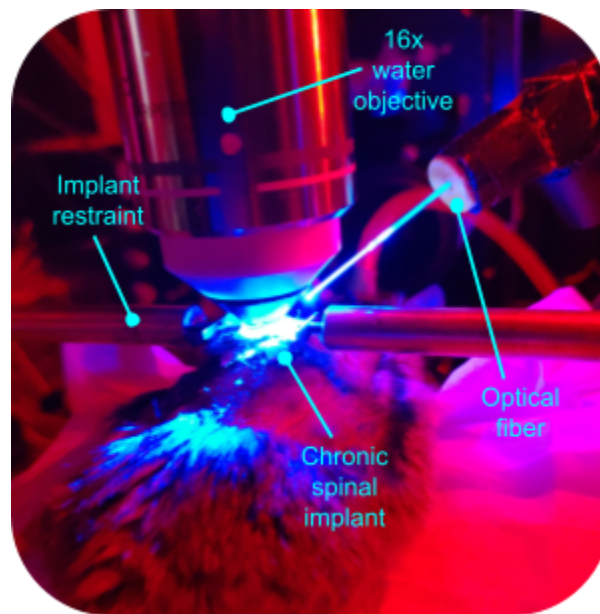
**Figure 23.** Hindpaw plantar opto-mechanical stimulation experiments involved randomly interleaved pulses of optical and mechanical stimulation in anesthetized or awake hindpaw-restrained animals. **(A)** Summary of the timing and driving voltage settings for the randomly interleaved opto-mechanical stimulation delivered at random time intervals every 9...12 s. **(B)** An illustration of a typical experimental animal with brain and spinal cord implants with hindpaw restrained over an optical fiber mounted on the piezoelectric bender for opto-mechanical stimulation.

Imaging and opto-mechanical stimulation were controlled and synchronized using either an OpenEphys or National Instruments (NI BNC-2110, NI PCI-6713 and USB-6008 for emCCD and BNC-2110 with PCIe-6353) data acquisition boards operated via MATLAB R2015b and OpenEphys software v0.5.5.3.

#### 2.7.7.3 Optogenetic stimulation of the spinal cord

The optogenetic stimulation of the spinal cord was performed by directly aiming an optical fiber at the spinal cord implant ([Fig. 24](#)). The important aspect of spinal cord optical stimulation was the use of flyback to reduce the artifacts during 2-photon imaging. Even though the imaging approach used for collecting the data reported in [Fig. 16C](#) relied on *GFP* filters that should block 470 nm light used for stimulating the spinal cord, the imperfect stop-band of the filters and high sensitivity of 2-photon

photomultiplier tubes (PMTs) results in artifacts when trying to apply optical stimulation during 2-photon imaging. Flyback stimulation approach relies on the fact that galvo scanning requires pausing the imaging during the data collection with PMTs to mechanically reposition the scanner between the scan lines. The transition time between the scan lines is defined as the line period and the stimulation is applied only during this transition to effectively reduce the signal contamination with the photons reflected back into the objective from the stimulation beam.



**Figure 24.** Direct spinal cord optical stimulation of the blood vessels labeled with fluorescent dextran was applied to study endogenous blood vessel sensitivity to blue (470 nm) light following tail-vein injection of dextran. Spinal cord was optically stimulated during 2-photon imaging under anesthesia using an LED-coupled optical fiber aimed directly at the spinal cord window under angle.

### 2.7.9 Image analysis

All microscopy images have been processed using either ImageJ (Fiji, v1.53f51) or MatLab R2021a (MathWorks Inc.). For all the multi-trial analyses (e.g. [Fig. 15](#), [16B](#),

[17](#), [18](#)) the data as plotted as a percentage change of baseline (i.e.  $dF/F$  % change) activity during 1 s interval preceding the stimulus:

$$dFF = \frac{\text{Signal} - \text{baseline}}{\text{baseline}} \times 100\%, \quad (1)$$

**Equation 1.** Equation for calculating the normalized signal dFF.

In the case of individual pinch response data in [Fig. 16A](#), the baseline for each neuron was defined as the full experiment-averaged activity within the area surrounding each neuron.

### 2.7.10 Statistical analysis

The analysis of animal weights reported in [Fig. 14](#) relied on multiple comparisons using the Bonferroni method. The significance threshold was set to  $p\text{-value} < 0.05/300 = 0.00017$ . The statistical analysis was performed using MatLab R2021a (MathWorks Inc.).

### 2.7.11 Publication counts

The publication counts across years in [Fig. 10](#) plotted using the data from the Web of Science. The search for brain and spinal cord microscopy publication counts relied on the following keywords: (i) Brain, mice, imaging, microscopy; (ii) Spinal, cord, mice, imaging, microscopy. Only the publications from the field of neuroscience were included and publications containing the keyword “MRI” were excluded from the analysis to reduce the presence of non-microscopy publications in the plotted counts.

## 2.8 Limitations

The animal weight analysis results in [Fig. 14](#) suffer from a small interaction effect from the fact that the surgeon's skillset was changing throughout different surgical preparations. Hence, the same exact preparation could easily result in significant weight changes at the earlier learning stage and not cause any weight change at the later stages. In our specific context, we observed significant differences only between the initially performed simpler surgical preparations and the more complex single surgery multi-site preparation. There is no reason to believe that these particular significant differences would have not been present in the absence of surgeon's skill interaction effects.

One of the limitations of this study is the lack of repetition of different responses across multiple animals. Albeit  $n > 3$  for every response example would be needed to make any claims about the biology that we observe, present work focuses on development of methodology rather than studying specific questions around the biology and the diversity of preparations presented here can be seen as the benefit compared to the high repetitions of fewer preparations across larger animal cohorts.

Another limiting factor is that we never observed the individual cell responses both in the brain and spinal cord within the same animal. Hence, most of our calcium imaging focused on imaging the field responses. There is no reason not to be able to see the individual cell dynamics across the brain and spinal cord at the same time using the tools presented here. The limiting factor comes from the fact that both the brain and spinal cord surgical preps have certain degree of variability and when we combine both at the same time, these variabilities multiply and the chances of finding the right

responsive areas becomes much harder, but as with all the prior methodologies, this can be streamlined by proper repetition and refinement of the protocols towards labeling the targets of interest both in the brain and spinal cord within the same animal.

Another important factor that could not be validated as part of this study was whether the vascular dilation effects presented here were caused by the thermal impact of the light pulses or the interactions of light with the endogenous optical receptors in the spinal cord. The exact temperature changes would depend on many factors that are difficult to control (e.g. distance between the fiber and the sample, stimulation beam spread, depth of anesthesia, the temperature and cleanliness of the water used for the water immersion objective). Albeit, the exact mechanisms cannot be determined at the moment, even if the thermal effects were partially involved, finding such robust thermal responses in the spinal cord would be a novel and useful finding.

One of the limitations is that the machined implants made of steel can be immediately implanted without the need for any post-processing and can be reused between the animals. As is described in methods, 3D printed implants require several additional processing steps before they can be implanted. Furthermore, we have not attempted reimplantation of 3D printed implants, but we never experienced any challenges with reimplanting the stainless steel implants.

One of the consistent observations throughout this work was that animals were gradually biting off the farthest most edges of the spinal cord chambers fabricated from Tough 1500\_v1 resin. This never affected the quality of the experiments in this work because this was a gradual process with millimeters wide pieces being removed over the course of months, this did not seem to affect the quality of the spinal cord window,



and the lengths of the remaining restraint wings were always sufficient to allow spinal cord restraint under anesthesia or attach the miniscope in back-unrestrained animals. However, we acknowledge that this could be a more significant issue for the studies relying more heavily on spinal cord restraint. One of our observations is that such loss of implant parts has not been observed with the implants fabricated from Dental SG and hence could be tested further for longevity.

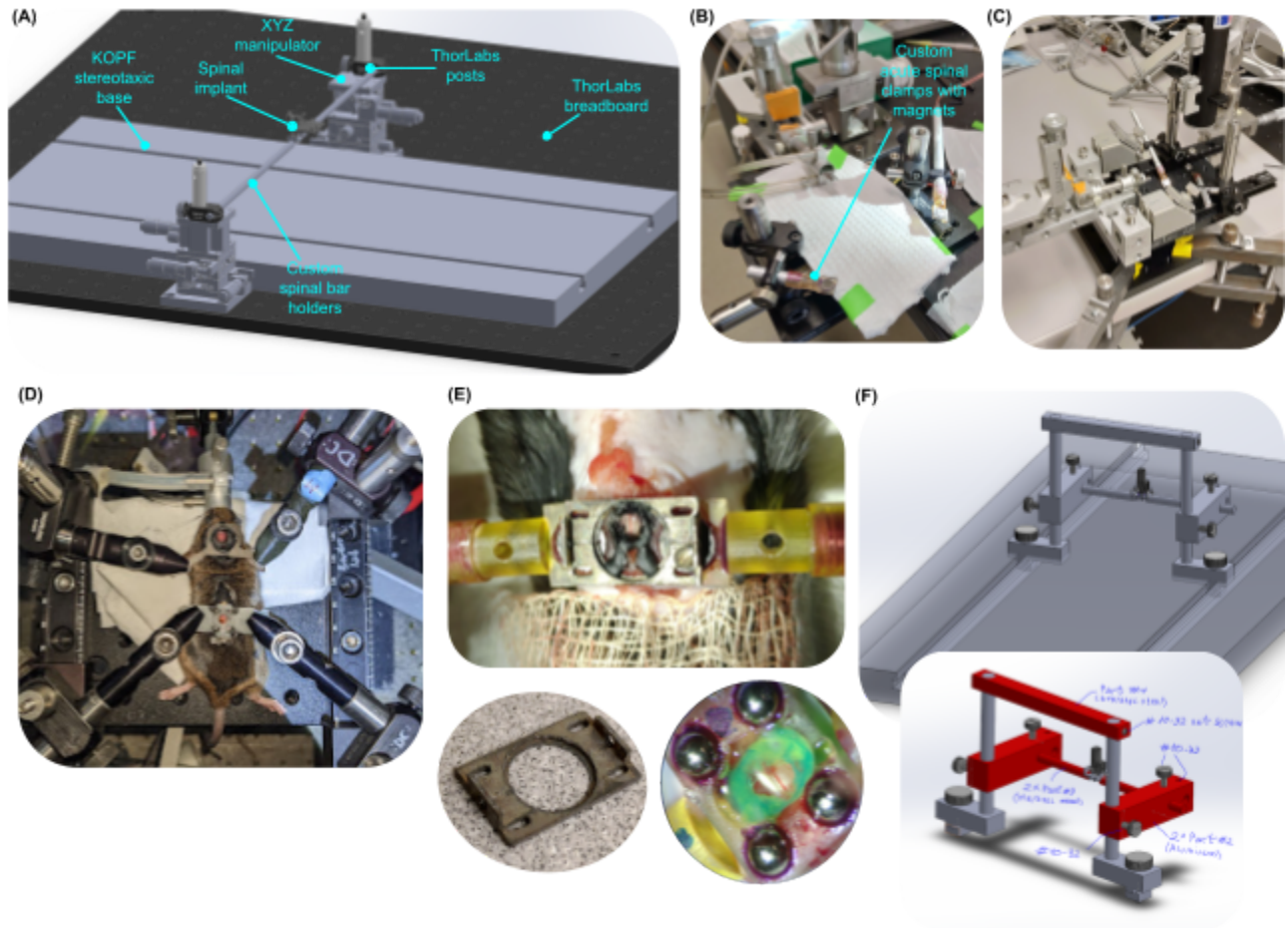
Furthermore, it is important to keep in mind that *Trpv1* receptor is expressed not only at the skin nerve endings, but also in the endothelium of blood vessels throughout the body (e.g. arterioles in the ears and urinary bladder), some areas of the brain (e.g. hypothalamus, somatosensory cortex, insula, amygdala, periaqueductal gray) [251], keratinocytes, Schwann cells [251], astrocytes [251], and the cells of the immune system [252], [253]. For instance, it has been reported that stimulation of endothelial Trpv1 can cause relaxation of vascular smooth muscle cells through the mechanisms involving nitric oxide and endocannabinoids [253]. In the present study, we used *Trpv1*-driven expression of channelrhodopsin-2 (*ChR2*) light-gated ion channels to specifically stimulate nerve endings innervating hindpaw's skin. To ensure the focality of optogenetic stimulation of the hindpaw, we use optical fiber placed near the surface of the skin to minimize beam divergence and we shield the optical fiber with black-color non-reflective materials (i.e. electrical tape atop of the hindpaw and black aluminum breadboard around the tip of the optical fiber). Albeit, these precautions should minimize any risks of confounding activations of *Trpv1* channels outside of the targeted hindpaw area, there is a possibility for photons to reach distant targets (e.g. animal's eyes) following multiple scattering events from the experimental hardware surfaces. However,

