

Optical Electrophysiology of Engineered Three-Dimensional Cortical Microtissues Using
Genetically Encoded Voltage Indicators

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B.Sc. University of Massachusetts Amherst, 2021

Thesis

Submitted in partial fulfillment of the requirements for the
Degree of Master of Science in Biomedical Engineering at Brown University

Providence, Rhode Island

May 2026

This thesis by William J. Ash III is accepted in its present form by the Department of
Biomedical Engineering as satisfying the thesis requirements for the degree of Master of
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Abstract

Engineered three-dimensional primary cortical microtissues provide a physiologically relevant in vitro model of cortical network organization, but their electrophysiological characterization has been limited to calcium imaging and extracellular multielectrode array recordings, which do not capture membrane potential dynamics or subthreshold voltage activity at single-cell resolution. This thesis investigates the integration of Voltron2, a chemigenetic genetically encoded voltage indicator, into an established three-dimensional cortical microtissue platform to enable optical electrophysiology at the level of individual neurons. Four primary objectives were addressed: validation of Voltron2 expression and dye labeling under Borton Lab culture conditions, optimization of adeno-associated viral titer for transduction, identification of acquisition parameters required for voltage signal detection, and development of an electrical stimulation platform compatible with simultaneous confocal imaging. Dual-vector AAV transduction produced sparse, neuron-specific Voltron2 expression, confirmed by immunohistochemistry, while viral titer was found to be a key determinant of optical separability, with higher titers producing overlapping fluorescence that limited single-cell resolution. Stimulus-evoked voltage responses were detected in individual neurons at later culture timepoints. In parallel, impedance characterization of a custom stimulation plate cover identified a frequency range that enabled reproducible and consistent field stimulation across electrode sites. Together, these results establish a framework for applying genetically encoded voltage indicators to three-dimensional neural microtissues and demonstrate the feasibility of resolving single-cell voltage dynamics in a physiologically relevant in vitro system.

Ch. 1 — Background

1.1 — Cortical Electrophysiology and the Need to Measure Voltage

The cerebral cortex is the seat of the brain's most sophisticated computation capacities, including sensory perception, motor planning, memory formation, and executive function. These capacities arise from the coordinated electrical activity of neurons organized in laminar circuits that span cortical columns and communicate across long-range projections. At the cellular level, information is encoded and transmitted through rapid, highly regulated changes in membrane potential caused by voltage differences maintained across the plasma membrane by the active and passive flux of ions, principally Na^+ , K^+ , and Cl^- . The resting membrane potential of a typical cortical neuron sits between -60 and -70 mV, reflecting the electrochemical gradients established by Na^+/K^+ -ATPase pumps, leak channels, and the selective permeability of the plasma membrane. When sufficient net depolarizing drive elevates membrane potential to threshold, voltage-gated sodium channels open in a regenerative positive-feedback loop that produces a rapid, stereotyped action potential (AP) lasting approximately 1-2 ms. This AP then propagates along the axon to drive synaptic release onto downstream neurons.^{1,2}

Yet the AP represents only the final output of a richer and more continuous computational process happening at the subthreshold level. Cortical pyramidal neurons receive thousands of excitatory glutamatergic and inhibitory GABAergic synaptic inputs distributed across apical and basal dendritic compartments that extend hundreds of micrometers from the soma. The resulting local voltage fluctuations of excitatory and inhibitory postsynaptic potentials (EPSPs and IPSPs), dendritic plateau potentials, and

slow oscillatory membrane potential modulations collectively determine whether and when the cell will fire. These subthreshold dynamics are not passive filters but rather active conductances distributed across the dendritic membrane. Voltage gated sodium and calcium channels, hyperpolarization activated cyclic nucleotide-gated (HCN) channels, and A-type potassium channels amplify, attenuate, or reshape synaptic signals as they propagate toward the axon initial segment. Human cortical layer 2/3 pyramidal neurons, for example, express graded calcium-mediated dendritic action potentials (dCaAps) that enable single neurons to classify linearly non-separable inputs. This computation is attributed to multilayer networks highlighting that subthreshold dendritic computation is not merely a passive integrative process but an active substrate for neural logic.^{3,4}

At a network level, groups of cortical neurons oscillate synchronously over frequency bands from slow delta rhythms (<1 Hz) associated with sleep to high frequency gamma oscillations (30-150 Hz) that correspond with attentive, active processing. These rhythms, which come from the interplay of recurring excitation and feedforward/feedback inhibition, support working memory maintenance, and bind distributed representations into coherent percepts. Disruption of these oscillatory dynamics underlies pathological states including epilepsy, schizophrenia, autism spectrum disorder, and Alzheimer's disease. Critically, these oscillations are generated at the level of individual cellular conductances and synaptic dynamics, a mechanistic substrate that is inaccessible to solely extracellular recording methods, which capture only the summed synaptic currents and spiking activity of neuronal populations without resolving the membrane voltage of individual cells.^{5,6}

The motivation to directly measure membrane voltage extends beyond fundamental circuit neuroscience into the domain of human disease modeling. The development of induced pluripotent stem cell (iPSC) technology has made it possible to derive patient-specific cortical neurons and to assemble them into three-dimensional organoids and microtissue platforms that recapitulate key features of early human cortical development, including the spontaneous emergence of synchronous network oscillations reminiscent of preterm human EEG activity.^{7,8} Exploiting these models for mechanistic disease studies and drug discovery requires electrophysiological measurement approaches that are compatible with their complex, optically scattering three-dimensional architectures and that can report activity from identified cell types without invasiveness of electrode implantation. Neither conventional extracellular recordings nor standard calcium imaging fully meet these requirements, motivating the development and application of genetically encoded voltage indicators (GEVIs) capable of reporting membrane potential dynamics within cellular resolution in dense, heterogeneous three-dimensional tissue.⁹

The following sections trace the conceptual and technical trajectory that has brought the field to present moment from the foundational techniques of patch-clamp electrophysiology to the emergence of optical voltage indicators, the specific development of Voltron chemigenetic GEVI platform, the biology and fabrication of engineered cortical microtissues, and the remaining challenges of combining optical electrophysiology with three-dimensional neural tissue models. Together, these sections establish the rationale and context for the experimental work presented in this thesis.

1.2 — Traditional Electrophysiological Techniques and Their Limitations

The patch-clamp technique, introduced by Neher and Sakmann in the late 1970s and recognized with a Nobel Prize in Physiology or Medicine in 1991, remains the gold standard for measuring ionic currents and membrane potential in individual neurons with high fidelity. In its whole-cell configuration, a fire-polished glass pipette with tip resistance between 2 and 10 M Ω is pressed against the neuronal membrane and suction is applied to rupture the underlying membrane patch, allowing the intracellular compartment to equilibrate with the pipette solution and permitting both current-clamp (voltage measurement under injected current) and voltage-clamp (current measurement at fixed voltage) recordings. This configuration provides direct, millivolt-resolution access to the full membrane potential waveform including resting potential, subthreshold depolarizations, individual synaptic potentials, and the precise timing of action potentials at a temporal resolution limited only by the amplifier bandwidth, typically within the range of tens of kHz.^{10,11}

Despite its unmatched signal quality, patch-clamp electrophysiology carries fundamental constraints that limit its applicability to the large-scale, long-duration, or three-dimensional recording scenarios demanded by modern systems neuroscience and tissue-based disease modeling. Manual whole-cell patching is technically demanding, requiring skilled operators to navigate a micropipette under visual guidance, achieve a giga ohm seal, and maintain recording stability for tens of minutes to hours. In practice, an experienced electrophysiologist can record from approximately 2-4 cells per day and attempts to record from more than a handful of cells simultaneously require specialized multipipette rings that remain labor-intensive and difficult to scale.^{11,12} The physical

insertion of a pipette into tissue also causes mechanical disruption and dialysis of the intracellular milieu, progressively altering the biochemical environment of the cell being recorded. In three-dimensional preparations such as brain slices and organoids, the mechanical constraints of pipette navigation further restrict recording to cells near the tissue surface, leaving deep layers inaccessible. Finally, patch clamp provides no intrinsic cell-type specificity. Without prior labeling or identification under fluorescence, the recorded cell type cannot be determined from the recording alone.^{12,13}

Sharp intracellular microelectrodes, thin tipped glass pipettes with tip resistances of 30-100 MΩ filled with a concentrated electrolytic solution, predate the patch clamp and were the primary tool for intracellular recording for several decades. They offer the advantages of recording membrane potential with minimal dialysis of the cytoplasm, making them better suited for studying intracellular signaling cascades and second-messenger dynamics in their native biochemical context. However, sharp electrodes impose significant electrical access resistance that introduces noise and distorts the recorded voltage waveform, particularly during fast action potentials. Like patch clamp, sharp electrodes are limited to single-cell or sparse multi-cell recordings and require substantial manual skill. Extracellular field potential recording with metal or silicon microelectrodes offers a complementary approach: these electrodes can be placed in or near neural tissue without seal formation, enabling stable, minimally invasive recordings of multi-unit spiking activity and local field potentials (LFPs) across timescales ranging from minutes to weeks. Field recordings have been invaluable for mapping circuit-level dynamics, identifying oscillatory phenomena, and studying plasticity in animals.^{14,15}

Multielectrode arrays (MEAs) scale the extracellular recording approach by arranging tens of thousands of electrodes in planar or three-dimensional configurations, enabling simultaneous recording of spiking activity from large neuronal populations. Modern complementary metal-oxide semiconductor (CMOS) MEA platforms achieve electrode densities of thousands per square millimeter with interelectrode pitches below 20 μm , approaching the scale of individual neuronal somata and even subcellular structures. These devices have been widely adopted for studying network-level activity in dissociated cultures, brain slices, and more recently, iPSC-derived neuronal preparations and organoids.^{16,17} Nonetheless, conventional MEAs share a fundamental limitation: they record extracellular field potentials, which represent the summed transmembrane currents of surrounding neurons and neuropil. This extracellular signal is substantially attenuated compared to the true intracellular membrane potential, and the translation between extracellular spike waveforms and intracellular voltage is confounded by the geometry and impedance of the cell-electrode interface. Consequently, planar MEAs are entirely blind to subthreshold synaptic potentials, dendritic dynamics, and the oscillatory membrane potential fluctuations that occur below the threshold for spike generation. These are precisely the signals most relevant to understanding how neurons integrate input and participate in circuit computation.^{18,19}

Extracellular recordings also lack inherent cell-type specificity: action potential waveforms from excitatory pyramidal neurons, inhibitory interneurons, and genetically defined subpopulations cannot be distinguished by spike shape alone, constraining mechanistic interpretation of population activity. Spike sorting algorithms can partially address this by clustering spike waveforms, but misclassification rates increase

substantially in dense tissue where the extracellular fields of neighboring neurons overlap. In addition to these signal-level limitations, planar MEA geometries are architecturally incompatible with the three-dimensional structure of cortical organoids and microtissues: electrodes arrayed on a flat substrate can contact only the outermost cell layer of a three-dimensional tissue, leaving the interior electrically invisible, which may contain the most developmentally mature or disease-relevant cell populations. Although flexible three-dimensional MEA scaffolds and shell-shaped electrode architectures have been developed to address this constraint, their fabrication complexity, limited scalability, and potential for mechanical disruption of the tissue have restricted widespread adoption.^{17,20}

Taken together, traditional electrophysiological approaches present a clear tradeoff landscape. Patch clamp and sharp electrodes provide the highest signal fidelity but at the cost of throughput, invasiveness, and scalability, while MEA-based extracellular recording offers population-level access but sacrifices sensitivity to subthreshold dynamics, cell-type specificity, and compatibility with three-dimensional tissue architecture. The unmet need for a measurable modality that can simultaneously provide single-cell resolution, access to the full voltage waveform including subthreshold events, cell-type specificity, and compatibility with dense three-dimensional preparations has been a principal driver behind the development of optical electrophysiology and, specifically, genetically encoded voltage indicators. The emergence of these tools is described in the following section.

1.3 — Emergence of Optical Electrophysiology

The desire to observe neuronal electrical activity across large populations of cells simultaneously without the spatial constraints inherent to electrode-based recording, motivated the earliest attempts at optical electrophysiology. The foundational insight was

that certain organic fluorophores redistribute or reorient within the plasma membrane in response to changes in the local electric field, altering their spectroscopic properties. This was established in the 1970s with the characterization of merocyanine and styryl voltage-sensitive dyes (VSDs).²¹ The synthetic compounds insert into the lipid bilayer and report membrane potential changes through electrochromic mechanisms, in which the electric field shifts the energy levels of the dye's ground and excited electronic states. This produces measurable changes in fluorescence intensity or emission spectrum with sub-millisecond response times. VSD imaging was quickly adopted for mapping cortical activity patterns, revealing the spatial organization of sensory representation and the propagation of epileptiform activity across the cortical surface with a spatial resolution and field of view inaccessible to individual electrodes.²²

Despite the early promise of VSD-based optical recording, several fundamental limitations constrained its widespread adoption and interpretability, particularly in intact mammalian brain tissue. Because VSDs are bath-applied or pressure-injected, they stain all membranes indiscriminately, including neuronal, glial, and vascular membranes alike, generating a large, heterogeneous background fluorescence that substantially degrades the signal-to-noise ratio of individual cellular responses. In dense neuropil, where neuronal processes, astrocytic processes, and myelinated axons occupy most of the tissue volume, the somatic fluorescence of individual neurons is rendered optically unresolvable against the neuropil background.²³ The lack of cell-type specificity means that signals from excitatory pyramidal neurons, inhibitory interneurons, and non-neuronal cells are superimposed without the possibility of post-hoc disambiguation. Additionally, VSDs are not amenable to long-term *in vivo* experiments because the dye must be

reapplied as the compounds are often phototoxic at the illumination intensities required for adequate signal-to-noise, and the necessity of craniotomy and dural removal introduces surgical variability and tissue trauma. These constraints collectively limited VSD imaging to acute preparations, populations-level averages, and superficial tissue layers.^{4,23}

In parallel with VSD development, genetically encoded calcium indicators (GECIs) emerged as a practical and widely adopted approach to fluorescence-based monitoring of neuronal activity. GECIs, notably the GCaMP family of sensors, couple a circularly permuted fluorescent protein to calmodulin and a calmodulin-binding peptide such that calcium binding produces a large, sustained increase in fluorescence. Delivered virally or expressed from transgenic loci, GECIs can be targeted to specific cell types via CRE-dependent expression systems, enabling population-level imaging of identified neuronal subpopulations with cellular resolution across large cortical areas.²⁴ The GCaMP6 generation of sensors introduced in 2013, demonstrated sufficient sensitivity to detect single action potentials in cortical neurons in vivo with signal to noise ratios that made population-scale two-photon calcium imaging practical across hundreds to thousands of simultaneously imaged cells. This achievement made calcium imaging the dominant modality for system-level neuroscience over the following decade.^{6,24}

However, the widespread adoption of GECI-based calcium imaging did not eliminate the need for voltage measurement but rather supported it through the emphasis of calcium imaging's limitations. The relationship between intracellular calcium concentration and membrane potential is indirect and nonlinear because calcium enters neurons primarily through voltage-gated channels that open during action potentials,

making calcium transients a proxy for suprathreshold firing while rendering the technique effectively blind to the richest and most computationally meaningful aspects of neuronal activity. Subthreshold synaptic potentials, which may modulate firing probability without themselves triggering spikes, produce no detectable calcium signal in most neuron types. Sustained subthreshold depolarization of 20 mV or more, the up states that characterize slow-wave sleep and recurrent cortical dynamics, are also invisible to calcium imaging.^{2,6} Even the fastest genetically encoded calcium indicators exhibit calcium decay kinetics on the order of hundreds of milliseconds, which limits the temporal precision with which spike timing can be inferred. This precludes reliable discrimination of individual spikes within bursts and restricts accurate measurement of neural oscillations above a few hertz. Furthermore, hyperpolarizing inhibitory events, which are critical for understanding feedforward and feedback inhibition in cortical circuits, do not produce any calcium signal at all. These gaps are not engineering limitations of current sensors but intrinsic biophysical constraints of the calcium-membrane potential relationship.^{6,25}

These limitations created a clear and compelling argument for genetically encoded voltage indicators, fluorescent reporters that, like GECIs, can be stably expressed in defined cell types from viral or transgenic constructs and imaged with standard microscopes, but respond directly to changes in membrane potential rather than to the downstream calcium signal. The conceptual framework for GEVIs was established in the late 1990s with the demonstration that voltage-sensing domains from voltage-dependent fluorescence changes in mammalian cells.²⁶ Early GEVIs suffered from small signal amplitudes of 1% $\Delta F/F$ per 100 mV or less, slow kinetics incompatible with action potential detection, and poor membrane trafficking that led to intracellular accumulation of the

indicator and elevated background fluorescence. The subsequent two decades saw iterative efforts across multiple design strategies, including fluorescent protein fusion to isolated voltage-sensing domains, circularly permuted fluorescent proteins inserted into voltage-sensing scaffolds, and opsin-based electrochromic FRET architectures, that progressively improved sensitivity, speed, and membrane localization.^{27,28}

The maturation of the GEVI field can be traced through a series of landmark indicators that together established optical electrophysiology as a practical tool for mammalian neuroscience. The ASAP family of circularly permuted GFP-based indicators, the Archon and QuasAr rhodopsin-based sensors, and the electrochromic FRET platforms including Ace-mNeon and Voltron demonstrated, for the first time, reliable detection of single action potentials with cellular resolution in intact brain tissue and in behaving animals.^{9,28,29} Critically, each of these indicators retained sensitivity to subthreshold voltage fluctuations, making them unique among activity reporters in their ability to capture the full dynamic range of neuronal membrane potential.^{27,28}

1.4 — Genetically Encoded Voltage Indicators (GEVIs)

Genetically encoded voltage indicators are fluorescent proteins or protein-dye conjugates that report changes in membrane potential as changes in fluorescence intensity or spectral properties. All GEVIs share a common architectural requirement, the voltage-sensing component must reside within or be coupled to the plasma membrane so that changes in transmembrane electric field are transduced into detectable optical signal. Beyond this shared constraint, GEVIs vary substantially in their molecular scaffolds, voltage-sensing mechanism, kinetic properties, signal amplitudes, spectral characteristics, and suitability for different experimental contexts. To be practically useful

in neurons, a GEVI must satisfy several performance criteria simultaneously. It must localize efficiently to the plasma membrane with minimal intracellular accumulation, as off-membrane fluorescence elevates background and reduces signal-to-noise ratio. It must produce a fractional fluorescence change ($\Delta F/F$) large enough to be distinguished from photon shot noise within the acquisition time of a single imaging frame. Its response kinetics must be fast enough to faithfully report the voltage events of interest, whether those are millisecond-timescale action potentials or slower subthreshold oscillations. And it must be sufficiently photostable to permit recordings lasting seconds to minutes without substantial loss of signal. ^{25,30}

The first class of GEVIs couples one or more fluorescent proteins to the voltage-sensing domain (VSD) of a voltage-sensitive phosphatase or ion channel subunit. In these indicators, voltage-driven conformational changes in the VSD are mechanically transduced to the attached fluorescent protein, altering its chromophore environment and changing its fluorescence. The prototypical example is ArcLight, which fuses the VSD of *Ciona intestinalis* voltage-sensitive phosphatase to a pH-sensitive variant of GFP. ArcLight produces fluorescence decreases of up to 35% per 100 mV, making it one of the most sensitive VSD-based indicators, but its activation kinetics are relatively slow (tens of milliseconds), limiting its ability to resolve individual action potentials at firing rates. The VSFPs (voltage-sensitive fluorescent proteins) represent an earlier and ongoing series of VSD-FP fusion indicators that established the general principle of optical voltage sensing and demonstrated that GEVIs could report subthreshold dynamics in cortical neurons in vivo. ^{2,25}

A major advance in VSD-based GEVI design came with the introduction of the ASAP (accelerated Sensor of Action Potentials) family. This family of GEVIs inserts a circularly permuted green fluorescent protein (cpGFP) into the extracellular S3-S4 loop of the *Gallus gallus* voltage-sensitive phosphatase VSD, rather than appending it to the C-terminus. This arrangement places the cpGFP where voltage-driven S4 helix movement can directly modulate the chromophore environment, producing faster and larger fluorescence changes than C-terminal fusion designs. ASAP1, the first member of this series, demonstrated on and off kinetics of approximately 2 ms, enabling faithful tracking of potential trains up to 200 Hz in single trials with a single amplitude of approximately 18-29% $\Delta F/F$ per 100 mV.³¹ Subsequent engineering produced ASAP2f, which showed larger responses in vivo, and ASAP3, which combined sub millisecond activation kinetics with a 51% fluorescence decrease across the physiological voltage range from -70 to +30 mV and the highest power-normalized signal-to-noise ratio among fluorescent protein-based GEVIs at the time of its introduction.³² The most recent member of the family, ASAP4, inverts the fluorescence-voltage relationship so that depolarization produces fluorescence rather than decreases, yielding fractional changes exceeding 180% and enabling single-trial spike detection in vivo with both one-photon and two-photon microscopy.³³

A second major class of GEVIs exploits the intrinsic voltage sensitivity of microbial rhodopsins, light-driven proton pumps whose retinal chromophore undergo protonation state changes in response to the transmembrane electric field, producing near-infrared fluorescence with a sensitivity of approximately 35% $\Delta F/F$ per 100 mV and sub-millisecond response kinetics. Archaeorhodopsin-3 (Arch) and its engineered derivatives

QuasAr1 and QuasAr2 were among the first rhodopsin-based GEVIs demonstrated to resolve single action potentials in mammalian neurons, and the Archon1 variant further improved brightness and membrane localization through large-scale directed evolution screening of over one million variants.^{34,35} A key practical limitation of rhodopsin-based indicators in their native form is their reliance on high-intensity illumination, typically red or near-infrared laser light, to generate sufficient photon flux from what are inherently dim fluorophores. This requirement raises concerns about phototoxicity during extended recordings and can complicate integration with optogenetic actuators that share overlapping excitation spectra. Soma-targeted variants of Archon, in which trafficking sequences restrict expression to the cell body, partially address the signal-to-noise problem by eliminating neuropil background from unresolved processes.³⁵

A third design strategy, electrochromic FRET or eFRET, couples a bright fluorescent protein donor to a rhodopsin acceptor whose absorption spectrum shifts with membrane voltage, thereby modulating FRET efficiency and producing voltage dependent changes in donor fluorescence. Because the readout fluorescence originates from the bright donor rather than the dim rhodopsin, eFRET indicators combine the kinetic speed of rhodopsin voltage sensing with the photon output of conventional fluorescent proteins. The Ace2N-mNeon indicator, which pairs the *Acetabularia acetabulum* rhodopsin with a bright mNeonGreen donor, demonstrated sub-millisecond kinetics and enabled recordings of fast spike trains in awake, behaving mice and *Drosophila*. The VARNAM family extended this approach to red-shifted spectra using mRuby3 as a donor, enabling spectral separation from green-channel optogenetic tools and improving the compatibility with all-optical experimental designs.^{27,36}

Each of these GEVI classes presents a distinct set of performance tradeoffs that must be weighed against the specific demands of the experimental system. Fluorescent protein-based indicators such as the ASAP family offer good brightness and compatibility with two-photon excitation but produce relatively small absolute $\Delta F/F$ values per action potential, typically in the range of 2-8% per spike under realistic imaging conditions in tissue, requiring low noise detectors and optimized acquisition setting for reliable single-trial detection. Rhodopsin-based indicators offer large intrinsic voltage sensitivity and fast kinetics but require high illumination intensity and, until recently, were poorly suited to two-photon imaging. FRET-opsin hybrids partially resolve this tradeoff but introduce complexity in probe delivery and add constraints on spectral compatibility with other indicators or actuators. In all classes, the concentration of GEVI molecules is fundamentally limited to the plasma membrane area accessible within the imaging volume, which is substantially smaller than the cytosolic volume available to calcium indicators such as GCaMP, placing an intrinsic upper bound on per-cell photon flux and making shot noise a dominant source of signal degradation, particularly at the fast frame rates required for voltage imaging.^{23,30}

These considerations collectively shaped the design goals that led to the development of Voltron, a chemigenetic GEVI that addresses several of the limitations inherent to fully genetically encoded indicators by replacing the fluorescent protein donor with a bright, photostable synthetic Janelia Fluor dye conjugated via a HaloTag self-labeling domain. By decoupling the voltage-sensing function from the fluorescent reporting function and allowing the synthetic dye to be selected based on its optical properties rather than constrained by available fluorescent protein variants, Voltron

achieves improvements in photostability and brightness that are particularly advantageous through for extended recordings in optically challenging environments such as three-dimensional neural tissue. The design, performance, and specific suitability of Voltron for the present experimental system are described in the following section.^{9,37}