in the cases where we employed the optogenetic stimulation on both parts of the body ([Fig. 17](#)) we would observe similar response components when stimulating either side of the body if the observed responses were driven by systemic stimulation via scattered photons. The fact that the observed responses are stimulation side-specific is evidence for the lack of confounding stimulation by the scattered photons. Epineural light delivery would be a further improvement towards restricting the photostimulation to peripheral axons [203].

Finally, a more broad limitation characteristic of most animal research is that the mechanisms identified in mice are not always directly translatable to humans [254]. This is especially true in the case of pain because of its complex biopsychosocial nature [110]. Even humans of different sex, socioeconomic, ethnic and comorbidity backgrounds respond variably to the same pain treatment [110]. The difference and variability in responses to treatment become even more different when we start comparing or attempting to translate the biological mechanisms across species such as mice and humans. Hence, when it comes to the field of pain it has been recommended to diversify the animal species being used as much as possible, for instance, by comparing the data from mice, rats and human tissue samples whenever possible [254]. Although the methodology developed as part of my dissertation work has not been tested in any other animal species besides mice, there is no reason to believe that this methodology cannot be translated to other species such as rats or even non-human primates after the appropriate design optimization and testing. Furthermore, it is important to note that some of the characteristics of neuropathic pain were shown to be conserved across the species. Hyperexcitability and spontaneous activity of DRG

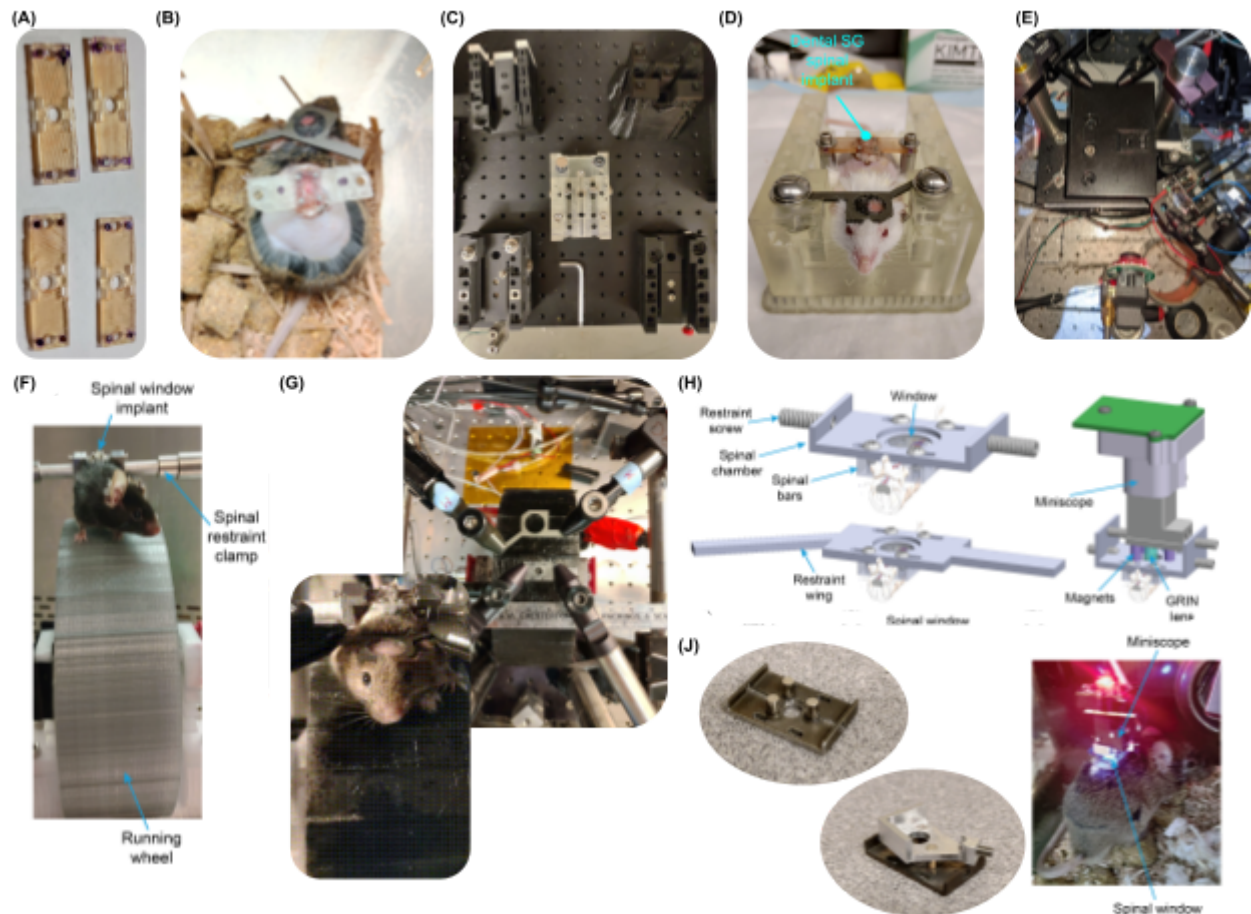
neurons found both in humans and mice afflicted with surgical injury-evoked pain [255] is one example of cross-species preserved features of pain and is relevant for the dynamic functional imaging techniques developed in my dissertation. These techniques are specifically tailored for studying the circuit mechanisms and in contrast with mechanisms pertaining to immune or endocrine systems, circuit mechanisms have a long history of transferability across species [256]–[258]. One particular example of such a cross-species conserved neural circuit mechanism pertaining to pain is the Gate Theory of Pain [209].

## 2.9 Supplements

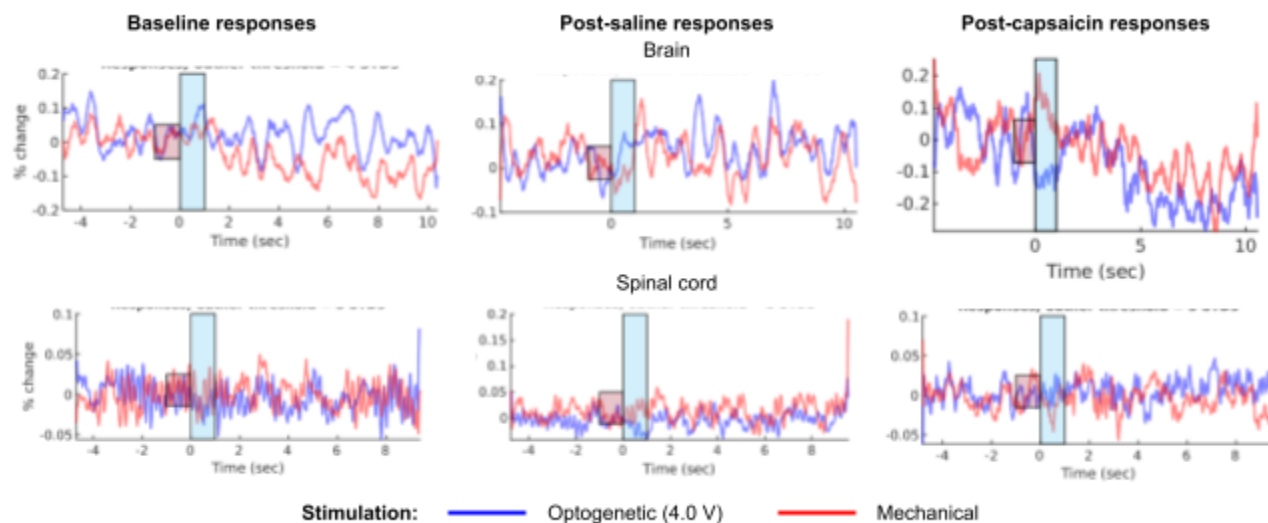


**Figure 25. S2-1.** Setup and implant designs and builds for chronic surgeries and acute imaging. (A) A 3D-rendered design of the chronic spinal cord surgical setup used for all the spinal surgeries described in this report. (B) One of the first versions of the custom acute surgical setups built for imaging during acute spinal cord surgeries. (C) The final version of the acute spinal cord surgical setup offers a more sturdy design with a higher number of degrees of freedom for the spinal cord manipulators. (D) An example of an imaging setup used for imaging of hindpaw plantar stimulation evoked brain-spine responses under anesthesia. (E) An example of the use of custom magnetic spinal cord clamps 3D-printed from Dental SG resin and applied for acute imaging using a customized spinal cord chamber. The custom acute implant was fabricated from thermally processed stainless steel that ensured strong magnetism to allow stable fixation in place via magnets in the spinal cord clamps. The custom implant had an 8 mm central aperture compared to 5 mm as in the design originally described by Farrar et al. 2012. Larger aperture allows more flexibility during the acute surgery and offers a larger FOV. Thermal processing and machining is a relatively costly process. Hence, the bottom right image shows a 3D-printed version of an acute implant that serves the same purpose as a machined implant. A 3D-printed version relies on glued-in magnets to ensure stable attachment to the spinal cord

clamps. The same custom acute spinal cord clamps were used as part of the acute setups in (B) and (C). (F) A 3D render of the setup that was designed and fabricated for manipulating the miniscope for acute spinal cord imaging.