1.5 — The Voltron Chemigenetic Voltage Indicator Platform

Voltron was introduced in 2019 as a chemigenetic voltage indicator engineered to overcome the principal photophysical limitations of fully genetically encoded GEVIs.⁹ The indicator is built on an electrochromic FRET architecture in which the voltage-sensing function is separated from the fluorescent reporting function. The voltage-sensing component is a truncated microbial rhodopsin derived from *Acetabularia acetabulum* (Ace2), whose retinal chromophore undergoes voltage-dependent shifts in its absorption spectrum as the transmembrane electric field changes. Rather than coupling this rhodopsin to a fluorescent protein donor as in Ace2N-mNeon and related eFRET indicators, Voltron replaces the fluorescent protein entirely with a HaloTag self-labeling protein domain fused to the intracellular face of the rhodopsin. The HaloTag domain covalently and irreversibly captures a synthetic Janelia Fluor (JF) dye that has been conjugated to a HaloTag ligand, placing the dye near the retinal chromophore. When the rhodopsin absorbs in the red-shifted state corresponding to a depolarized membrane, FRET from the JF dye to the retinal acceptor is enhanced, quenching dye fluorescence and producing a decrease in the observed signal. Membrane hyperpolarization reduces FRET efficiency, restoring dye fluorescence. The result is a voltage-dependent fluorescence change that reflects the thermodynamic state of the retinal rather than the photophysics of a fluorescent protein.^{9,27}

The key innovation enabling Voltron's superior performance is the substitution of synthetic JF dyes for fluorescent proteins. The JF dye series, developed at Janelia Research Campus, is based on rhodamine scaffolds in which the classical N,N-dimethylamino groups are replaced with four-membered azetidine rings.³⁸ This structural substitution substantially increases both the quantum yield and the photostability of the resulting fluorophore by restricting the conformational flexibility of the amino substituents, reducing non-radiative decay pathways. Compared to common fluorescent proteins such as GFP and its derivatives, JF dyes are substantially brighter on a per-molecule basis and bleach much slower under continuous illumination. JF525, the dye used most with Voltron, has a peak excitation near 525 nm and a quantum yield of approximately 0.91, and it is cell-permeable in its lactone form which allows it to cross plasma membranes and reach HaloTag-expressing proteins in the intracellular compartment. The dye covalently bonds to labels the HaloTag domain within minutes and excess unbound dye is simply washed away. This leaves a clean, membrane-localized fluorescent signal with minimal cytoplasmic background.^{38,39}

The photostability advantage of Voltron relative to existing GEVIs is quantitatively substantial. Under equivalent illumination conditions in primary neuron culture, Voltron outperformed Ace2N-mNeon and ASAP2f in mean time to bleach by more than an order of magnitude.⁹ This improvement translates directly into practical gains in experimental capability. In mouse cortex, Voltron enabled continuous single-trial recording of action potentials and subthreshold voltage signals from dozens of neurons simultaneously over 15 minutes of uninterrupted imaging, a duration that would be impossible to sustain with fluorescent protein-based indicators under equivalent acquisition conditions. The

extended recording window is particularly relevant for studies requiring stable baseline measurements, drug application time courses, or the correlation of voltage dynamics with behavioral events that unfold over tens of minutes as opposed to seconds. Beyond duration, the higher photo budget per molecule also improves signal-to-noise ratio at a given frame rate, since the fractional fluorescence change per action potential can be detected with greater statistical confidence when baseline fluorescence is high and stable.^{9,28}

In terms of voltage sensitivity and kinetic performance, Voltron525 produces a fractional fluorescence decrease of approximately 23% per 100 mV under field stimulation conditions in cultured neurons, with sub-millisecond activation kinetics inherited from the Ace2 rhodopsin voltage-sensing domain. This combination of sensitivity and speed allows Voltron accurately to report individual action potential waveforms, distinguish closely spaced spikes in burst firing, and capture the subthreshold depolarization that precedes action potential threshold. The indicator also reports graded subthreshold signals, including synaptic potentials and slow oscillatory membrane potential fluctuations, with a linear fluorescence-voltage relationship across the physiological voltage range, making it well suited for quantitative comparison of voltage dynamics across cells and conditions.

^{9,40}

Following the initial characterization of Voltron, subsequent engineering efforts produced improved variants with enhanced performance. Voltron2, generated through site saturation mutagenesis of the rhodopsin domain at 40 positions followed by automated patch-clamp screening of more than 2,700 variants, carries a single A122D substitution that increases fluorescence response to a single action potential by

approximately 65% relative to the original Voltron and improves subthreshold sensitivity by approximately threefold.⁴⁰ A separate line of engineering produced Positron, which introduces three-point mutations into the proton transport pathway of the Ace2 rhodopsin to invert the polarity of the fluorescence response, yielding fluorescence increases rather than decreases upon depolarization. Positive-going indicators offer practical advantages in some imaging scenarios because fluorescence increases are easier to detect above background decreases, and they eliminate potential confusion between bleaching artifacts and true voltage signals. The availability of both polarities within the same chemigenetic scaffold further expands the versatility of the Voltron platform.⁴¹

For practical deployment in neuronal populations, Voltron is typically delivered via adeno-associated viral (AAV) vectors under cell-type specific promoters such as hSyn, which restrict expression to neurons. A soma-targeting variant incorporating a trafficking sequence from the Kv2.1 potassium channel restricts Voltron expression to the cell body, dramatically reducing fluorescence from axonal and dendritic processes that would otherwise contribute background signal and complicate extraction of somatic voltage traces.^{9,42} In the context of three-dimensional neural tissue models, the use of a dual-vector AAV strategy in which a CRE-dependent Voltron construct is co-delivered with a CRE recombinase under the same or a complementary promoter allows for sparse labeling. This improves single-cell optical separability within densely packed cellular constructs and reduces the contribution of out-of-focus labeling processes to background fluorescence. It is a practical requirement for resolving individual voltage traces in tissue where cells are closely organized and optical sectioning is limited by scattering. These delivery and targeting strategies, put together with the photostability and brightness

advantages of the JF dye system, establish Voltron as a highly suitable platform for optical electrophysiology.^{8,9}

1.6 — Engineered Three-Dimensional Cortical Microtissues

For decades, dissociated neuronal monolayers on planar glass or plastic substrates have served as the workhorse of cellular neuroscience, enabling patch-clamp characterization of ion channel properties, MEA-based measurement of network activity, and high throughput pharmacological screening. Despite these contributions, two-dimensional cultures impose fundamental constraints that limit their fidelity as models of cortical tissue physiology. Neurons plated on flat surfaces are constrained to extend processes in a single plane, reducing synaptic connectivity relative to the omnidirectional axonal and dendritic arborization that neurons exhibit *in vivo*. Cell density is typically far below the values found in living brain tissue, where cells are packed at densities 10^5 cells per mm^3 . The absence of a three-dimensional extracellular matrix (ECM) deprives neurons of the mechanical, biochemical, and structural cues that *in vivo* ECM provides, including laminin, fibronectin, and heparan sulfate proteoglycans that guide neurite outgrowth, support synapse formation, and regulate glial morphology and function.^{43,44} Astrocytes, which in two-dimensional cultures typically adopt a flattened, proliferative morphology distinct from their stellate, process-bearing *in vivo* phenotype, play critical roles in supporting neuronal survival, regulating extracellular ion and neurotransmitter homeostasis, and modulating synaptic strength. Three-dimensional culture conditions have been shown to promote the transition of astrocytes toward a more physiologically relevant morphology, with meaningful implications for the fidelity of neuron-glia interactions in the model.^{44,45}

Three-dimensional neuronal cultures address many of these limitations by allowing cells to organize into spatially compact, mechanically contiguous structures in which synaptic connectivity can develop in all directions, cell-cell contact occurs at densities closer to those of living tissue, and endogenous ECM components accumulate within the tissue volume as they are secreted by resident neurons and glia. Multiple studies have documented that neurons in three-dimensional configurations exhibit longer neurite outgrowth, higher survival rates, faster network maturation, and electrophysiological properties that more closely resemble those of neurons in brain slices than in two-dimensional cultures.^{45,46} Three-dimensional cultures also display increased complexity of spontaneous network activity, including synchronized bursts, local field potential-like fluctuations, and structured temporal dynamics that reflect the interplay of excitatory and inhibitory populations within a spatially constrained microcircuit. These network properties emerge earlier in development and are more stable to over extended culture periods in three-dimensional cultures than in two-dimensional cultures. This suggests that the three-dimensional architecture fundamentally enhances the functional organization of the *in vitro* network.^{46,47}

Within the broad space of three-dimensional neural tissue models, two main strategies have been pursued. iPSC-derived cerebral organoids recapitulate developmental processes of human brain organogenesis, including the formation of cortical layers and ventricular zone-like structures, and have enabled studies of human-specific features of neural development and neurological disease that are not available in rodent models.⁸ However, organoids suffer from substantial batch-to-batch variability arising from the stochastic nature of stem cell differentiation, poorly defined heterogenous

cellular compositions, and the prolonged culture period of many months required to reach functional maturity. These properties limit the experimental throughput and reproducibility needed for systematic studies of electrophysiology or controlled pharmacological interventions. Primary cortical microtissues, formed by the self-assembly of dissociated neonatal rat cortical cells into compact three-dimensional tissues, offer an alternative that trades the development complexity of organoids for well-defined cellular composition, rapid maturation within two to four weeks, and higher inter-tissue reproducibility.^{48,49}

The specific microtissue platform used in this work was developed in the Borton laboratory at Brown University, building on approaches pioneered in the Hoffman-Kim laboratory, and is described in detail by Brown, Atherton, and Borton.^{48–50} Primary cortical are harvested from neonatal P1 to P2 rat cortices by papain dissociation, then seeded into custom-machined non-adherent agarose microwell arrays. The low-attachment properties of agarose prevent cells adhering to the substrate and instead promote self-aggregation, resulting in compact, trampoline-like microtissues with diameters of approximately 400 to 800 μm . The injection-molded agarose microfeature design positions the forming microtissue approximately 350 μm from the bottom of the culture plate, placing it within the working distance of high-numerical-aperture objectives on an inverted confocal microscope. This geometry enables live imaging without physical manipulation of the tissue that could introduce mechanical disturbances or alter the culturing environment and has been validated for use with virally delivered fluorescent indicators, transgenic donor animal lines, and a range of live confocal imaging protocols.⁵⁰

The dissociated primary cortical cell suspension contains a mixture of excitatory glutamatergic pyramidal neurons, inhibitory GABAergic interneurons, astrocytes,

microglia, and oligodendrocyte precursor cells in proportions broadly reflecting those of the neonatal rodent cortices. Over the first two weeks in vitro, cells within the microtissue extend processes, form synaptic contacts, establish gap junctions, and develop spontaneous electrical activity that can be detected by calcium imaging beginning around day in vitro (DIV) 14. Longitudinal calcium imaging studies in the Borton laboratory have demonstrated that microtissues undergo progressive functional remodeling from week two through week four in vitro, with increasing synchrony, strengthening of community structure within the functional network, and the development of correlated burst activity characteristic of mature cortical microcircuits.^{48,49} The glial cell populations within the microtissue, particularly astrocytes and microglia, contribute to structural maintenance and the regulation of synaptic dynamics, and their presence make the primary microtissue model more representative of intact cortical tissue than purely neuronal cultures. The endogenous ECM that accumulates within the microtissue volume further supports the structural coherence and functional maturation of the network.^{50,51}

The primary cortical microtissue platform is compatible with AAV-mediated transgene delivery, allowing fluorescent reporters, calcium indicators, and subsequently voltage indicators to be expressed under cell-type specific promoters within the three-dimensional tissue. This compatibility with viral tools, combined with the transgenic accessibility of primary rodent tissue, makes the platform well suited for disease modeling experiments in which genetic modifications can be introduced either through transgenic donor animals or through viral constructs applied to the microtissue in culture.^{48,50} Prior work from the Borton laboratory has used this platform with GCaMP-based calcium imaging to characterize how acute neuroinflammatory insults alter functional connectivity

and microcircuit community structure, and to model the innate cellular response to implanted neural recording devices. The present thesis extends the capabilities of this platform by applying Voltron-based optical voltage imaging to access direct membrane potential readouts from neurons within intact three-dimensional microtissues, a capability that calcium imaging cannot provide and that has not previously been characterized in this or analogous primary microtissue systems. ^{48–50}

1.7 — Challenges of Optical Voltage Imaging in 3D Neural Tissues

Applying optical voltage imaging to three-dimensional neural tissue introduces a set of challenges that do not exist, or exist in far milder form, in dissociated two-dimensional cultures. The most fundamental of these is light scattering. Biological tissue is optically heterogenous: lipid membranes, myelinated processes, organelles, and ECM components all present refractive index discontinuities that scatter photons away from their original direction. As excitation light penetrates tissue, the fraction of photons that remain ballistic, traveling along their original path to the focal volume, decreases exponentially with depth according to the Beer-Lambert relationship, with an attenuation length that correlates with their wavelength. In conventional single-photon confocal microscopy using visible wavelengths near 488 to 561 nm, the usable imaging depth in dense neural tissue is typically limited to 100 to 150 μm before scattered photons degrade the signal-to-background ratio below the level required for reliable cellular resolution. ^{52,53} Two-photon microscopy, which uses near-infrared excitation near 800 to 1400 nm and confines fluorescence generation to the diffraction-limited focal volume through a nonlinear excitation mechanism, can penetrate substantially deeper, typically 500 to 800 μm in cortical gray matter, but introduces its own constraints including the need for high

peak laser power, risks of photothermal tissue damage after prolonged illumination, and voltage insensitivity of both rhodopsin-based and eFRET GEVIs such as Voltron under two-photon excitation.^{32,53}

The signal-to-noise requirements for voltage imaging are substantially more demanding than for calcium imaging, and this disparity becomes even more severe in three-dimensional tissue where scattering amplifies background fluorescence. A calcium indicator such as GCaMP6f responding to a single action potential produces a fractional fluorescence change of approximately 25% that persists for hundreds of milliseconds, giving the detector hundreds of frames over which to accumulate photons from the signal. A voltage indicator responding to the same action potential produces a fractional change of similar magnitude but lasting only 1 to 3 ms, confining all the single photons to one or two imaging frames. The number of photons collected from a membrane-localized GEVI per frame is already fundamentally limited by the small membrane area within the imaging volume, and at the frame rates of 200 to 1000 Hz required to resolve voltage transients, shot noise from this limited photon count becomes the dominant noise source.^{54,55} In three-dimensional tissue, scattered out-of-focus fluorescence from labeled processes outside the focal plane contributes additional background photons that are indistinguishable from the signal of interest, further degrading the signal-to-background ratio. This out-of-focus contamination is particularly problematic in microtissues where Voltron-expressing neurons are densely packed and their axonal and dendritic processes extend in all directions through the tissue volume.^{55,56}

Photobleaching poses a related and compounding challenge. Because voltage imaging requires continuous illumination at frame rates far above those used for calcium

imaging, the total photon dose delivered to the sample per unit of experimental time is correspondingly higher. Most fluorescent protein-based GEVIs bleach to half their initial brightness within seconds to minutes of continuous illumination at the intensities needed to achieve adequate signal-to-noise for action potential detection.²³ In three-dimensional tissue, where illumination must penetrate through multiple layers before reaching cells of interest, the optical power at the tissue surface must be increased relative the power in thin preparations, accelerating photobleaching of indicator molecules at shallower depths even as deeper cells are being imaged. The photostability advantage of Voltron relative to fluorescent protein-based indicators is therefore especially valuable in three-dimensional preparations. The substantially extended photon budget of JF dyes, which bleach an order of magnitude more slowly than comparable fluorescent proteins at equivalent illumination conditions, directly translates into longer usable recording windows and higher cumulative photon yields per experiment.^{9,38}

Voltron presents a unique practical challenge in three-dimensional tissue that does not arise in monolayer cultures: the synthetic JF dye must penetrate through the tissue volume to reach and label HaloTag-expressing cells throughout the microtissue. In two-dimensional cultures, dye applied to the culture medium rapidly equilibrates across the thin cell layer and labels surface-accessible HaloTag domains within minutes. In a microtissue several hundred micrometers in diameter, diffusion of the dye from the tissue surface to the interior is governed by both the diffusion coefficient of the dye through the extracellular space and the binding kinetics of the HaloTag labeling reaction. If binding at the surface is rapid relative to diffusion, a gradient of labeled indicator can form, with surface cells brightly labeled and interior cells poorly labeled.^{8,9} This penetration

challenge is mitigated by the fluorogenic properties of JF dyes in their lactone form, which are cell-permeable and exhibit low fluorescence until they bind their HaloTag targets, reducing non-specific background from unbound dye during the labeling incubation. Optimizing the dye concentration and incubation time to allow adequate penetration without oversaturation of surface cells is an empirical parameter that must be established for each tissue geometry, representing an additional step in protocol development for three-dimensional Voltron experiments.^{8,38}

The acquisition speed requirements of voltage imaging create a fundamental tension with the optical sectioning provided by confocal microscopy. Laser scanning confocal systems achieve sectioning by using a pinhole to reject out-of-focus fluorescence, but the serial pixel acquisition process imposes an upper limit on frame rate that depends on the number of pixels scanned, the scan speed of the galvanometric or resonant mirror system, and the dwell time required per pixel for adequate signal. At the frame rates of several hundred hertz needed for action potential detection, the usable field of view in resonant-scanner confocal mode must be substantially reduced, typically a narrow region of interest only spanning a small number of cells to maintain the necessary acquisition speed.^{56,57} Widefield single-photon imaging with a high-speed scientific CMOS camera avoids this trade-off by illuminating the entire field simultaneously and capturing all pixels in parallel, achieving kilohertz frame rates across large fields of view, but sacrifices optical sectioning entirely and is therefore severely limited in thick tissue where out-of-focus background from scattered light and unlabeled processes overwhelms the voltage signal. Advanced optical configurations that combine targeted illumination with confocal gating or light-field approaches offer potential paths

toward reconciling these competing demands. However, they require specialized hardware not yet routinely available for three-dimensional tissue preparations.^{55–57}

These overlapping optical, photochemical, and biophysical constraints collectively define the design space for voltage imaging experiments in three-dimensional cortical microtissues. A practical experimental approach must balance imaging depth against signal-to-noise, field of view against frame rate, illumination intensity against photobleaching, and dye penetration against surface labeling heterogeneity. The primary microtissue platform used in this thesis was specifically designed for imaging using single-photon confocal imaging to maximize access to the tissue interior. The choices of Voltron as the voltage indicator and the JF dye as the reporter reflect these optical constraints directly, as the photostability of the dye system address the photobleaching limitation and the single-photon excitation compatibility of Voltron permits the use of confocal microscopy for background rejection. The integration of electrical stimulation to evoke voltage responses, rather than relying solely on spontaneous activity detection, further relaxes the sensitivity requirements.^{9,38,48}

1.8 — Knowledge Gaps and Motivation for This Work

The preceding sections have traced the conceptual and technical trajectory of optical electrophysiology from its origins in voltage-sensitivity dye imaging through the emergence of genetically encoded voltage indicators, the development of the Voltron chemigenetic platform, and the parallel maturation of engineered three-dimensional cortical microtissues as functional in vitro models for cortical circuitry. Taken together, this

body of work establishes a measurement system in which Voltron-expressing neurons within a three-dimensional microtissue report their membrane potential dynamics as fluorescence changes in a photostable synthetic dye, enabling non-invasive, cell-type-specific, and spatially resolved voltage imaging across an architecturally complex tissue designed to resemble the organization of living cortical tissue. Yet, despite the convergence of these independently mature technologies, their integration has not been previously demonstrated, and many questions stand between individual components and a validated experimental platform.

The first and most fundamental gap is that Voltron has not previously been characterized in three-dimensional, self-assembled primary cortical microtissues. The original description of Voltron by Abdelfattah et al. established its performance in dissociated two-dimensional neuronal cultures and in acute in vivo preparations in mice, zebrafish, and *Drosophila*, but three-dimensional tissue models were not examined.⁹ The biological environment of a self-assembled primary microtissue differs from both of these methods in ways that are relevant to Voltron performance. The dense cell packing, presence of endogenous ECM, optical scattering introduced by the tissue volume, and reduced accessibility of HaloTag domains in interior cells all represent variables that must be investigated before the indicator can be confidently applied in this context. A further uncertainty specific to the Borton laboratory platform is whether differences in the cell culture dissociation and maintenance protocols between the original Voltron characterization studies and those used for primary cortical microtissue affect transduction efficiency, transgene expression levels, or the brightness and photostability of JF dye labeling. These media-related variables must be decoupled from tissue

geometry effects before the performance of Voltron in three-dimensional tissue can be meaningfully interpreted.

The second gap concerns the optimization of AAV-mediated Voltron delivery in three-dimensional microtissues. The dual-vector CRE-dependent expression strategy used in this work requires co-transduction of each target neuron with two AAV constructs, and the transduction efficiency of both vectors must be high enough to produce sparse but reliable Voltron expression throughout the tissue volume. The relationship between viral titer, expression density, and optical separability of individual cells in the densely packed three-dimensional tissue have not been established for this indicator or tissue geometry. Too low a titer produces insufficient expression for reliable voltage detection; too high a titer produces overlapping fluorescent signals from densely labeled processes that cannot be resolved as individual cell traces. The optimal titer window must be determined in the specific microtissue context, and the maturation timeline of Voltron fluorescence from AAV transduction to stable expression in three-dimensional tissue may differ from the timelines reported for two-dimensional cultures.

A third gap involves the acquisition protocol parameters required to detect Voltron voltage signals in three-dimensional tissue with adequate signal-to-noise using the Olympus FV3000 resonant-scanner confocal system. The relationships among imaging frame rate, pixel size, digital zoom, line averaging, laser power, and the resulting signal-to-noise ratio for Voltron voltage transients had not been previously established for this specific instrument-indicator combination. The failure to detect spontaneous voltage signals in the initial two-dimensional imaging experiments conducted during this work demonstrated that optimizing these acquisition parameters is an important prerequisite to

successful voltage imaging, not something that can be inherited from published protocols developed on different instruments and in different tissue preparations. Defining and validating acquisition parameters for both structural and dynamic voltage recordings is a critical methodological step that underpins the reliability and interpretation of all subsequent voltage imaging results.

The fourth gap lies in the development a of reliable stimulation method compatible with simultaneous confocal voltage imaging of three-dimensional microtissues. While electrical field stimulation of dissociated neuronal cultures using MEA or electrode systems is well established, the extension of this approach to intact three-dimensional microtissues surrounded by agarose introduces consideration that have not been systematically addressed. The geometry of the stimulation electrodes relative to the tissue, the impedance characteristics of the electrode-medium interface, the spatial uniformity of the electric field across a tissue, and the potential for stimulation artifacts to contaminate the fluorescence recording channel all represent practical engineering challenges requiring characterization.

Against this background of unresolved questions, the present thesis pursues four interconnected aims. First, to validate Voltron expression and JF dye labeling in primary cortical neurons under the Borton laboratory cell culture maintenance conditions, establish that media formulation differences do not adversely affect indicator performance and providing a two-dimensional baseline against which three-dimensional results can be compared. Second, to characterize Voltron-based optical voltage recordings in three-dimensional primary cortical microtissues following dual-AAV transduction, including the optimization of titer, dye concentration, and imaging acquisition parameters. Third, to

identify and optimize imaging parameters for acquisition of Voltron voltage signals via the Olympus FV3000 confocal imaging system. Fourth, to develop and validate stimulation methods for reliable, spatially uniform field stimulation via impedance spectroscopy and finite element analysis modeling, and to demonstrate its use in evoking voltage signals detectable by Voltron imaging within the microtissue.

The broader significance of this work extends beyond the specific preparation and indicator use. Primary cortical microtissues, occupy a strategically valuable position in the landscape of in vitro neural models, offering greater physiological relevance than dissociated monolayer cultures while maintaining the reproducibility, defined cellular composition, and experimental accessibility that iPSC-derived organoids currently lack. Demonstrating that membrane potential measurements are achievable in this preparation using a chemigenetic voltage indicator opens a capability that calcium imaging cannot provide: the ability to time and potentially quantify action potentials within the three-dimensional cortical model. This is also equally as significant from the three-dimensional microtissue perspective for use in disease modeling platforms, in which electrophysiological phenotypes associated with channelopathies, neurodegenerative conditions, and neuro inflammation could be characterized at a level of membrane potential rather than through indirect calcium methods. The work described in the following chapters addresses the technical and biological questions that must be answered before broader applications can be explored.