**Figure 26. S2-2.** A variety of brain-spine restraint and implant setups have been designed, built and tested towards finding the most optimal configurations. (A) Spinal cord implants 3D-printed from Dental SG resin with the magnets in the lateral wings for optimal restraint. (B) An example of an animal with a spinal cord implant 3D-printed from Dental SG resin. (C) 5 generations of custom designed and 3D-printed setups for brain-spine restraint. (D) An application example of the custom brain-spine setup to restrain an awake animal. (E) An example of the setup used for awake imaging (the design closely mimics the design originally described by Black et al. 2020). (F) An example of a mouse spine-restraint on a running wheel with the implant restraint hardware replicating the design by Farrar et al. 2012. (G) A design of a running wheel with the simultaneous brain-spine restraint. (H) 3 design iterations of the spinal cord implants to accommodate different forms of restraint and imaging using miniscope v3.2. (I) The example of the spinal cord implant chamber with integrated magnets to allow the attachment of the miniscope v3.2 via an intermediate baseplate. The rightmost image shows an animal with the miniscope v3.2.



**Figure 27. S2-3.** Capsaicin injections in the hindpaw did not evoke changes in brain-spine activity. Capsaicin injections have been performed in 3 animals without evoking detectable changes in brain-spine activity. This is an example of right hindpaw plantar stimulation with responses imaged under anesthesia using 1-photon miniscope 76 days after the spinal cord surgery. The spinal cord data is plotted for the same animal and same region of interest as the spinal cord data in [Fig. 17](#). The capsaicin data was collected as part of the terminal procedure.

## 2.10 Acknowledgements

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# CHAPTER 3

## 3. Miniaturized Devices for Bioluminescence

### Imaging in Freely Behaving Animals

#### 3.1. Preface

This chapter is composed of an article I published in 2020 on the redesign and first ever use of a wearable miniature microscope for bioluminescence imaging *in vivo*. This article reported on the first steps towards making possible bioluminescence imaging in freely behaving animals and bringing all the theoretical benefits of bioluminescence imaging outlined in the Introduction to this dissertation into the hands of behavior scientists.

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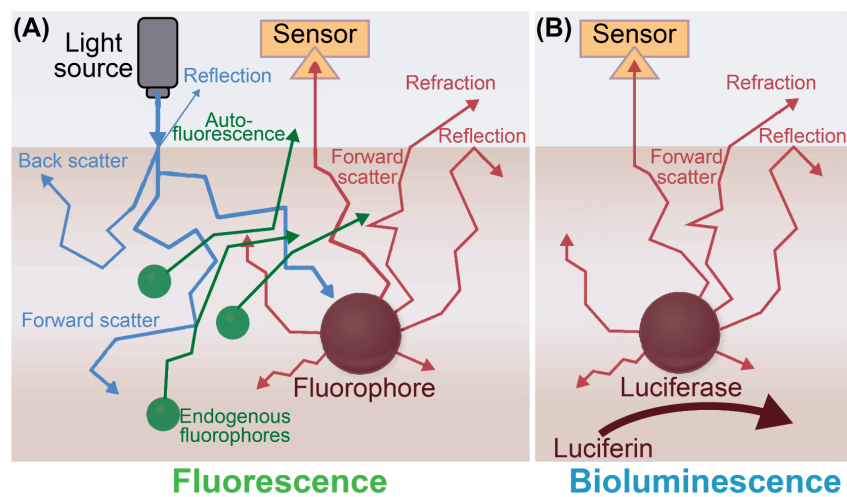
### 3.4. Abstract

*In vivo* fluorescence miniature microscopy has recently proven a major advance, enabling cellular imaging in freely behaving animals. However, fluorescence imaging suffers from autofluorescence, phototoxicity, photobleaching and non-homogeneous illumination artifacts. These factors limit the quality and time course of data collection. Bioluminescence provides an alternative kind of activity-dependent light indicator. Bioluminescent calcium indicators do not require light input, instead generating photons through chemiluminescence. As such, limitations inherent to the requirement for light presentation are eliminated. Further, bioluminescent indicators also do not require excitation light optics: the removal of these components should make a lighter and lower cost microscope with fewer assembly parts. While there has been significant recent progress in making brighter and faster bioluminescence indicators, the advances in imaging hardware have not yet been realized. A hardware challenge is that despite potentially higher signal-to-noise of bioluminescence, the signal strength is lower than that of fluorescence. An open question we address in this report is whether fluorescent miniature microscopes can be rendered sensitive enough to detect bioluminescence. We demonstrate this possibility *in vitro* and *in vivo* by implementing optimizations of the UCLA fluorescent miniscope v3.2. These optimizations yielded a miniscope (BLmini) which is 22% lighter in weight, has 45% fewer components, is up to 58% less expensive, offers up to 15 times stronger signal and is sensitive enough to capture

spatiotemporal dynamics of bioluminescence in the brain with a signal-to-noise ratio of 34 dB.

### 3.5. Introduction

Microscopic imaging of fluorescent indicators to track neural activity is an increasingly important tool in systems neuroscience. Over the last 9 years, portable microscopes that allow free behavior while collecting 1-photon signals have become regularly employed in freely behaving animals. This key advance became possible with the introduction of miniature microscopy (or ‘miniscopes’) in 2011 by Schnitzer and colleagues [16]. The adoption of this technology was accelerated around 2016 when a group from the University of California in Los Angeles (UCLA) released their first version of an open-source miniature microscope most commonly known as the UCLA miniscope [17]. Ever since its release, the miniscopes have been used by over 400 different groups to answer questions in domains ranging from memory representation to olfactory processing [30].



**Figure 28.** Illustration of the factors contributing to noise in the case of fluorescence (A) and bioluminescence (B).

Despite their impact, fluorescence-based imaging tools are fundamentally limited by their need to project light onto the brain. Common problems that limit imaging sessions are photobleaching and phototoxicity. The signal-to-noise ratio (SNR) for localizing discrete signals in the tissue is also fundamentally limited due to autofluorescence from unintended sources, and non-homogeneous illumination due to the scattering of excitation light ([Fig. 28A](#)). These issues become further exacerbated in the case of miniscopes, due to smaller form and cost factors compared to traditional benchtop microscopes. The weight requirement of mobile devices implanted in mice [259] inevitably constrains the optics, imaging quality and experimental duration attainable with epifluorescence miniscopes.

Bioluminescent molecular tools offer a promising alternative to fluorescent indicators. Bioluminescence is a form of “cold-light” generated via chemiluminescent reaction between a luciferase (an enzyme) and luciferin (a substrate). Many forms of bioluminescence in nature require a second factor to produce photons, such as increased levels of adenosine triphosphate [260] or calcium concentration, [261] and there has been a recent increase in the synthesis of optimized luciferases for calcium sensing [262].

Bioluminescent indicators create light within the cell of interest, and do not require excitation light, creating several benefits. Bioluminescent cells do not suffer from photobleaching, phototoxicity, or excitation scattering, and these point sources do not create autofluorescence ([Fig. 28B](#)). As such, the bioluminescent indicator strategy significantly reduces the noise ‘denominator’ of the SNR, offering more definitive localization and increased imaging depth [95]. Also, because excitation light is not

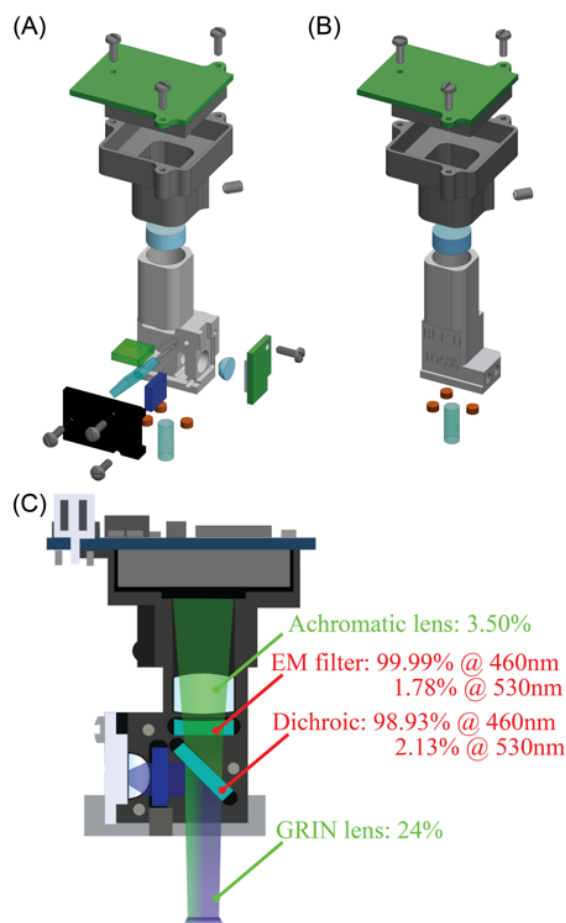
needed, the design of the miniscope can, in concept, be made significantly simpler and lightweight by removing components associated with excitation. These gains are important when considering the challenges of *in vivo* mouse imaging.

Natural bioluminescent indicators were among the first employed in neuroscience [263]. However, native bioluminescent sources had two key limitations. First, many photoproteins required achieving a complex molecular configuration, causing fast rundown of the signal. These molecules also often had slow time constants and lower total signal output. These issues have largely been addressed by recent synthetic bioluminescent indicators, but whether their signal strength is sufficient for large-scale neural imaging *in vivo* remains a major question.

To directly address this key question, we systematically modified the miniscope design for bioluminescence, and tested its capacity for detecting bioluminescent signals *in vitro* and *in vivo*. We found that this modified miniscope could robustly detect these signals, and when compared to a conventional system, showed a significant increase in signal detectability, reduction in complexity, and decrease in weight. This progress represents a key step in the refinement of this approach, which can prove transformative to an important imaging approach.

## 3.6. Methods

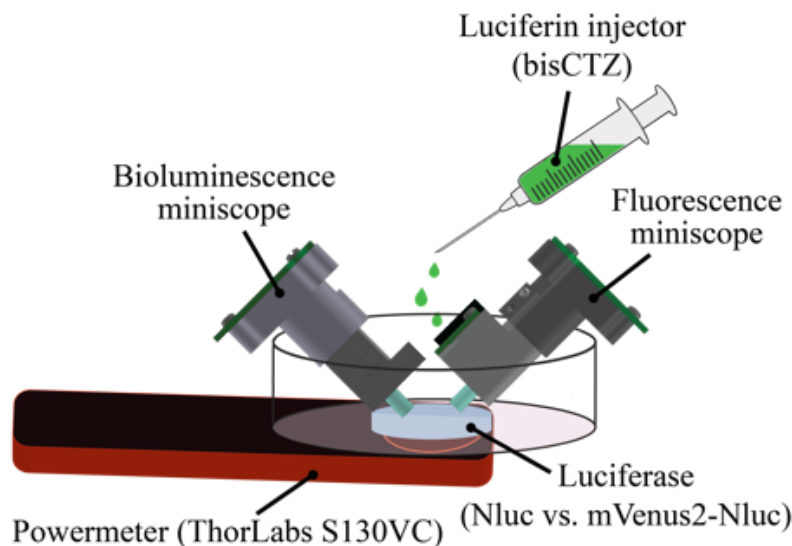
### 3.6.1. Design of bioluminescence miniscope (BLmini)



**Figure 29.** (A) An epifluorescence UCLA miniscope v3.2 [9] and (B) the optimized bioluminescence miniscope (BLmini) v1.0. (C) Cross-section of a fluorescence UCLA miniscope highlighting the percentage of total light losses caused by the optical components along the emission light path. The components marked in red were removed as part of design optimization for bioluminescence imaging. EM = emission.

The chemiluminescent generation of bioluminescence removes the need for excitation light optics, allowing beneficial simplification of the microscope's architecture. In the case of UCLA miniscope v3.2, we removed the excitation light emitting diode's (LED) printed circuit board (PCB), filters, dichroic mirror and filter set cover ([Fig. 29](#)).

### 3.6.2. Arrangement for the *in vitro* comparison of the standard UCLA miniscope and the BLmini



**Figure 30.** Experimental setup used for in vitro comparison of fluorescence and bioluminescence miniscopes. Bioluminescence signal was simultaneously tracked using a powermeter underneath a Petri dish and two miniscopes immersed in the luciferase solution.

The ability to detect light of the same intensity was compared between the two miniscopes as shown in [Fig. 30](#). Both miniscopes were placed into a Petri dish with gradient-index (GRIN) lenses (#64-519, Edmund Optics Inc.) dipped into a luciferase solution containing 150  $\mu\text{L}$  of either Nanoluc (NLuc, also known as NanOgluc; synthesized by Twist Bioscience as a part of 10,000 Free Genes Project) or mVenus2-Nluc, corresponding to 460 nm and 530 nm peak emission wavelengths, respectively. To initiate bioluminescence emission,  $\sim 150$   $\mu\text{L}$  of luciferin (bis-coelenterazine (bisCTZ)) diluted to 10-15  $\mu\text{M}$  was added. All measurements were done inside a custom-made light-tight chamber. An optical powermeter (PM100D with S130VC sensor, ThorLabs) was used to track the bioluminescence emission and control the background light inside the dark enclosure.

Miniscopes' and powermeter's signals were synchronized in time using LED flashes. By default, the UCLA miniscope's excitation LED is constantly turned on, even when set to the lowest power. To avoid confounding light contamination, the excitation

LED was disconnected from the sensor PCB during these measurements. Using UCLA miniscope's software, both miniscopes were configured to sample at 5 frames-per-second (FPS; exposure time ~200 ms) and the gain set to its maximum of 64, corresponding to a gain of 4 of the complementary metal–oxide–semiconductor (CMOS) sensor (MT9V032C12STM). To compute light intensities using acquired miniscope movies, the pixel intensity was averaged across all the pixels and normalized by the baseline pixel intensity.

### 3.6.3. *In vivo* comparison of the UCLA miniscope and the BLmini

*In vivo* testing was done on signals emanating from a mouse expressing mNeonGreen tethered to *EkL9H* luciferase (i.e. a shrimp-based luciferase variant also referred to as *NCS2*) following the injection of *pAAV-hSyn-Kozak-NCS2* virus in primary somatosensory cortex (SI) 11 weeks prior (craniotomy centered at A/P = -1.25 mm and M/L = 3.25 mm relative to Bregma, injection depth = 0.35 mm). Immediately prior to the experiment, dura was removed around the imaging area and a delivery pipette (34G, #207434, HAMILTON Inc.) brought into contact with the surface of the cortex, causing it to dimple. The injection of 1  $\mu$ L of h-CTZ (2.36 mM, Cat # 3011, NanoLight Technologies Inc.) was carried out at a rate of 1.25  $\mu$ L/min. A GRIN lens (GT-IFRL-180\_inf\_50-NC) was mounted on the BLmini and was lowered to touch the surface of the brain near the injector's needle. Using ambient light, we acquired brightfield images of the cortical surface to verify the location of the GRIN lens within the craniotomy based on vascular landmarks.

The miniscope was configured for image acquisition at 1 FPS and software gain was set to its maximum value of 64. The miniscope was powered 40 min prior to



imaging onset to minimize the impact of thermal noise. All measurements were conducted inside a custom-made dark enclosure with an animal under isoflurane anesthesia. An electron multiplying charge-coupled device (emCCD) camera (Ixon 888, Andor Inc.) with a Navitar Zoom 6000 lens system (Navitar, 0.5x lens) was used to acquire higher amplification cortical maps before and after imaging with BLmini. The exposure time of the camera was set to 10 s, and the electron multiplying gain was set to 30. Images from the BLmini and emCCD camera were aligned manually based on vascular landmarks and used to verify the correspondence between the locations of the peak signals detected by two cameras. The SNR was calculated using the following equation:

$$SNR = 20 \times \log_{10} \frac{BL_{max}}{STD(BL_{base})}, \quad (2)$$

**Equation 2.** SNR equation.

where  $BL_{max}$  is the peak bioluminescence value,  $BL_{base}$  is the baseline bioluminescence signal (i.e. the pixel grayscale values during 150 s before the injection and STD is the standard deviation. Both  $BL_{max}$  and  $BL_{base}$  were calculated for the same manually selected region-of-interest (ROI).

All procedures were conducted in accordance with the guidelines of the National Institute of Health and with approval of the Animal Care and Use Committee of Brown University.

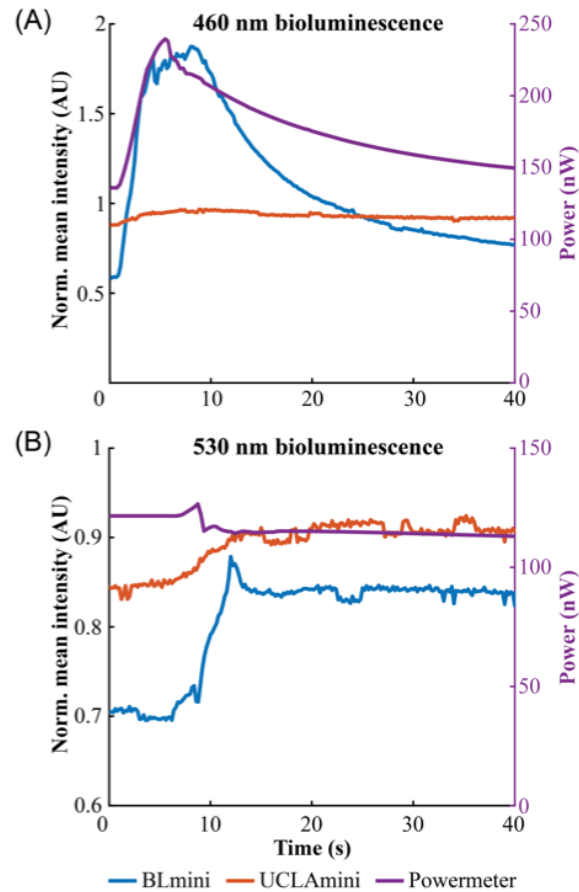
### 3.7. Results

### 3.7.1. Greater signal strength, reduced mass, cost, power consumption and assembly complexity of the BLmini

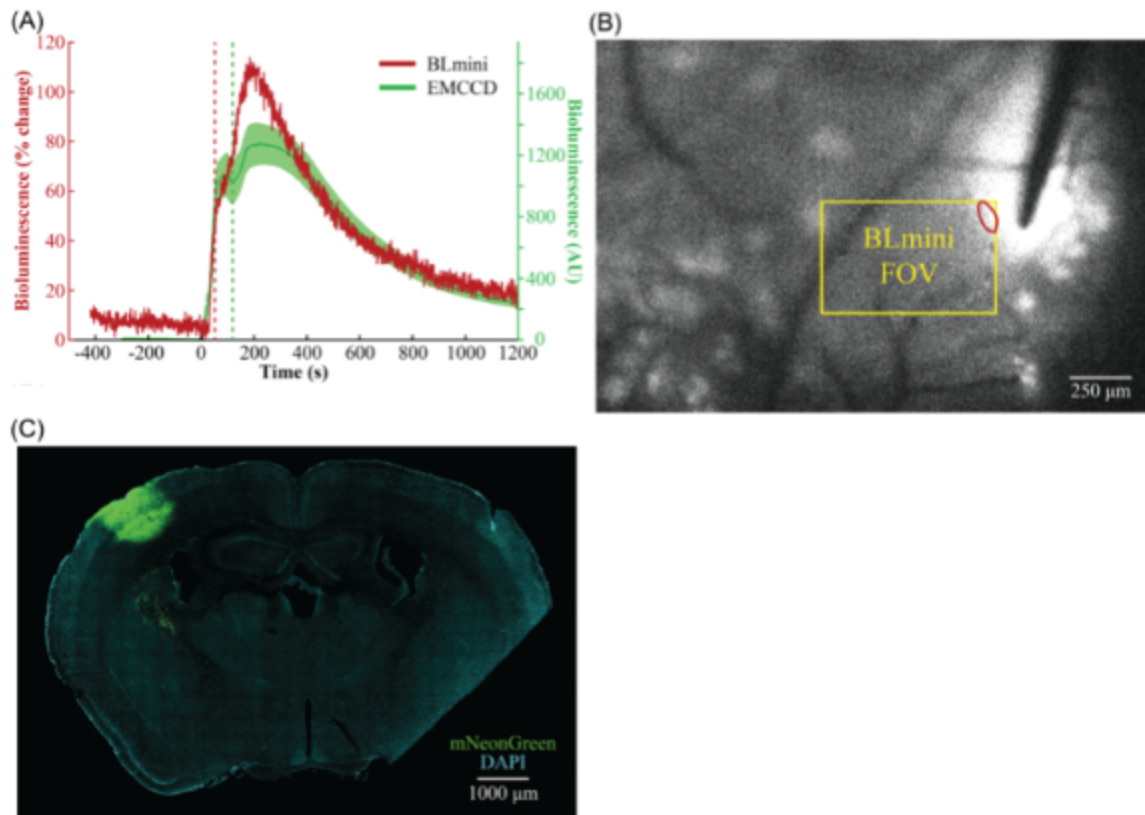
[Table 2](#) summarizes characteristics of two miniscopes. The signal strength comparison was based on the experiment summarized in [Fig. 30](#) and results are shown in [Fig. 31](#). Both miniscopes were able to detect bioluminescence from both constructs, but the BLmini showed a significantly stronger signal. The power budget in [Table 2](#) was estimated for a wire-free version of UCLA miniscope made public on 5/21/2019 [250].

|                             | <b>UCLA miniscope</b>                            | <b>BLmini</b>                                   |
|-----------------------------|--------------------------------------------------|-------------------------------------------------|
| <b>Mass</b>                 | 3.2 g                                            | 2.5 g                                           |
| <b>Number of components</b> | 22                                               | 12                                              |
| <b>Cost/scope</b>           | \$1263 (1-4 scopes)<br>\$547 ( $\geq 20$ scopes) | \$526 (1-4 scopes)<br>\$443 ( $\geq 20$ scopes) |
| <b>Signal strength</b>      | x @ 460nm<br>y @ 530nm                           | ~15x @ 460nm<br>~3y @ 530nm                     |
| <b>Power consumption</b>    | 1,100 mW (wired)<br>296 mW (wire-free)           | 957 mW (wired)<br>148 mW (wire-free)            |

**Table 2.** Summary of Comparison of the Conventional UCLA miniscope and the BLmini.



**Figure 31.** Results of in vitro bioluminescence measurements using the BLmini (blue), fluorescence UCLA miniscope (UCLAmini; orange) and powermeter (purple) for two luciferase constructs. Besides differences in wavelengths ((**A**) for 460 nm from Nluc and (**B**) for 530 nm peak from mVenus2-Nluc), two different luciferases had different emission intensities as can be seen from powermeter data.