Ch. 2 — Materials and Methods

2.1 — Two-Dimensional Primary Cortical Tissue Dissociation

Primary cortical neurons were obtained from neonatal Sprague-Dawley rats at postnatal day 1 or 2 (P1–P2). All procedures were conducted under Brown University IACUC-approved protocols. Twenty-four-well glass-bottom plates (Cellvis P24-1.5H-N) were coated overnight with poly-D-lysine (PDL; 0.05 mg/mL in sterile water), rinsed three times with sterile water, and air-dried before use.

On the day of culture, cortices were dissected under sterile conditions in ice-cold HBSS-based dissection buffer. Meningeal membranes were removed under a stereomicroscope, and cortical tissue was minced into approximately 1 mm³ pieces. Tissue was digested in papain solution (Worthington) supplemented with Benzonase nuclease at 37°C for 30 minutes with periodic agitation. Following digestion, papain was removed by aspiration and tissue was mechanically dissociated by serial trituration in DMEM/FBS trituration media using a serological pipette followed by a P-1000 micropipette. The cell suspension was filtered through a 40 µm strainer and centrifuged at 90xg for 8 minutes. The pellet was resuspended in plating media (phenol red-free MEM supplemented with glucose, transferrin, insulin, L-glutamine, and heat-inactivated FBS). Viability was assessed by Trypan Blue exclusion using a Countess automated cell counter.

2.2 — Two-Dimensional Neuronal Culture Preparation

Dissociated cortical cells were seeded at approximately 500,000 cells/mL in PDL-coated 24-well glass-bottom plates. Voltron2 was introduced at DIV 0 by nucleofection using a Lonza 4D-Nucleofector (program CU 110, P3 primary neuron solution with Supplement 1). Plasmid DNA was prepared at 0.5 µg in 4 µL of endotoxin-free water per

sample. Following electroporation, cells were allowed to recover briefly in plating media before seeding.

Cultures were maintained in parallel under two media conditions to evaluate the effect of formulation on Voltron2 expression: the Borton Lab's standard cortical culture media (CCM) and NbActiv4 (BrainBits), the formulation used in the laboratory where Voltron2 was originally developed. Approximately 2.5-3 hours after seeding, a wash step was performed with pre-warmed phenol red-free MEM, followed by replacement with 1 mL per well of 90:10 NbActiv4/plating media. Subsequent twice-weekly media changes used 50% exchanges of NbActiv4 only. Cultures were maintained to DIV 15-16 prior to live imaging.

2.3 — Cell Culture Media Formulations

A central variable in adapting Voltron-based optical electrophysiology to the Borton lab platform was cell culture preparation and maintenance differences. The original Voltron characterization studies were conducted using NbActiv4 (BrainBits, LLC), a commercially formulated neuronal maintenance medium, whereas Borton lab's established cortical microtissue protocol utilizes a serum-free chemically defined formulation designated cortical culture media (CCM). Differences in supplement composition, osmolarity, and the presence or absence of serum-derived factors can influence viral transduction efficiency, transgene expression level, and the brightness of membrane-localized fluorescent indicators.^{58–60} To determine whether media composition alone affected Voltron expression or JF dye fluorescence performance, both formulations were evaluated in parallel using cells from the same dissociation, plated in the same vessel, and transduced under identical conditions.

The Borton lab CCM formulation served as the primary long-term maintenance medium for cultures designated for three-dimensional microtissue experiments and as one of the two conditions evaluated in two-dimensional culture. CCM was prepared using Neurobasal A medium without phenol red as the base, supplemented with B-27 neuronal supplement, GlutaMax dipeptide supplement, and penicillin-streptomycin. CCM is serum-free and chemically defined, which reduces glial overgrowth, supports long-term neuronal survival and synaptic maturation, and yields more stable spontaneous electrophysiology activity compared to formulations containing serum. At 24 hours post plating, plating media in CCM-designated wells was replaced with CCM, which was then maintained with half-volume changes every two to three days for the duration of the culture period.

The Abdelfattah Lab media condition used NbActiv4 (BrainBits, LLC) as the primary neuronal maintenance medium, reflecting the formulation employed in the original Voltron studies. NbActiv4 is a commercially prepared supplement designed to enhance neuronal activity and network maturation and has been reported to support robust spontaneous firing in dissociated cortical cultures.⁶¹ For the initial 24-hour period post plating, all wells received the same MEM-based plating media described in section 2.1. At the first media change, NbActiv4-designated wells received a transitional 90/10 formulation consisting of 90% NbActiv4 supplemented with 10% plating media by volume. This blended formulation was used for the first post-seeding media change to support cell recovery.

By maintaining both media conditions within the same 24-well plate using cells from a single dissociation, the experimental design minimized batch-to-batch biological variability and isolated media composition as the primary experimental variable. Wells A1

through D3 received CCM and wells A4 through D6 received NbActiv4-based formulation. Voltron expression was assessed in both conditions by confocal imaging of JF-525 and JF-608 labeled cultures, and cellular composition was characterized by immunohistochemistry using antibody panels targeting neurons, microglia, astrocytes, and oligodendrocyte precursor cells. This comparison established whether the Borton lab CCM environment was permissive for Voltron expression and JF dye uptake prior to scaling the platform to three-dimensional microtissues, where CCM is the standard maintenance formulation.

2.4 — Voltron Delivery in Two-Dimensional Cultures by Electroporation

Voltron2 was introduced into dissociated cortical neurons at DIV 0 by nucleofection using a Lonza 4D-Nucleofector system. Prior to electroporation, plasmid DNA was prepared at 0.5 µg in 4 µL of endotoxin-free, sterile cell culture grade water per sample. Cells were resuspended in Lonza P3 Primary Cell Solution supplemented with Supplement 1 at a ratio of 2 mL P3 to 444 µL Supplement 1, with 20 µL of the P3+S1 solution used per sample. The P3+S1 solution was allowed to warm to room temperature prior to use and the mixed solution was used within three months of preparation. Electroporation was performed in batches of eight samples using the Lonza 4D-Nucleofector strip system at program CU 110, the validated setting for primary neuronal cells. Following the electrical pulse, 80 µL of plating media was immediately added to each strip well, and cells were transferred to 1.5 mL recovery tubes containing 150 µL of pre-warmed plating media and allowed to recover for approximately one minute before seeding. Cultures were seeded at approximately 500,000 cells per mL and returned to the incubator. A post-seeding media change was performed 2.5 to 3 hours after plating,

consisting of an MEM wash step followed by replacement with the 90% NbActiv4 / 10% plating media maintenance formulation described in Section 2.3.

2.5 — Janelia Fluor Dye Conjugation (JF-525 and JF-608)

Voltron2 reports membrane voltage through electrochemical fluorescence resonance energy transfer (eFRET) between its Ace2 rhodopsin domain and a synthetic fluorophore covalently conjugated to the fused HaloTag self-labeling protein domain. Fluorophore delivery therefore requires incubation of live, expressing cultures with a membrane-permeable HaloTag ligand, which diffuses into the cell and undergoes an irreversible covalent bond with the HaloTag protein. The JF dye series was selected due to its superior brightness and photostability relative to more conventional rhodamine fluorophores.

Two dye variants were used in parallel to enable dual-channel characterization of Voltron2 labeling across cultures and imaging conditions. JF525 which exhibits an excitation maximum near 525 nm and a quantum yield of approximately 0.91,³⁸ was the primary dye used for live voltage imaging given its spectral compatibility with the 488 and 514 nm laser lines available on the Olympus FV30000 confocal system and for its better signal-to-noise characteristics. JF 608, with excitation shifted toward the 561 nm laser line, was used in parallel to assess dual-channel labeling efficiency and to evaluate spectral crosstalk between the two dye conditions under the same acquisition parameters. Both dyes were prepared in concentrated stock solutions in anhydrous dimethyl sulfoxide (DMSO) and stored at -20°C protected from light until use.

Dye labeling was performed on live cultures at DIV 14 through DIV 16, a window selected to coincide with established spontaneous network activity and confirmed Voltron2

expression. JF525-HaloTag ligand and JF608-HaloTag ligand were diluted into a final concentration of 100 nM in warm culture media and applied to all wells. Cultures were incubated at 37°C and 5% CO₂ for 30 minutes to allow dye diffusion across the plasma membrane and covalent HaloTag conjugation to be completed. Following incubation, unbound dye was removed by three sequential washes with warmed culture media, each allowed to equilibrate for 10 minutes before aspiration, to minimize residual extracellular fluorescence that would otherwise elevate background in imaging.

Following the final wash, cultures were equilibrated at 37°C and 5% CO₂ for a minimum of 15 minutes before confocal imaging to allow thermal and optical stabilization of the preparation. The dual-dye labeling strategy served two purposes. Firstly, it allowed confirmation that Voltron2 expression and HaloTag accessibility were consistent between CCM and NbActiv4 media conditions. Secondly, it provided a spectral redundancy check for membrane localization and soma-targeting of the Voltron2 construct. For three-dimensional microtissue labeling, the same dye preparation and wash protocol was applied with the addition of an extended pre-wash incubation step to allow for dye diffusion through the tissue volume prior to the wash series, as described in section 2.8.

2.6 — AAV Construct Design and Dual-Vector Voltron Delivery Strategy

Sparse, neuron-specific expression of Voltron2 in three-dimensional cortical microtissues was achieved using a dual adeno-associated virus (AAV) strategy that restricts indicator expression through Cre recombinase-dependent genomic inversion. Density of labeled cells is a critical parameter for voltage imaging: at high transduction levels, overlapping membrane fluorescence of neighboring cells can impact the ability to separate individual somata and reduces the signal-to-noise of single-cell $\Delta F/F$ traces. A

CRE dependent architecture decouples the total viral load from the density of expressing neurons, because only cells that receive both vectors simultaneously and co-express CRE and the FLEX constructs will produce functional Voltron2 proteins, allowing for the CRE to act as a tunable dial for expression density while keeping the Voltron2 titer independently sufficient for robust transgene delivery.

Two AAV vectors were used in combination. Firstly, AAV1-hSYN-CRE-eGFP, drives neuronal expression of the CRE recombinase fused to enhanced green fluorescent protein under the human synapsin promoter, enabling neuron-specific delivery and simultaneous visualization of transduced cells via eGFP fluorescence. Secondly, AAV1-hSyn-FLEX-Voltron2-ST, encodes the Voltron2 indicator in a under the same hSyn promoter. In the absence of CRE, Voltron2 is maintained in an antisense orientation and is not translated. Upon CRE-mediated recombination, the sequence is permanently inverted into the sense orientation, resulting in stable Voltron2 expression. The soma-targeting (ST) suffix designates inclusion of the Kv2.1 proximal restriction and clustering motif, which restricts Voltron2 membrane traffic to the soma and proximal dendrites and concentrating the indicator to the cell body. This reduces neuropil contamination of voltage signals.^{9,40}

To identify the optimal viral titer for three-dimensional microtissue transduction, two titer conditions were evaluated in a side-by-side comparison at the time of initial seeding (DIV 0). A higher-titer condition used 1×10^9 genomic copies per well (gc/mL) of AAV1-hSyn-CRE-eGFP and 1×10^{10} gc/mL of AAV1-hSyn-FLEX-Voltron2-ST, representing a 100-fold reduction in CRE dose and a corresponding reduction in the frequency of dual-transduced expression neurons. Both vectors were co-applied simultaneously in the viral

inoculum at the time of microtissue formation. Expression density, fluorescence quality, and voltage response to electrical stimulation were assessed by confocal imaging following JF dye conjugation.

2.7 — Three-Dimensional Cortical Microtissue Fabrication and Agarose Microfeature Seeding

Three-dimensional cortical microtissues were fabricated following the protocol established by Brown et al. for scaffold-free primary cortical microtissue generation using injection-molded agarose microfeatures.⁵⁰ Agarose microfeatures were molded into the bottom of wells of non-treated 24-well glass bottom plates (CellVis P241.5HN) using a custom-machined stainless steel injection mold. A 2% (w/v) agarose solution was prepared by adding 1 g of autoclaved ultrapure agarose (ThermoFisher 16-500-100) to 50 mL of sterile phosphate-buffered saline (PBS) and heated in the microwave in 30-second increments until boiling and full dissociation. One milliliter of hot agarose was drawn into a luer-locking syringe and injected through the stainless-steel mold into the bottom of each well, then allowed to cool on ice until solidified. The mold was carefully removed, leaving a molded microfeature within 200 μm of the plate bottom with six intact agarose pegs per well.