**Figure 32.** (A) Time courses of bioluminescence emission measured using BLmini (red) and EMCCD camera (green) in two different animals. Miniscope imaging involved topical administration of h-CTZ and emCCD imaging involved cortical injection of CTZ. The injections were made at time point zero and finished at the time points marked by the dashed lines. (B) An image showing a bioluminescence map acquired using the more sensitive emCCD camera immediately following miniscope measurements after removing the BLmini with the GRIN lens from the EMCCD's FOV. Yellow ROI corresponds to the location of the BLmini's FOV and the smaller red region corresponds to an ROI for which the mean intensity was plotted in (A). (C) Confocal microscopy image showing viral expression of mNeonGreen tethered to EkL9H luciferase on the coronal brain slice.

### 3.7.2. BLmini captures spatiotemporal dynamics of bioluminescence during the initial *in vivo* measurements

The BLmini was also able to capture the spatiotemporal dynamics of *in vivo* bioluminescence with the SNR of 34 dB, as shown in [Fig. 32](#). For comparison, we overlaid time courses acquired using the BLmini and EMCCD in a different experiment

([265]; [Fig. 32A](#)). The signal captured by the BLmini also tracked more subtle features of the known bioluminescence response. These properties include an early slowing in photon production during substrate injection, thought to be due to the inhibition of photon production by the breakdown products.

A clear difference between the emCCD's and miniscope's signals lies in the baseline signal magnitude prior to the injection of h-CTZ. The CMOS sensor employed in the miniscope does not rely on any form of cooling and consequently suffers from significantly stronger thermoelectric background noise, as well as anisotropic distribution of noise across the pixels characteristic for any CMOS sensor.

By measuring the decaying bioluminescence signal using an emCCD in the present experiment after the removal of the BLmini from the field-of-view (FOV), we were able to verify the areas where we should anticipate the strongest signal ([Fig. 32B](#)). The area showing highest SNR within the miniscope's frames aligned well with the peak bioluminescence intensity area identified via emCCD, and corresponds to the viral injection site verified via histology ([Fig. 32C](#)).

### 3.8. Discussion

The data shown here, enabled by substantial modification of miniscope design, confirm that standard bioluminescent signals generated *in vivo* can be reliably detected. Further, the proposed benefits of design modification were realized: the BLmini is more sensitive, lighter, and simpler to assemble. Given the ongoing molecular innovation in bioluminescent indicators, and in miniscope sensitivity and resolution, this area of

technology development is well-positioned to fully take advantage of the benefits of the bioluminescent strategy.

Reduction in power consumption offered by the removal of the excitation LED offers even greater experimental benefits for wire-free miniscope imaging. The version of the miniscope utilized for the present work relied on an ultrathin coaxial cable to communicate between the data acquisition system and the miniscope. However, alternative designs have recently been released that allow wire-free collection of imaging data by replacing a wired data link with an SSD card [266], [267]. The UCLA wire-free design is compatible with the simplified optics presented here. Further, the wire-free fluorescence miniscope requires ~296 mW. Approximately half of this power is consumed by the current driver powering the LED, so its removal cuts the power consumption in half and prolongs battery life. A 45 mAh LiPo battery weighing 1.1 g allows continuous fluorescence imaging for ~20 min. When using bioluminescence indicators, this time can be extended up to 40 min, or the battery weight can be reduced to ~0.5 g to offer the same experimental duration of 20 min.

One key next step will be the refinement of SNR by using better imaging sensors [268], improved transmission optics and brighter bioluminescence constructs. CMOS sensors offering higher sensitivity are widely available and, as seen in [Fig. 29C](#), optimization around the objective GRIN lens alone can help to further mitigate 24% of light loss. We did not attempt a detection of calcium indicators in the current study, focusing instead on this initial existence proof. Another key next step will be systematic testing of such signals.

In the present report we demonstrate early results on detectability of bioluminescence using a modified UCLA miniscope optimized for bioluminescence imaging (BLmini). Removal of the excitation optics allowed us further to reduce the mass, cost, power consumption and assembly complexity of miniscopes. Even with the minimal modifications presented here, we were able to capture spatiotemporal dynamics that typically can be observed when using a high-end emCCD camera. Not only were we able to detect bioluminescence using a much simpler, smaller, lower-cost camera, we were able to do so at a much higher frame rate (as fast as 5 fps compared to 0.1 fps used with emCCD [265]), paving the way towards measurements of calcium dynamics via bioluminescence indicators undergoing active development. Transitioning to bioluminescence miniature microscopy can enable testing of a broader spectrum of hypotheses.

### 3.9. Acknowledgments

Authors would like to thank Daniel Aharoni for support and valuable guidance in miniscope optimization. We also thank Kimani C. Toussaint Jr. for valuable discussions around empirical testing of miniscopes, as well as Jill Juneau for valuable discussions about electrical circuit design and troubleshooting. This work was supported by grants from the National Science Foundation (NSF NeuroNex 1707352) and the National Institute of Health (NIH R01 NS108414). We thank all members of the Bioluminescence Hub ([www.bioluminescencehub.org](http://www.bioluminescencehub.org)) for helpful discussions.

# CHAPTER 4

## 4. Conclusions and future work

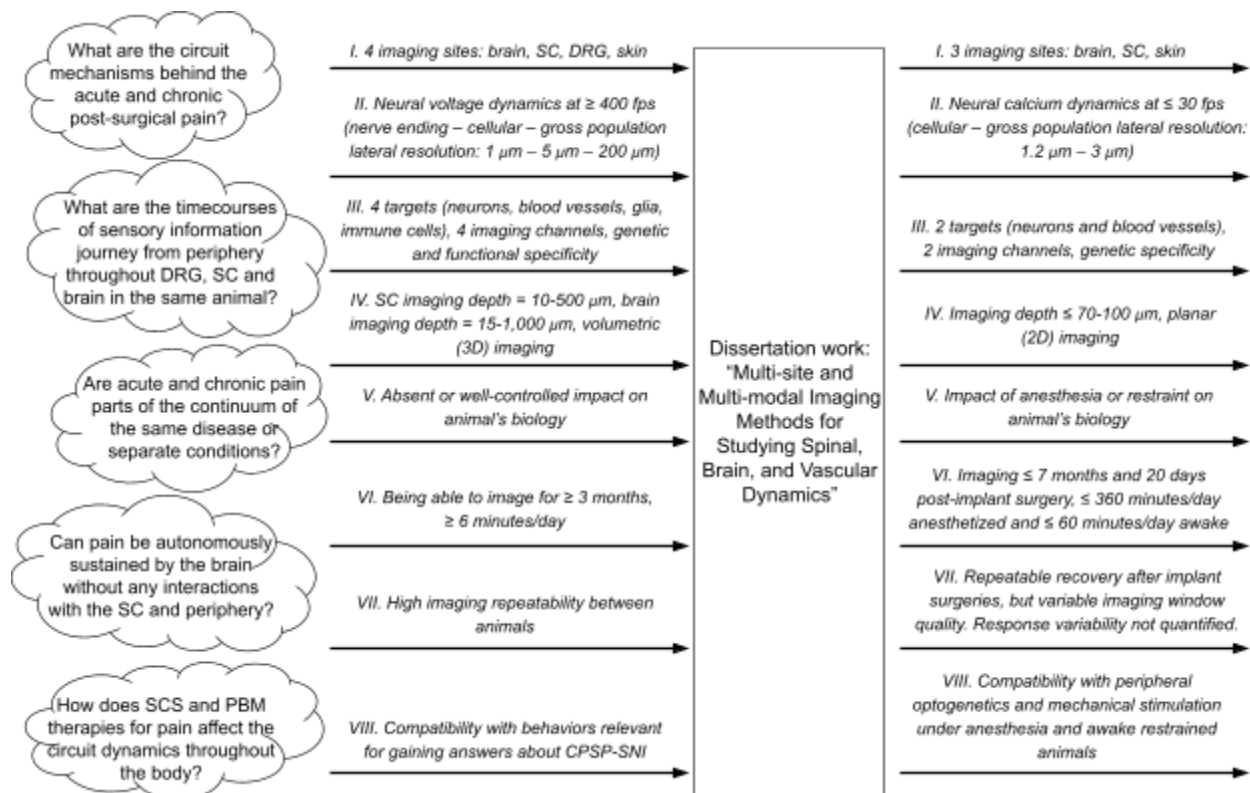
### 4.1. Conclusions

Chapter 2 of the present work describes the development of tools and methodology for gaining optical access to the multiple parts of the body in the same animal. The unique novel contributions of this part of my dissertation work include: (i) development of the low-cost, easy-to-fabricate and easy-to-customize implants universally applicable for either brain or spinal cord imaging, (ii) developing a custom surgical setup for spinal cord surgeries that offers finer control over the spinal cord during chronic surgeries, (iii) demonstrating that the same mouse can tolerate well two surgeries for the brain and spinal cord window implants, (iv) demonstrating that single surgery across both brain-spinal cord foci is feasible and can be applied for functional studies involving perfusion and calcium dynamics, (v) first ever demonstration of brain and spinal cord imaging of the responses evoked by the optogenetic stimulation of thermo-chemical nociceptors (*Trpv1*-expressing) and low-threshold mechanoreceptors (*Cacna1h*-expressing), (vi) first ever imaging of spinal cord pinch responses in transgenic *SOM x GCaMP6s x tdTomato* animals, (vii) development of the methodology of imaging across the brain-spinal cord continuum in the same animal, (viii) first ever demonstration of simultaneous imaging of 3 parts of the body (i.e. the brain, spinal cord



and the hindlimb), (ix) first ever demonstration of spinal cord vascular dilations being reliably induced by blue light in wild type animals.

Chapter 3 of the present thesis describes my progress towards imaging of low-noise low-signal bioluminescence using low-cost low-sensitivity miniature microscopes up to this date applied only towards fluorescence imaging. My unique novel contributions to the field in this chapter include: (i) first demonstration of the ability to image bioluminescence using a low-sensitivity miniature microscope, (ii) designed a new miniscope (BLmini v1.0) for imaging bioluminescence, (iii) first demonstration in practice of the benefits of bioluminescence compared to fluorescence towards simplifying the microscopy hardware.



**Figure 33.** The input (goal) specifications of the imaging method were defined at the beginning of this dissertation based on the knowledge gaps surrounding acute and chronic pain. The

output specifications of the developed imaging methodology present a significant advancement of pain researcher's toolbox. SC = spinal cord, DRG = dorsal root ganglion, SCS = spinal cord stimulation, PBM = photobiomodulation, CPSP-SNI = chronic post-surgical pain caused by sciatic nerve injury.

The overarching goal of this thesis was to contribute towards significant advancement of the imaging methodology by meeting the specifications defined in [Fig. 33](#) and, ultimately, bringing the basic and translational science pain questions outlined in Chapter 1 within the reach of the scientific community. The targeted (input) and achieved (output) specifications are summarized in [Fig. 33](#). The status of each specification can be summarized as follows:

- I. *Number of imaging sites:* in this dissertation I demonstrated and systematically documented the methodology for imaging simultaneously up to 3 sites. This is a significant advancement over current state-of-the-art 1 imaging site. For the skin imaging, I demonstrated the ability to image perfusion in the hindpaw. However, the same approach is readily transferable to other parts of the body. This has been validated during this dissertation work by imaging the skin in the ears using the same exact hardware and software as used for imaging the hindpaw (data not reported). DRG imaging has not been pursued in this dissertation, however, given the recent development of a chronic DRG implant [38] and my experience with spinal cord surgeries and implant design, it seems as a feasible and important future step to develop an implant for simultaneous DRG and spinal dorsal horn imaging within the same animal. **I foresee developing simultaneous DRG and spinal dorsal horn surgical preparation as the most challenging step in achieving 4-site imaging to fully satisfy this specification.**

II. Spatio-temporal resolution: in this dissertation I implemented calcium rather than voltage imaging. In the case of neurons, calcium has a slower temporal scale than voltage both in the brain and spinal cord, with spinal cord lamina I calcium events being slower than hippocampal CA1 pyramidal neurons [269]. As calcium signals have significantly slower temporal scales than voltage signals (on the order of 500 ms for calcium [269], [270] vs. 1 ms for voltage [214]), 30 fps is sufficient to image calcium and vascular dynamics, and is the default maximum frame rate supported by UCLA miniscope v4.4. 1.2  $\mu\text{m}$  resolution was determined via 2-photon (900 nm excitation) imaging of a calibration slide using 20x objective (LUCPLFLN20X, Olympus Inc.) with an additional optical zoom of 2x, and 3  $\mu\text{m}$  resolution was determined via 1-photon (470 nm excitation) imaging of a calibration slide using 5x objective (#46-143, Edmund Optics Inc.). These were typical objectives used in this work for calcium and vascular benchtop microscopy. UCLA miniscope v4.4 offers comparable lateral resolution of  $\sim 1.6 \mu\text{m}$ . It must be noted however that the actual lateral resolution decreases for deeper targets and the specific values provided here are the estimates of resolution at the surface rather than deep in tissue. Finally, I do not list the resolution output specifications for neural endings because I did not image individual nerve endings in my work. However, it should be noted that the methods developed in my dissertation should be directly translatable towards imaging calcium dynamics in the individual nerve endings in the skin based on the similarity of microscope hardware specifications presented here and reported for imaging nerve endings in [79]. **I foresee achieving voltage rather than**

**calcium imaging as the most challenging future step in satisfying this specification.** Even though previously developed voltage imaging methods [3] in principle should be directly translatable to multi-site imaging, this has never been done before and would require different microscopy hardware and analysis software than reported in this document. Special consideration would also need to be given to efficient handling and analysis of large datasets: 30 minutes of 1-site 1-channel miniscope imaging at 30 fps in this work generated 10 GB of compressed image data, when imaging voltage at 400 fps with the same FOV 30 minutes of imaging would yield ~130 GB, and scaling this to 3 imaging sites, as was done in my dissertation, would yield ~400 GB per 30 minutes (800 GB/hr or 13 GB/min).

- III. *Imaging targets, channels and specificity:* in this dissertation I imaged up to 2 targets simultaneously - neural calcium and blood vessels - and hence used up to 2 optical channels for imaging (i.e. channels sensitive to wavelengths surrounding green and red visible colors). To achieve genetic specificity, I tested a variety of genetic targets throughout my work (*PV*, *SOM*, *NDNF*, *CaMKIIa*, *hSYN*, *Tie2*, *Ly6g*, *Trpv1*) using AAV viruses and transgenic mice. Most of the results throughout my work were acquired using *CaMKIIa* and *hSYN* promoter AAV viruses. I have not explored glial imaging, however, I explored immune imaging in the periphery and the spinal cord. I attempted transgenically labeled neutrophil imaging acutely in the spinal cord and in the punch injury in the ear, as well as white blood cell imaging in the dextran-labeled blood vessels in the spinal cord (data not shown). Albeit targeting of cells based on their functional

phenotype would be valuable (e.g. by identifying the sub-populations of neurons responsive to nociceptive vs. innocuous stimuli, we could optogenetically suppress specifically nociceptive cells and excite specifically innocuous information processing cells, and assess its impact on pain during different stages of pain chronicity). **I foresee achieving simultaneous labeling and imaging of 4 targets as the biggest future challenges in fully meeting this specification.** Ultimately, the complexity of labeling needed to answer any given question will depend on specific imaging targets (e.g. as part of my spinal cord work I imaged white blood cells as shadows inside the blood vessels loaded with fluorescent dextran dye, hence if a given study requires imaging of blood flow and tracking of white blood cells, both targets could be acquired using a single imaging channel).

- IV. *Imaging depth and volume:* the imaging depth of 70-100  $\mu\text{m}$  is based on the deepest imaging achieved throughout this dissertation work, which involved imaging right underneath the spinal dorsal vein. The spinal dorsal vein has been reported to be 70-100  $\mu\text{m}$  deep [188]. Furthermore, the majority of my experiments involved planar (2D) imaging, with only a few trials of volumetric (3D) imaging of blood vessels. The main caveat of 3D imaging is the decrease in the sampling rate or the decrease in the FOV to accommodate additional imaging planes. **I foresee achieving high imaging depth at more than 1-site to be the most significant challenge in meeting this specification.** 500  $\mu\text{m}$  deep SC imaging with cellular resolution currently can be achieved only using 3-photon microscopy [97] and reaching 1,000  $\mu\text{m}$  depth in the brain requires at least

2-photon microscopy. Even though 2-photon microscopes are gradually becoming more accessible, the cost of 3-photon microscopes still limits their use to very few laboratories in the world. Multi-photon imaging of two sites within the same animal simultaneously would inevitably require even more customized and more costly microscope designs. Hence, more affordable and simple techniques like bioluminescence imaging are especially valuable as we scale up imaging to many targets and sites.

- V. *Impact on animal's biology:* even though throughout this dissertation multiple animals have been imaged during unrestrained behavior (data not reported), all the results presented in this thesis involved either anesthesia (i.e. isoflurane) or head-restraint. Isoflurane is known to affect neuronal [37] and vascular function [53], and restraint is known to induce stress in mice [271]. Even though at the basic level these are known impacts on animal's biology, these effects cannot be considered well-controlled. Furthermore, the impact of restrained and anesthesia on an animal's biological dynamics has never been investigated in the case of multiple implants compared to a single implant. In the present study, we are the first to assess the impact of multiple implants on an animal's weight while carefully monitoring for qualitative signs of stress. Even though we have not tested the impact of surgeries on animal's central nervous system (e.g. by directly imaging CNS function), the lack of significant changes in weight and absent qualitative stress signs suggest that the methodology reported in this thesis has the impact on animal's biology comparable to the procedures involving 1-site imaging. **I foresee achieving the absent impact on animal's biology as**

**the biggest (and maybe impossible) challenge towards satisfying this specification.** However, eliminating the anesthesia and restraint, minimizing surgical invasiveness and increasing surgical repeatability, and minimizing imaging invasiveness (e.g. by imaging through the skull or between the vertebrae, and relying on intrinsic signals) are the examples of important advancements that allow better control over undesirable biological confounds.

- VI. Imaging chronicity: even though the maximal post-implant pre-imaging duration achieved in this thesis significantly exceeds the pre-defined specification (i.e. 3 months vs. 7 months and 20 days), it is important to note that as part of my dissertation work I did not methodically track the same exact neurons, blood vessels or regions of interest throughout the entire 7 months and 20 days. Rather, I was able to image responses within different regions of interest of the implanted window as late as 7 months and 20 days after the surgery. The methodology developed in this dissertation offers access to neurovascular dynamics across brain-spinal cord for significant durations spanning acute and chronic stages of pain. However, in assessing the role of individual cell dynamics in development of pain it is particularly important to be able to track the same cells and blood vessels throughout months. **I foresee achieving the ability to image the same exact functional units in 4 sites for  $\geq 3$  months as a significant future challenge.** This will be especially a challenge in specific preparations, for instance, requiring restraint (especially by the vertebral column, which has a more limited long-term stability compared to the head restraint because of higher mobility of the animal's back). On the other hand, in the

preparations allowing imaging under anesthesia, methodology developed here is readily translatable for long-term tracking of the same targets.

- VII. *Imaging repeatability*: it has been demonstrated in this dissertation that different animals recover consistently well after multiple surgeries needed for multi-site imaging, as well as after multi-site imaging sessions. It was also demonstrated that different animals are consistently responsive to the stimuli employed in this study. However, the repeatability of the response features across animals has not been assessed in my dissertation. It can be summarized qualitatively that responses and the quality of implanted windows seemed to be variable between animals. However, the observed variability makes perfect sense given that as part of the methodological development in this dissertation it was required to test as many iterations as possible to identify what works and what does not rather than studying the specific circuits and questions. Hence, it was not aimed (especially at the beginning) to precisely replicate the spinal levels of the implanted windows (the implants were attempted across all the spinal levels between L1-L6) or the specifications of AAV injections (both for brain and spinal injections a variety of injection rates, depths, volumes, injection counts and XY coordinates have been tested). By fixing these variables right from the beginning, there was a risk of converging to conditions that would yield poor imaging results right from the beginning. **I foresee the repeatability of the implant procedure between different surgeons as a significant potential challenge in fully satisfying this specification.** Repeatability between the surgeons becomes particularly challenging once the complexity of the surgery increases and

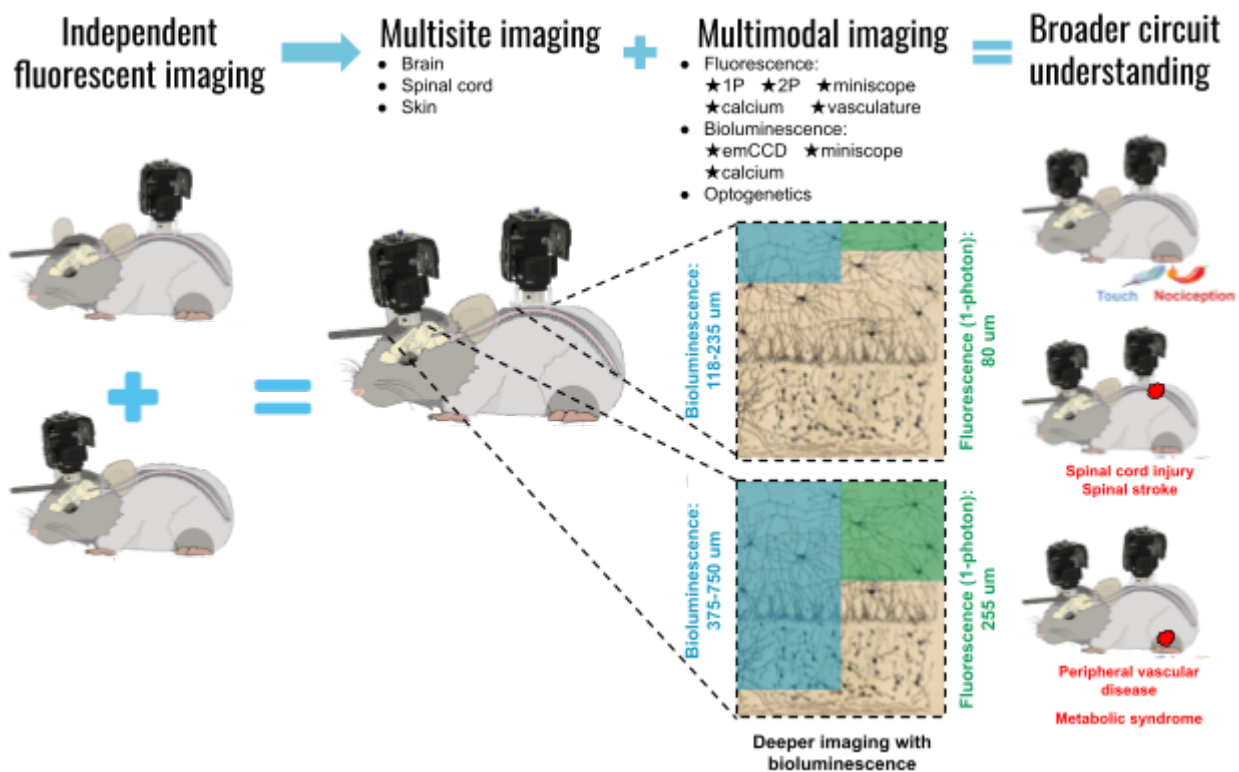


achieving repeatable, high quality imaging across 4-sites can involve a significant amount of surgical skill, for instance, in the cases where chronic invasive access is needed to the brain, spinal cord and DRG within the same animal. Continuing the development of carefully documented protocols as presented here is part of the process of fully satisfying this specification.