One milliliter of CCM was added to each molded well to allow medium diffusion into the agarose, and plates were kept in the incubator at 37°C w/ 5% humidity. Two sequential 1 mL media changes were completed over the two days prior to seeding of any cells. Immediately before seeding, the full 1 mL of media was removed from each well. The cell suspension was adjusted to 2.7×10^8 cells per mL and 10 μL were seeded into each well. Plates were placed in the incubator for 25 minutes, after which 1 mL of CCM was added dropwise into each well. Successful microtissue formation was

confirmed at 24 hours post seeding by distinct tissue contraction around the agarose pegs. A full 1 mL of media changes was performed every other day following seeding.

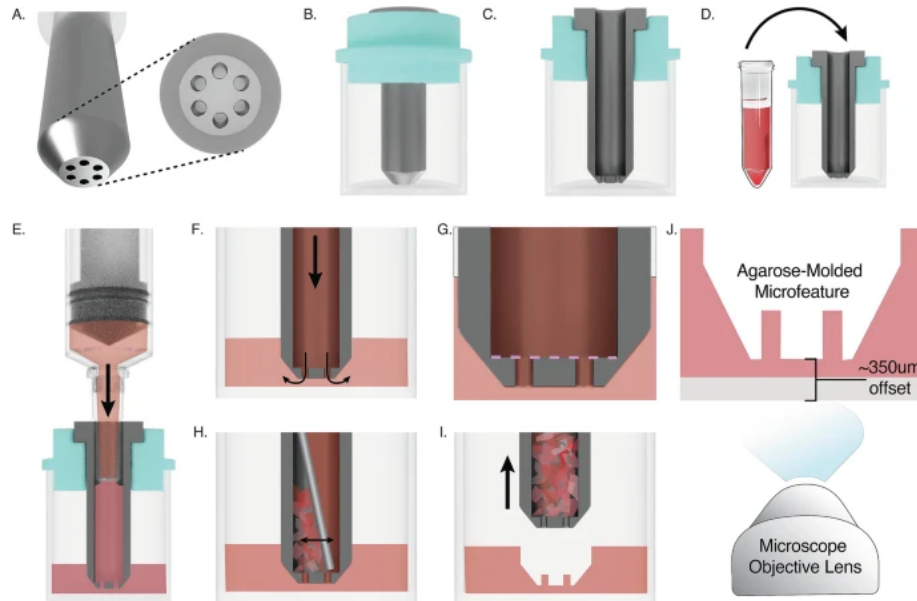


Figure 1: Agarose molding process using machined stainless-steel injection molds. (A-C) Stainless-steel injection mold orientation in cell culture plate well with a custom stainless-steel lift piece. (D-F) Molten agarose is injected into the mold and flows into the well through the stainless-steel injection mold holes. (G, J) Agarose is left to cool and form a solid microfeature $\sim 350\ \mu\text{m}$ from the imaging surface. (H, I) Removal of stainless-steel injection mold. Agarose is broken apart inside the stainless-steel mold, detaching the “pegs” to allow for mold removal without jeopardizing microfeature integrity. (Figure adopted from Brown et al.)

2.8 — AAV Co-Transduction of Three-Dimensional Microtissues

All AAV-related procedures were carried out in accordance with the University guidelines for handling biosafety level 1 materials. Although AAV viruses are non-pathogenic in humans, all virus material was handled within a certified biosafety hood with proper personal protective equipment worn at all times. All pipette tips, serological pipettes, 15 mL conical tubes, and microcentrifuge tubes used during transduction experiments were subsequently soaked in 10% bleach solution for a minimum of 10 minutes following use. The biosafety hood was sprayed with 10% bleach and wiped down

10 minutes later at the beginning and end of any viral handling, followed by a 15-minute UV light exposure.

AAV viral vectors were procured from the Abdelfattah laboratory at Brown University and aliquots were stored at -80°C. At DIV 1, microtissues were co-transduced with AAV1-hSyn-CRE-eGFP and AAV1-hSyn-FLEX-Voltron2-ST via a complete 1 mL CCM media change containing both vectors diluted to their working concentrations. Both vectors were diluted together in CCM, and the full 1 mL volume was applied per well. Subsequent media changes followed the every-other-day schedule described in Section 2.7. Details regarding the viral titer conditions evaluated are described in Section 2.9 and Section 3.6.

2.9 — Viral Titer Selection and Optimization

All AAV procedures were performed per Brown University Environmental Health and Safety guidelines. Both CRE and Voltron2 AAVs were procured from the Abdelfattah lab, and all aliquots were stored at -80°C. DIV 1, microtissues were co-transduced with AAV1-hSyn-CRE-eGFP and AAV1-hSyn-FLEX-Voltron2-ST via a complete 1 mL CCM media change containing both vectors. For the titer evaluation experiment, high titer wells received 1×10^9 gc/mL of the AAV1-hSyn-CRE-eGFP and 1×10^{10} gc/mL of the AAV1-hSyn-FLEX-Voltron2-ST. Low-titer wells received 1×10^7 gc/mL of the AAV1-hSyn-CRE-eGFP and 1×10^8 gc/mL of the AAV1-hSyn-FLEX-Voltron2-ST. Both vectors were diluted together in CCM and a 600 μ L volume was applied to each well. The viral cocktail was allowed to incubate for 3 days before being aspirated; subsequent media changes followed the every-other day schedule post transduction.

2.10 — Confocal Live Imaging Setup and Acquisition Parameters (Olympus FV3000)

Live imaging was performed on an Olympus FV3000 laser scanning confocal microscope equipped with a resonant scanner and a stage incubation chamber maintained at 37°C with 5% CO₂. Prior to sample loading, the stage incubator was pre-warmed, and the CO₂ supply was confirmed active. Plates were loaded and an initial survey scan was performed using the Galvano scanner in one-way mode at 20–40% laser power to locate regions of interest containing Voltron2-expressing cells. Once a suitable focal plane was identified, the scanner was switched to resonant mode. Where roundtrip scanning was enabled to increase frame rate, a motion-blur artifact arising from bidirectional scan line alternation was corrected by adjusting the roundtrip correction offset in the LSM panel until scan lines were correctly aligned. Time series acquisitions were configured in the Series panel with the interval set to FreeRun and cycle count set to achieve the desired recording duration. For Z-stack acquisitions, the Z Section panel was used to determine step size. Following each session, all files were transferred to the lab drive.

Multiple acquisition configurations were used across experiments, selected based on the preparation type, expression density, and temporal resolution requirements of each recording context. Configurations used for gathering the specific results discussed within this thesis are documented below.

Configuration 1: Two-Dimensional Spontaneous Voltage Recording. Two-dimensional cultures were imaged using the UPLSAPO 60XS silicone immersion objective at 4.52x digital zoom. A reduced rectangular ROI of 512x33 pixels was drawn around the somatic membrane of individual Voltron2-expressing neurons, yielding a pixel

size of 0.0917 μm and a physical field of view of 46.9x3.0 μm . The resonant scanner operated in roundtrip mode at a frame interval of 2.53 ms, corresponding to an effective acquisition rate of approximately 395 Hz. Two channels were acquired simultaneously: Channel 1 was excited at 561 nm to capture Voltron2-bound JF dye fluorescence, and Channel 2 was acquired in greyscale as a reference channel. A total of 65,535 frames were acquired per session, spanning approximately 165.8 seconds of continuous recording.

Configuration 2: Three-Dimensional Titer Evaluation and Manual Stimulation.

Three-dimensional microtissues in the titer evaluation experiment were imaged using the resonant scanner without line averaging. Two frame intervals were used within the same preparation to assess the effect of acquisition rate on signal detectability. At a 10 ms frame interval, a single resolvable cell was identified and used for $\Delta F/F_0$ analysis. At a 30 ms frame interval from the same preparation, six individually resolved cells were identified within the imaging field and used for multi-cell $\Delta F/F_0$ analysis. JF585-labeled Voltron2 fluorescence was excited at 561 nm. Imaging was performed at DIV 28.

Configuration 3: Three-Dimensional Programmed Stimulation. For the programmed stimulation experiment, microtissues were imaged at 30x magnification using the resonant scanner at a frame interval of 19.14 ms over a total acquisition duration of 7,713 ms (403 frames). JF585-labeled Voltron2 fluorescence was excited at 561 nm. This frame interval was selected to balance temporal resolution with per-frame photon collection in the high-titer preparation, where individual cell boundaries were resolvable but expression density was higher than in the titer evaluation. Six regions of interest corresponding to

individual cell somata were defined manually using the LSM ROI tool and used for $\Delta F/F_0$ extraction.

Timelapse files from all configurations were analyzed in Fiji (ImageJ). Files were imported as hyperstacks using the Bio-Formats importer with XYZCT stack order. Freehand ROIs were drawn around individual cell somata and added to the ROI Manager. The multi-measure function was applied across all frames to extract mean gray value per ROI per timepoint, and the resulting table was exported as a CSV file for downstream signal processing as described in Section 2.15.

2.11 — Stimulation Electrode and Custom Stimulation Plate Cover Design and Fabrication

Initial validation of stimulus-evoked Voltron2 voltage responses was performed using the Abdelfattah laboratory's custom stimulation electrode, where the electrode was placed within a well and waveform stimulation was administered during confocal imaging. This system established that Voltron2 expressed in dissociated cortical cultures could produce detectable $\Delta F/F$ responses time-locked to the stimulus, confirming indicator functionality prior to the development of the stimulation plate cover.

A custom stimulation plate cover was designed to deliver reproducible biphasic electrical stimulation to three-dimensional cortical microtissues during confocal imaging. The cover was designed to fit the Cellvis P24-1.5H-N 24-well glass-bottom plate and was 3D printed. Two 22 AWG solid wire electrodes were integrated per well in a bipolar configuration, with electrode tips positioned to descend into the well and contact the culture media when the cover was seated on the plate. The fixed cover geometry ensures consistent electrode-to-tissue spacing across wells and experiments, reducing variability

in the delivered electric field. Electrode leads were routed through the cover body and terminated in connectors compatible with the stimulation waveform generator used for experiments described in Section 2.12. The cover was designed to maintain an unobstructed optical path below the plate for the 20x objective. The electrode geometry and inter-electrode spacing were informed by the well dimensions of the P24-1.5H-N plate and validated by finite element analysis of electric field distribution as described in Section 2.13. Prior to use, the cover and electrodes were sterilized and confirmed to be free of media contamination between experiments.

2.12 — Electrical Stimulation Parameters

Electrical stimulation was delivered using an A-M Systems Model 4100 Isolated High Power Stimulator connected to the stimulation electrodes described in Section 2.11. Asymmetric biphasic pulse waveforms were used for all stimulation experiments. Waveform parameters were configured using the train events page of the AM4100 companion software, accessed over an ethernet connection. Two stimulation configurations were used across experiments, distinguished by delivery mode and current amplitude.

Manual Stimulation (Titer Evaluation, Section 3.5.1). In the initial titer evaluation experiment, stimulation was delivered manually using the Abdelfattah laboratory custom electrode positioned within the culture well. The stimulator was operated by hand, with individual pulses triggered using the single trigger button on the front panel during simultaneous confocal acquisition. A current amplitude of 10 mA was used, with a phase duration of 180 μ s. This configuration established that stimulus-evoked Voltron2 $\Delta F/F_0$ responses were detectable prior to the development of the custom stimulation plate cover.

Programmed Stimulation (Section 3.5.2). For the programmed stimulation experiment, stimulation was delivered via the custom stimulation plate cover described in Section 2.11 using computer-controlled asymmetric biphasic pulses. Parameters were configured in the software train events page with a current amplitude of 100 mA, a phase duration of 180 μ s, an inter-phase delay of 10 ms, and a stimulus period of 1,000 ms. Electrodes were positioned in the agarose outer well area for all recordings. Eight stimulus events were delivered across the recording duration, triggered via software during simultaneous confocal acquisition on the Olympus FV3000.

2.13 — Stimulation Plate Impedance Characterization Methods

The electrical output and spatial uniformity of the custom stimulation plate cover were characterized prior to in vitro experiments using a function generator and oscilloscope. The plate cover was seated on a Cellvis P24-1.5H-N plate filled with CCM at volumes equivalent to those used during imaging sessions. Measurements were taken across eight electrode sites distributed across the plate. A sinusoidal input of 100 mVpp was applied to each electrode pair and the output voltage was recorded across the frequency range of 10 Hz to 100 kHz. The peak-to-peak output voltage at each electrode site was measured at each frequency step to assess signal attenuation and spatial variation across the plate.