- VIII. *Compatibility with behaviors:* in this dissertation I demonstrated the ability to image multiple sites in the context of peripheral optogenetic, pharmaceutical and mechanical stimulation under isoflurane and awake restrained behaviors. I also demonstrated the ability to image the spinal cord in the case of direct optical stimulation under anesthesia, as well as multi-site imaging of background calcium dynamics in awake unrestrained animals during the transition from anesthesia to awake state and during the awake exploration of the homecage (data not shown). The behaviors implemented in this dissertation can already offer access to the new deeper insights into processing of noxious and innocuous stimuli as was illustrated in [Fig. 9](#). However, the repertoire of accessible behaviors should be expanded even further. **I foresee achieving the compatibility of 4-site imaging with stimulation (for instance, optical, mechanical, electrical, etc.) in freely behaving animals as a significant future challenge in fully satisfying this specification.**

In summary, my thesis presents a first detailed development of methods towards being able to study the brain and spinal cord within the same animal. Developed surgical and imaging procedures were applied to demonstrate the ability to image both the spinal cord and the brain within the same animal sequentially and simultaneously

presenting the types of datasets that have not been accessible to scientists before. The multi-site imaging approach presented here is not limited to a single imaging modality. I demonstrate that this methodology can be used with benchtop 1-photon and 2-photon microscopes, and wearable 1-photon miniscopes to image both the vascular and calcium dynamics chronically for over 6 months after surgery. My contribution further expanded towards a more long-term development of new calcium imaging indicators based on bioluminescence in pursuit of theorized benefits of improved SNR and imaging depth. I demonstrated the ability to image bioluminescence with redesigned miniscopes *in vivo* and showed in practice the simplification of microscopy hardware. Altogether, this thesis will help the forthcoming scientists to broaden our understanding of circuits in health and disease.



**Figure 34.** Visual summary of the dissertation: the progress towards multi-site and multi-modal imaging broadens circuit understanding and improves the quality of science by measurement

and control of foci spanning brain and spinal cord within the same animal. The background images of neurons were originally created by Santiago Ramón y Cajal and are in the [public domain](#).

## 4.2. Limitations

One of the holy grails and my dream as a student and a scientist was to see whether the endogenous light sensitivity effects (such as the ones demonstrated in [Fig. 16C](#)) have any distant effects throughout the body away from their focal origin site. For instance, does the local modulation of vascular dilations in the spinal cord affect in any way neurovascular dynamics in any part of the brain? Within the constraints of this dissertation all the spinal cord vascular modulation experiments were performed without concurrent imaging of the brain and vice versa, I analyzed some of the brain vascular data available in the lab which did not have associated spinal cord data. Furthermore, it has been shown that for the pulmonary arteries the endogenous blue light sensitivity effects are potentiated by inhibiting G protein-coupled receptor kinase (GRK2) [106], [107]. It has been my long-standing dream to investigate the effects of the GRK2 inhibitor on the endogenous effects of blue light. Besides the potential of GRK2 inhibitor to enhance the endogenous light responses, awake state over anesthetized state (as was studied in this dissertation) holds a promise for demonstrating more potent responses [53]. Hence, the ideal experiment which I never had a chance to pursue would involve the use of GRK2 inhibitor in awake animals.

Another limitation was the studying of the endogenous effects of light on the penetrability of the blood-spinal barrier. This is a limitation of this work only in my personal view only because the tools that I developed are well positioned to investigate this question and I had a chance to pursue only preliminary experiments. However, I

concluded that to address this question more meticulously I would need to beam shape the stimulation to the specific blood vessels and image the blood vessels in 3D to obtain a detailed spatial profile of the flow of dyes through the blood vessel walls without any ambiguity (especially in the spinal cord, where even under anesthesia there is a lot of tissue motion). The pursuit of questions around the modulation of blood-spinal barrier via endogenous light sensitivity requires a significant investment into refining the vascular imaging methods that were available to me and was not a compatible task with the bioluminescence and multi-site imaging work.

Another personal limitation of this study is that I took it as a personal challenge to demonstrate that we can image calcium responses to whisker stimulation using bioluminescent indicators in awake animals. The extensive efforts have led me to conclude that although the bioluminescent indicators that I tested thus far showed many promising putative responses, the extremely low signal makes it challenging to claim the reliable detection of calcium with bioluminescence. Hence, my quest for observing the undeniable bioluminescent calcium responses remains incomplete. It remains my dream to continue the pursuit of bioluminescent indicators, including the indicators for voltage and neurotransmitters, and experimentally assessing all the benefits of bioluminescence imaging over other imaging modalities.

Another limitation is that as part of the demonstration of multimodality of our methodology, I was hoping to demonstrate our ability to image the immune system responses. I attempted imaging neutrophil recruitment to the wound in the ear and the spinal cord in C57BL/6-Ly6g(tm2621(Cre-tdTomato)Arte) animals with transgenically labeled neutrophils [272]. However, in the cases of both animals I was not able to

reliably identify the recruitment of neutrophils. The only immune cells that I managed to image successfully throughout my work were the putative white blood cells rolling down the walls of the vessels in the spinal cord.

Finally, the challenges with tissue fibrosis limiting the lifespan of spinal cord windows present a significant concern for me as a developer. My main source of discomfort comes from the fact that our assessment of window quality similarly to others [33] is qualitative and primarily relies on visually observing the morphological changes or observing the loss of signal. A more ideal approach would involve systematic measurements of fibrosis for different surgical preparations to identify the best approach. However, this is a substantial study on its own, which hopefully will be undertaken by me or others in the future.

### 4.3. Future work

A futuristic dream of my ideal experiment involves imaging the **neuro-glio-vasculo-immune dynamics at the same time** not just in the brain and spinal cord, but **throughout the entire body**. A more realistic and yet short-term futuristic is the goal outlined in this dissertation of achieving **4 imaging sites**. At this stage, 4-site imaging is a futuristic goal akin to how 2-site and 3-site imaging were futuristic goals at the beginning of my dissertation. The involvement of different parts of the body in every action scales up or down depending on the bodily needs during any given action, but even with this scaling in mind, every action can be considered as a full mind-body experience. Hence, in order to develop a full mechanistic understanding of any process happening in animal's body, we need to understand not only how each part

of the body functions by itself, but also how these parts interact and function together as parts of a single system. How do different parts communicate together and what happens when this communication is altered? It would be fascinating to assess how far the limit can be pushed on the number and complexity of surgical procedures and the number of imaging implants within a single animal without adverse effects on animal's health. However, at some point increasing the count of implants and surgical impactfulness on biology becomes infeasible. Hence, the further away we look into the future, the greater is the need for non-invasive methods of monitoring bodily dynamics throughout the entire body.

An important avenue of future work in the domain of functional microscopy across multiple parts of the body involves the need of **efficient labeling of targeted structures and processes**. As we go beyond 2 implant sites, the surgical process for focal viral injections would become more challenging. Transgenic animals, systemic viruses and retrograde labeling become more important. For systemic or retrograde labeling it could be beneficial to have viruses encoding the **conjunction of bioluminescence and fluorescence** because the more implants we add, the harder it gets to synchronize the time courses of good expressions at each site. The viruses employed in my work already involved such conjunction, however, bioluminescent molecules were dependent on calcium and fluorescence was static. In the molecular design I am describing here, the bioluminescence would be static and fluorescence functionally variable (e.g. calcium dependent). Using bioluminescence, we could track the trajectory of viral expression outside of the body and perform implantation only once each site reaches the appropriate level of expression (similar to tumor tracking by Figley

et al. 2013 [245]). This could be useful even in the case of systemic viruses. If systemic viral expression is not perfectly repeatable for a given animal model or the viral labeling protocol simply has not been established yet to achieve repeatability, the bioluminescence imaged outside the body could be used to correlate the strength of bioluminescent signal with the quality of expression of fluorescent proteins (an indirect estimate correlated with the quality of fluorescent signal during imaging). Once the bioluminescent signal reaches a certain relative level in the part of the body of interest, the scientist can have an informed confidence in the quality of fluorescence expression and know the timeline for surgical intervention. Of course, the expression of additional proteins becomes an additional experimental confound, however, such expression of multiple fluorescent proteins is a common practice, there is no reason to expect any unique adverse effects from bioluminescent proteins compared to the effects of fluorescent proteins. There is also room for future developments of **temporally controlled** expressions of such **combo fluorescent-bioluminescent constructs** to constrain the bioluminescent expression only until the expression of fluorescent molecules reaches the right level. One can even fantasize about photoswitchable configuration where once the fluorescent molecules reach sufficiently strong expression, they could be excited by bioluminescent molecules sufficiently to induce fluorescent emission of sufficient energy to permanently photoswitch OFF the bioluminescence.

It also seems important to continue improving on the surgical preparation involving **a single surgery for brain-spinal cord implants** because it yields more consistent labeling and tissue healing timelines between the implant sites and reduces the number of cycles the animal has to undergo a surgical procedure. Relatedly, similar

single-surgery developments would be beneficial for other sites of interest, for instance, a single surgical procedure for brain, spinal cord and DRG.

On the behavior side, the vertebral, head and hindpaw restraints offer the best stability for optomechanical plantar stimulation of the hindpaw and 3-site restraint can be readily applied in anesthetized animals, but applying the **hindpaw plantar stimulation with 4-site imaging in freely moving animals** is the ultimate best case experimental scenario. In the present study I relied on multi-functional implant design that allows interchangeable imaging in either vertebrally restrained or freely moving animals to study sensory information processing with the hindpaw plantar stimulation. The restraint reduces the risk of hindpaw displacement from the optomechanical stimulator. The experimental design involving the restraint by the head, the vertebral column and the hindpaw was inspired by the previous work done in our lab on mouse self-reported nociceptive behavior [126]. However, the spinal cord studies summarized in [Table 1](#) show a clear trend towards imaging in the animals with freely moving vertebral columns using wearable microscopes rather than restraining the back. This trend contrasts the dominant approaches that were pursued in the literature at the beginning of my PhD which involved restraining the vertebral column [33], [37]. Achieving a stable spinal preparation requires a significant amount of skill and labor, and an animal's restraint reduces the longevity of even the best crafted implants because of the additional forces generated by the animal under the vertebral restraint conditions. The amount of forces exerted over the implant by restraint can be significantly reduced by extensive, gradual habituation to restraint. One example of previously reported restraint approaches involved the habituation to the head restraint

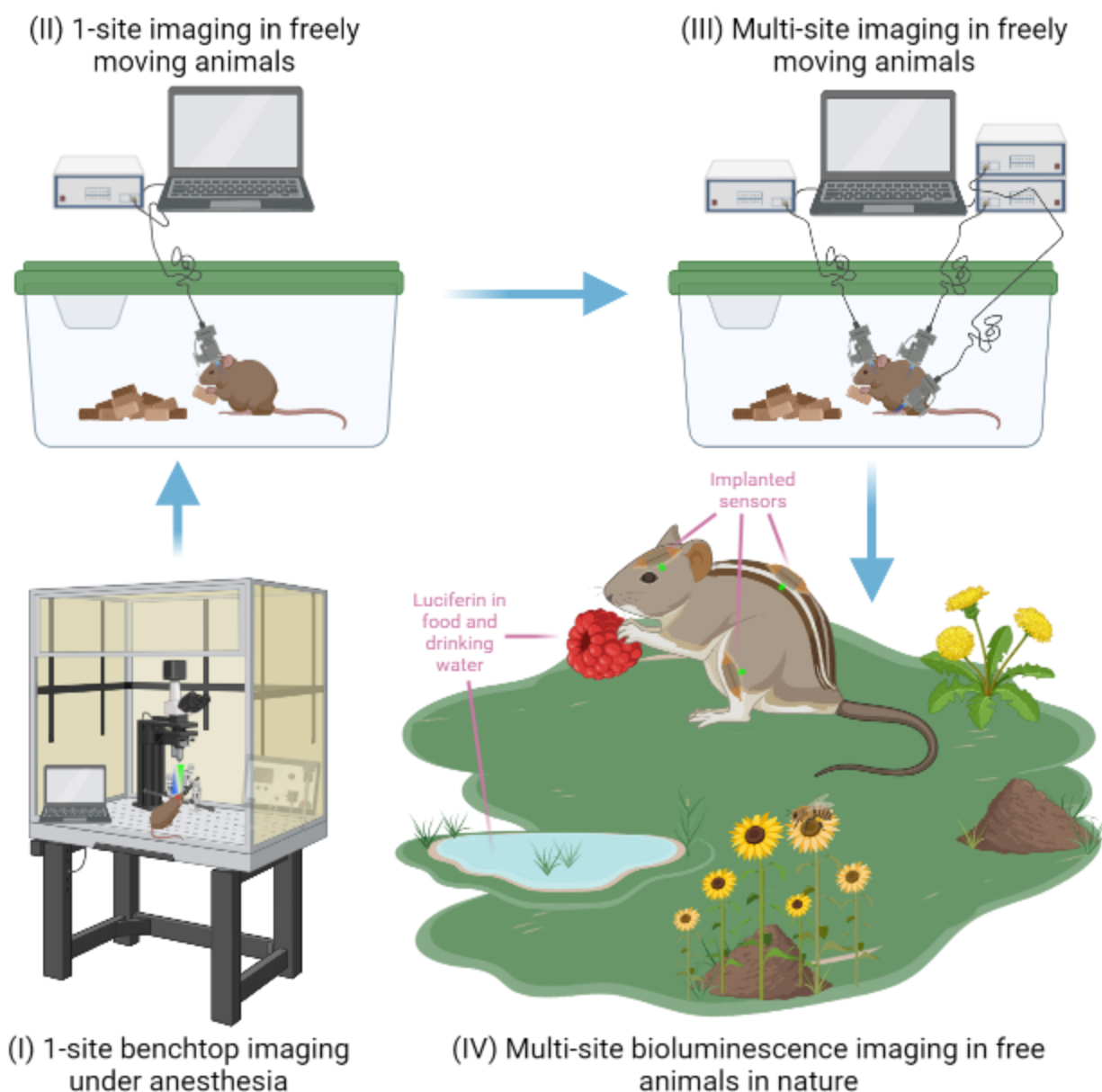


first, followed by the habituation to vertebral restraint [37]. In this example of gradual head-vertebral restraint, head implant was used only for restraint and did not provide an access to the brain for imaging. However, habituation for vertebral restraint can require a significant investment of time and labor because of sensitivity of the quality of spinal windows presented here to external forces (this is a qualitative observation from my dissertation work). Hence, I predict that keeping the animal free of any restraint is the best way of achieving the longest possible spinal cord window longevity. It is important to keep in mind though that it becomes challenging to apply consistent hindpaw plantar stimulation in unrestrained animals when studying the responses to hindpaw skin inputs [126]. The more ideal and naturalistic experimental design would involve imaging in freely moving animals with an implanted optogenetic stimulator [68], [69], [273]–[276] or it would be even better to avoid surgical confounds by applying optogenetic stimulation through the floor while tracking animal's paws in space [277].

In the realm of bioluminescent work, it is important to continue developing and testing new **more sensitive microscopes** and **higher quantum yield bioluminescent indicators in awake animals with systemic administration of luciferin**. Given how similar were the vibrissal responses that I imaged in awake animals using systemically administered fluorofurimazine (FFZ) to responses imaged in the same animals using fluorescent indicators (data not shown), it feels that the development of the bioluminescent indicators is close to yielding clear calcium responses in awake behaving animals. However, based on the current state-of-the-art, it seems that *in vivo* bioluminescent imaging with individual neuron-resolution and sub-second temporal resolution with systemic administration of the luciferin is still far away from being

achievable. Similarly, achieving imaging of bioluminescence in freely behaving animals will require substantial development of more light-tight wearable microscopes-implant systems and more sensitive cameras with better cooling. One promising direction of imaging hardware development includes the use of single-photon avalanche diode (SPAD) array cameras [278]. On the implant hardware side, it could also be beneficial to utilize the **soft cranial windows for repeatable direct cortical injections** of luciferin. Direct cortical injections offer the brightest bioluminescence, but in our hands are usually implemented terminally due to high invasiveness. Using less invasive injectors and soft cranial windows could expand the utility of every animal and offer chronic multi-day studies. Beyond repeatable direct cortical injections, it is important to explore other luciferin administration routes that could extend the chronicity of bioluminescence experiments even further, for instance, **imaging chamber implants with microfluidics system for perfusion, transparent hydrogels with controlled release of luciferin placed under the imaging window, systemic pumps for luciferin infusions, drinkable forms of luciferins** [279], [280], and **synthesizing luciferins inside the body without the need for exogenous administration** [281]. Finally, my biggest dream is to be able to image anything within any part of the body without any significant negative impact on natural biological dynamics. I foresee an important place for bioluminescence in our journey towards achieving this dream. Because bioluminescence imaging can be done using the simplest possible optics (e.g. some bioluminescence imaging in my work was done using a smartphone) and the ability to trigger the emission of bioluminescence via natural sources, for instance, via drinking water, bioluminescence imaging seems to be the perfect modality for the development

of **truly miniature, wireless, fully implantable microscopes**. Animals implanted with such microscopes could engage in behaviors of any nature and complexity indistinguishable from the animals found in the wild. Development of flat microscopes with the optical hardware replaced with computation [282], [283] is an already existing example of feasibility and progress in this direction for fluorescence imaging. Bioluminescence however would not require the additional computations to account for missing optical hardware and, as was estimated in Chapter 3, could decrease the power consumption for a wireless miniscope by half. Realizing the full potential of bioluminescence could further benefit from expanded scientific exploration of unique combinations of different physical modalities, for instance, applying ultrasound (or other physical phenomena, ultrasound being the best established example) for **focal release of luciferins across the blood-brain-barrier** [284], [285], enhancing the intensity (by reducing light scattering) and increasing spatial resolution of bioluminescence using optical wavefront modulation via ultrasound (aka. **ultrasound-enhanced chemiluminescence**) [286], [287] or microwaves (e.g. signal enhancement by increasing tissue temperature via microwaves) [288], and **measuring the ultrasound signal generated by bioluminescent molecules** (similar to how this has been demonstrated for fluorescence imaging of calcium indicators [289], except in the case of bioluminescence the imaging setup in principle can be significantly simplified because of the lack of need for any optical components and hence the lack of need for advanced custom setups combining lasers with ultrasound detectors; the specifications of fluorescence, bioluminescence and optoacoustic imaging have been compared by Pirovano et al. J Nucl Med. 2020 [290]).



**Figure 35.** In the past two decades the state-of-the-art functional imaging relied on benchtop microscopy of a single target under anesthesia (I). Recent development of wearable miniature microscopes allowed the transition towards imaging during more naturalistic behaviors, albeit still of a single part of the body (II). Present thesis work paves the way towards multi-site imaging using benchtop and wearable microscopes in anesthetized and freely moving animals (III). Tools like bioluminescence microscopy hold a lot of promise for a brighter future where we can study the biological dynamics of animal body in their natural habitat (IV). Created with [BioRender.com](https://www.biorender.com/).

Finally, more near future experiments of my dream, which make sense based on the progress presented here, include **calcium imaging simultaneously in the brain, spinal cord and the nerve endings in the skin** to better understand the mechanisms of pain and sensory information processing. After establishing the imaging at 3 sites, the logical and necessary next step is to transition away from calcium towards voltage imaging. Once we can **image voltage at 3 sites**, we can start transitioning towards expanding the imaged targets both in terms of systemic diversity (e.g. immune, nervous and vascular system at the same time), cellular diversity (e.g. parvalbumin and somatostatin neurons) and the different parts of the body. For instance, dorsal root ganglia imaging in conjunction with the brain, spinal cord and the skin seems very feasible based on the methodology presented in my dissertation. In the realm of pain and other diseases in general, I am particularly fascinated to apply the multi-site imaging approaches to gain better understanding of currently poorly understood modulatory interventions, such as photobiomodulation therapy [291], focused ultrasound therapy [292], [293], sonodynamic therapy [294], [295], spinal cord stimulation for back pain [152]–[154], purposeful harnessing of the placebo effect [296], [297], transcutaneous vagus nerve stimulation [298], bimodal sound and body stimulation [299], transcranial alternating current stimulation [300], and unique personalized combinations of non-invasive therapies (e.g. exercise + mindfulness + photobiomodulation). I consider the above modulatory interventions as particularly good questions to study using multi-site imaging approaches that I developed not only because of my personal fascination about non-invasive, understudied, non-mainstream interventions, but also because these kinds of interventions involve rather complex

mechanisms that likely require looking at multiple targets and multiple parts of the body at the same time (as does pretty much any process in the body).

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