Electrode impedance was estimated using a voltage divider approximation to characterize frequency-dependent behavior of the electrode-electrolyte interface. At low frequencies, output was attenuated consistent with capacitive electrode-electrolyte interface effects. Peak gain was observed near 1 kHz, with a stable plateau at higher frequencies and no oscillatory instability. Spatial uniformity across the eight measured

electrode sites was assessed by computing the coefficient of variation (CV) of output voltage at each frequency. CV decreased from approximately 8% at 10 Hz to approximately 1-2% at 1-3 kHz, reflecting a transition from interface-dominated to bulk-resistance-dominated behavior. No electrode-specific bias or edge-dominated variation was detected. The 1-3 kHz range was identified as the optimal operating window for reproducible stimulation, demonstrating maximal gain stability and minimal inter-electrode variability.

2.14 — Fixation and Immunohistochemistry

Microtissues were fixed and immunostained following the whole microtissue immunostaining protocol developed by Atherton, Brown, and the Borton laboratory.⁵⁰ On Day 0, a fixative solution of 4% paraformaldehyde and 8% sucrose was prepared by combining 5 mL of 40% paraformaldehyde (Formalin), 4 g sucrose, and 45 mL PBS. Microtissue trampolines were fixed in this solution for between 2 to 12 hours, then rinsed briefly with PBS to remove excess PFA, followed by three 1-hour PBS washes. Samples were stored in fresh PBS at 4°C if not proceeding to staining on the same day.

On Day 1, a blocking solution was prepared consisting of 0.4 g BSA, 1 mL normal goat serum (NGS), 8.9 mL PBS, and 0.1 mL Triton X-100. The agarose gel was removed by cutting a close square around each trampoline, placing the sample in a glass scintillation vial with PBS, and heating on a hot plate at 200°C with frequent agitation until the agarose melted (after approximately 5 minutes). The agarose-free trampoline was poured into a 35 mm petri dish, the solution was immediately removed, and two quick warm PBS washes were performed before transferring to a clean 48-well plate using a P1000 pipette with the tip cut halfway. Samples were blocked in 150-200 μ L of blocking

solution per well for 2 hours on a shaker at level 3. Primary antibody solutions were prepared in blocking solution at the following dilutions: mouse anti-NeuN 1:500, mouse anti-Iba1 1:25, rabbit anti-GFAP 1:200, and rabbit anti-NG2 1:200. Primary antibody solution (150-200 μ L per well) was applied overnight at room temperature on a level 3 shaker with the plate parafiled and covered with aluminum foil.

On Day 2, a PBT wash solution was prepared (0.2% Triton X-100 in PBS; 0.1 mL Triton X-100 per 50 mL PBS) and the primary antibody solution was removed. Samples were washed once briefly with PBT, then twice for 2 hours each with 500 μ L PBT on the shaker at level 3, followed by one 2-hour wash with 500 μ L blocking solution. Secondary antibody solutions were prepared at 1:800 dilution in blocking solution using AlexaFluor 635 anti-rabbit (for GFAP and Iba1 cocktails) and Cy3 anti-mouse (for NeuN and Iba1 cocktails). Secondary antibody solution (150-200 μ L per well) was applied overnight at room temperature on a level 3 shaker, parafiled and covered with foil.

On Day 3, the secondary antibody solution was removed. Trampolines were washed briefly with PBT to remove excess secondary, then washed four times for 1 hour each with 500 μ L PBT on the shaker at level 3. DAPI solution was prepared at 1:1000 in PBT and applied at 300 μ L per well for 1 hour on the shaker at level 3 at room temperature. DAPI solution was removed and samples were returned to PBS.

On Day 4, optical clearing was performed immediately before imaging. Clearing solutions were prepared cold: 25% formamide/10% PEG was made by combining 50% formamide in PBS with 20% PEG (8000 MW, w/v in water) 1:1 (vol:vol); 50% formamide/20% PEG was made by combining 100% formamide stock with 40% PEG 1:1 (vol:vol). PBS was removed and samples were incubated sequentially in 25% formamide/10% PEG for 10

minutes, then 50% formamide/20% PEG for 5 minutes, then a second incubation in 50% formamide/20% PEG for 15 to 60 minutes, all on a room temperature shaker. Cleared samples were imaged immediately.

2.15 — Image Analysis and $\Delta F/F$ Signal Processing

Fluorescence timelapse files acquired on the Olympus FV3000 were imported into Fiji (ImageJ) as hyperstacks using the Bio-Formats importer with XYZCT stack order. Freehand ROIs were manually drawn around individual cell somata of interest and added to the ROI Manager. The multi-measure function was applied across all frames to extract the mean gray value per ROI and each row corresponds to one timepoint. This table was then exported as a CSV file for downstream signaling.

In python, raw fluorescence traces $F(t)$ were converted to $\Delta F/F_0$ using a rolling baseline correction. The baseline $F^0(t)$ was estimated at each timepoint as a low percentile of the fluorescence signal within a sliding temporal window, capturing the slowly varying baseline fluorescence while excluding transient activity-related increases. $\Delta F/F_0$ was then computed as $((F(t)-(F^0(t))/ F^0(t))$. A four-frame smoothed running average was applied to each $\Delta F/F_0$ trace to reduce high-frequency shot noise while preserving the temporal structure of voltage transients. Stimulus-evoked responses were identified as $\Delta F/F_0$ deflections correlated to the enacted electrical stimulus delivered by the A-M Systems Model 4100 as described in Section 2.12.

2.16 — Optical Flow Analysis

Dense optical flow was computed across the full 403-frame timelapse recording using the Farnebäck algorithm as implemented in OpenCV (Python). The algorithm estimates per-pixel displacement between consecutive frames by fitting polynomial

expansions to local pixel neighborhoods, producing a dense two-dimensional displacement field at each frame transition. Key parameters were set as follows: pyramid scale 0.5, pyramid levels 4, window size 15, iterations 3, polynomial neighborhood size 7, and polynomial sigma 1.5. Per-frame mean displacement magnitude was computed as the square root of the squared sum of the x and y displacement components, averaged across all pixels in the field of view. Stimulus-locked displacement averages were derived by epoching the mean displacement trace around each detected stimulus event, using a window of 10 pre-stimulus and 20 post-stimulus frames, and averaging across all six events. Full-trace Pearson correlations between per-frame displacement magnitude and each cell's $\Delta F/F_0$ trace were computed using `scipy.stats.pearsonr`. Motion contamination scores were computed per cell per stimulus event as the ratio of peak displacement magnitude to peak absolute $\Delta F/F_0$ within a ± 12 -frame window centered on each stimulus onset and averaged across events to yield a single per-cell contamination score.

2.17 — XY Motion Correction

A reference frame was constructed by averaging the first 50 frames of the timelapse recording, representing the pre-transient baseline fluorescence distribution prior to any stimulus-evoked activity. For each subsequent frame, a Farnebäck displacement field was computed between the reference frame and that frame using the parameters described in Section 2.16. Each frame was then warped back to the reference coordinate space by remapping pixel coordinates using the estimated displacement field, with bilinear interpolation and reflected border handling applied via `cv2.remap`. Fluorescence traces were extracted from the motion-corrected stack by computing the mean pixel intensity within each cell ROI at every frame, identical in procedure to the raw

extraction described in Section 2.15. $\Delta F/F_0$ was recomputed from the corrected traces using the same rolling 8th-percentile baseline correction and 4-frame uniform smoothing described in Section 2.15. Motion correction addresses lateral XY displacement only and does not account for z-axis focal plane drift. All optical flow and motion correction procedures were implemented in Python using OpenCV and scikit-image.

Ch. 3 — Results

3.1 — Two-Dimensional Media Comparison: Brightfield Morphology and Culture Density

The mixed primary cortical cells plated were plated at approximately 500,000 cells per mL and were monitored by brightfield microscopy following the media change at 24 hours post plating. Both the CCM and NbActiv4 conditions supported cell adhesion and survival across the culture period. Under brightfield imaging, CCM wells exhibited a lower overall cell density with individual neurons and their extending processes clearly visible in contrast to the sparse background. This observation is consistent with the serum-free formulations established role in suppressing glial proliferation. NbActive4 wells displayed a markedly higher cell density with a more confluent cellular background. These morphological differences between conditions were apparent by the time of initial imaging and persisted throughout the culture period.

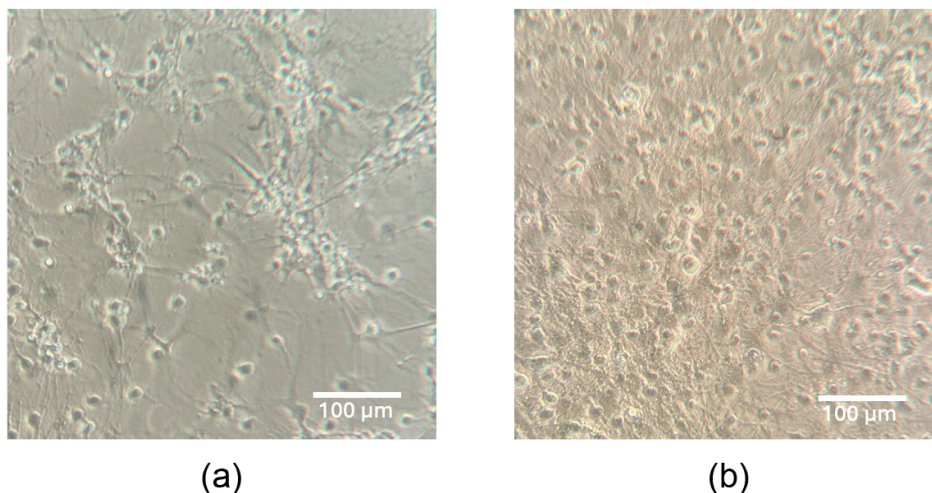


Figure 2: Brightfield comparison of primary cortical cultures under CCM and NbActive4 media conditions. Representative brightfield images of mixed primary cortical cultures at DIV 14 under (a) CCM (Nerubasal A/B27, serum-free) and (b) NbActive4. CCM cultures exhibit lower cell density with individual resolved neuronal somata and extended processes. NbActive4 cultures showed markedly higher cell density with a confluent cellular background.

These differences in culture density and morphology between CCM and NbActive4 conditions established a clear structural baseline for evaluating Voltron2 performance across formulations. To determine whether these media-dependent differences extended to transgene expression and indicator labeling, cultures were subsequently assessed by fluorescence imaging following JF dye conjugation to the Voltron2 HaloTag domain.

3.2 — Two-Dimensional Voltron2 Expression Check and JF Dye Labeling

Voltron2 expression was confirmed in both media conditions by fluorescence imaging of JF-525 and JF-608 HaloTag-conjugated dye labeling at DIV 14 to DIV 16, acquired on the Abdelfattah laboratory Nikon Eclipse Tiw microscope prior to confocal live imaging sessions. In both CCM and NbActive4 cultures, Voltron2-expressing neurons exhibited characteristic soma-targeted membrane fluorescence consistent with motif incorporated in the Voltron2 construct, with additional labeling extending into proximal

dendritic processes. CCM neurons displayed bright, soma-concentrated dual-channel labeling with well-resolved dendritic arbors extending across the field of view in both the JF-525 (green) and JF-608 (red) channels. NbActiv4 neurons showed a more diffuse labeling pattern with neurite-dominant fluorescence distribution, though somatic signal was comparable in intensity across conditions. Dye co-localization confirmed the presence of Voltron2-HaloTag protein accessible to both JF-525 and JF-608 ligands in both media conditions. Critically, the density of Voltron2-expressing neurons appeared consistent across CCM and NbActiv4 wells, with neither condition producing an excess of labeled cells that would cause signal crowding nor a deficit that would limit the number of recordable neurons per field. This balance in expression density between conditions further supported the conclusion that media composition did not differentially influence AAV transduction efficiency or transgene expression levels. These results established that neither CCM nor NbActiv4 adversely affected Voltron2 transduction efficiency, transgene expression, or HaloTag accessibility for dye conjugation. Representative fluorescence images of JF-525 and JF-608 labeling across both conditions are shown in Figure 3, with scale bars of 100 μm .

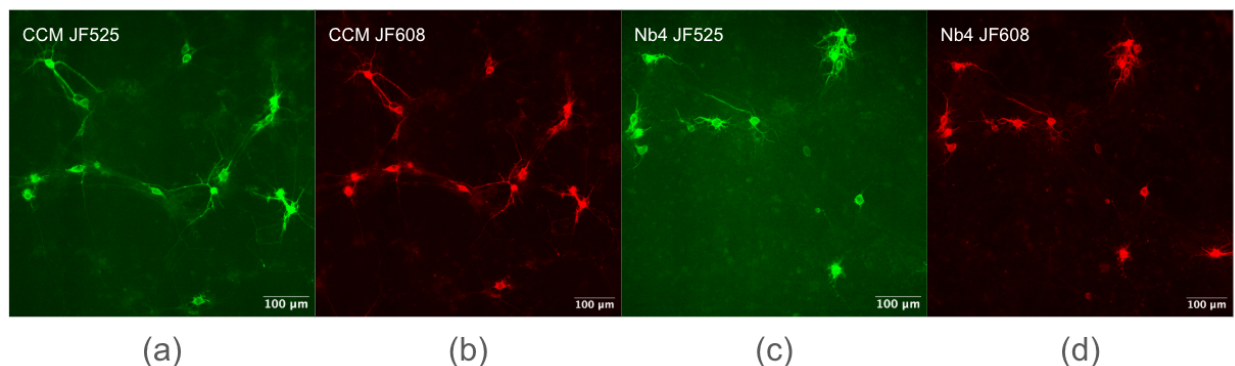


Figure 3: Voltron2 expression confirmed in 2D cortical cultures under CCM and NbActiv4 conditions via dual JF dye labeling. Fluorescence images of Voltron2-

expressing primary cortical neurons labeled with JF-525 and JF-608 at DIV 14 under CCM (a, b) and NbActiv4 (c, d) conditions. CCM neurons exhibit bright soma-concentrated labeling with well-resolved dendritic processes in both channels. NbActiv4 neurons display more diffuse neurite-dominant fluorescence with comparable somatic signal intensity.

3.3 — Live Two-Dimensional Spontaneous Voltage Recording Attempt

Live voltage recording was attempted in 2D cultures at DIV 14 to DIV 16 using the Olympus FV3000 resonant scanner in roundtrip mode with the UPLSAPO 60XS silicone immersion objective lens. Acquisitions were collected at a 4.52x digital zoom across a 512x33 pixel ROI containing the somatic membrane of a neuron expressing Voltron2, yielding a pixel size of 0.0917 μm per pixel and a physical field of view of 46.9x3.0 μm (Figure 3a). The resonant scanner operated at a frame interval of 2.53 ms, corresponding to an effective acquisition rate of approximately 395 Hz. A total of 65,535 frames were acquired per recording session, spanning a continuous imaging duration of approximately 165.8 seconds. Two channels were acquired simultaneously. Channel 1, excited at 561 nm, captured Voltron2-bound JF dye fluorescence, and channel 2 was acquired in greyscale as a reference channel. Mean fluorescence was extracted from the Voltron2 channel and processed using a rolling 8th-percentile baseline correction with a 1,000-frame window, followed by 4-frame uniform smoothing, to yield $\Delta F/F_0$ expressed as a percentage. The resulting trace showed high-frequency fluctuations with a noise floor standard deviation of 2.47% $\Delta F/F_0$ (3b, c). No spontaneous voltage transients above the noise floor were detected across the full 165.8-second recording in either media condition.

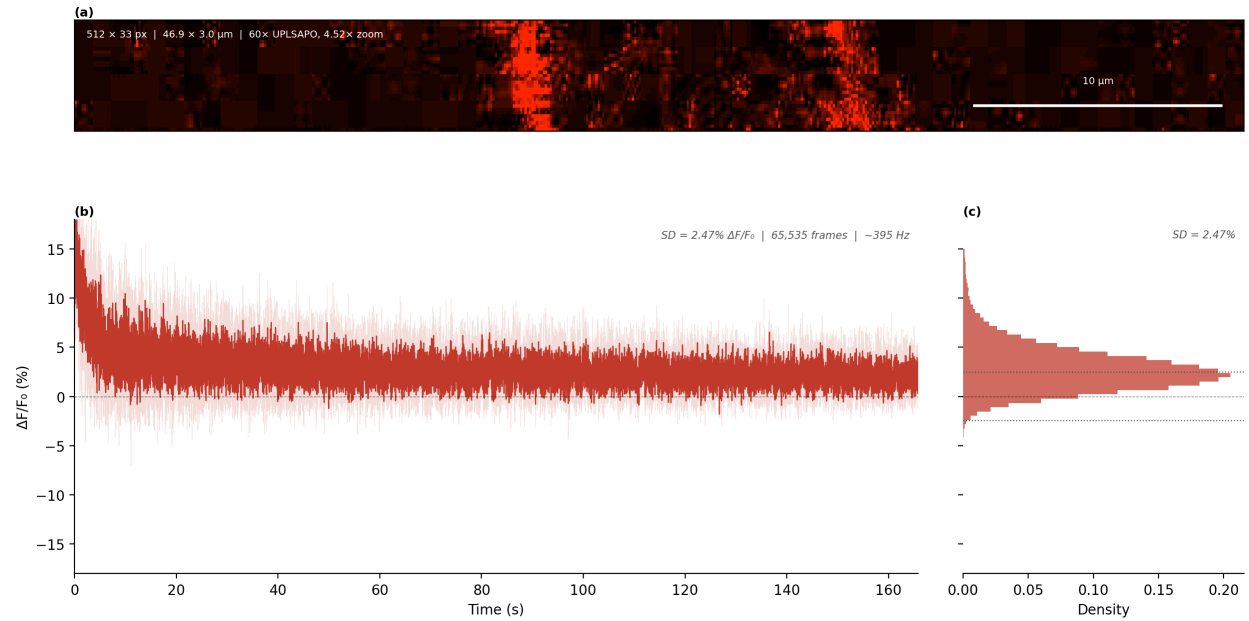


Figure 3: Voltron2 acquisition ROI, $\Delta F/F_0$ trace, and amplitude distribution from 2D spontaneous baseline recording. (a) Single-frame image of the CH2 acquisition ROI (512x33 px, 46.9x3.0 μm) showing Voltron2-expressing neurons as punctate fluorescent structures. Contrast enhanced for display. Scale bar, 10 μm. (b) Rolling 8th-percentile baseline-corrected $\Delta F/F_0$ trace from the Voltron2 channel acquired at ~395 Hz across 165.8 seconds at DIV 14–16. Raw signal and 4-frame smoothed trace overlaid. (c) Probability density histogram of $\Delta F/F_0$ amplitudes. Dotted lines indicate ± 1 SD (2.47% $\Delta F/F_0$). No spontaneous voltage transients were detected above the noise floor across the full recording duration.

3.4 — Two-Dimensional Immunohistochemistry

Following live imaging, two-dimensional cortical neuron cultures were fixed at DIV 19 and processed for immunohistochemistry (IHC) to characterize the cellular composition of each culture condition and to assess the compatibility of Voltron fluorescence with downstream antibody labeling. Four antibody cocktails were applied across both CCM and NB4 media conditions shown in Figure 4: Cocktail 1 (C1) targeting neuronal nuclei with mouse anti-NeuN primary and goat anti-mouse CY3 secondary; Cocktail 2 (C2) targeting microglia with mouse anti-Iba1 primary and goat anti-mouse CY3 secondary; Cocktail 3 (C3) targeting astrocytes with rabbit anti-GFAP primary and

goat anti-rabbit AF635 secondary; and Cocktail 4 (C4) targeting oligodendrocyte precursor cells (OPCs) with rabbit anti-NG2 primary and goat anti-rabbit AF635 secondary. CCM cultures received three replicates per cocktail (wells A1-A3, B1-B3, C1-C3, and D1-D3, respectively). NB4 cultures received two replicates of C1 (A4-A5) and C2 (B4-B5), and single replicates of C3 (A6) and C4 (B6). All imaging was performed on a confocal microscope at 10x magnification with z-stack maximum intensity projections (MAX Z), with representative fields additionally acquired at 4x optical zoom for higher-magnification assessment of co-localization.

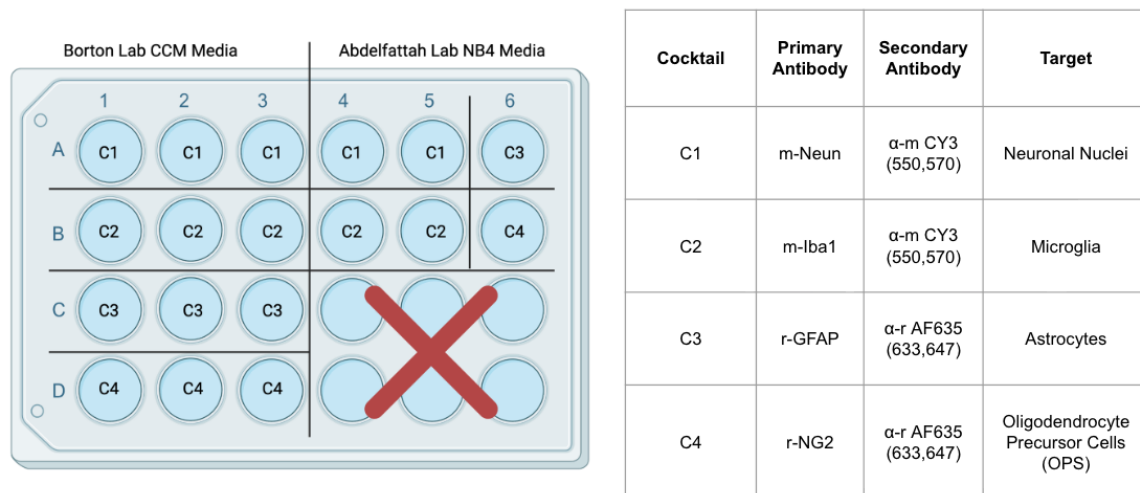


Figure 4: Two-dimensional IHC experimental design: plate layout and antibody cocktail assignments. A 24-well plate schematic showing the distribution of four immunohistochemistry antibody cocktails across CCM (columns 1-3) and NB4 (columns 4-6) media conditions at DIV 19. CCM wells received three replicates per cocktail: C1 (anti-NeuN, rows A1-A3), C2 (anti-Iba1, rows B1-B3), C3 (anti-GFAP, rows C1-C3), and C4 (anti-NG2, rows D1-D3). NB4 wells received two replicates of C1 (A4-A5) and C2 (B4-B5), and single replicates of C3 (A6) and C4 (B6). NB4 rows C and D were unused (marked). Table (right) summarizes primary antibody, secondary antibody with excitation/emission wavelengths (nm), and cellular target for each cocktail. C1: mouse anti-NeuN, goat anti-mouse CY3 (550/570 nm), neuronal nuclei. C2: mouse anti-Iba1, goat anti-mouse CY3 (550/570 nm), microglia. C3: rabbit anti-GFAP, goat anti-rabbit AF635 (633/647 nm), astrocytes. C4: rabbit anti-NG2, goat anti-rabbit AF635 (633/647 nm), oligodendrocyte precursor cells (OPCs).

3.4.1 — Neuronal Identity: NeuN Labeling (Cocktail 1)

NeuN IHC was performed to assess the neuronal identity of Voltron-expressing cells across both media conditions (Figure 4). In CCM replicates (A1-A3), DAPI staining revealed a dense, healthy nuclear distribution at DIV 19 (Figure 4, panels i, v). CY3 NeuN signal was robust across all three replicates, labeling cells with morphologically complex soma and extended processes consistent with mature cortical neurons (Figure 4, panels iii, vii). However, interpretation of Voltron fluorescence in C1 wells is confounded by the spectral properties of the goat anti-mouse CY3 secondary antibody (excitation/emission 550/570 nm). CY3 emission overlaps directly with the detection window of the mCherry Voltron JF608 channel (560/608 nm), rendering the JF608 channel uninterpretable as a Voltron signal in all C1 wells. Additionally, the CY3 excitation tail extends toward the JF525 emission peak (495/525 nm), raising the possibility of partial contamination of the EYFP Voltron channel as well. As a result, neither Voltron channel can be used as an independent readout of expression in CY3-labeled wells, and co-localization between Voltron and NeuN cannot be unambiguously established from this cocktail alone without spectral unmixing.

Despite this spectral constraint, the spatial distribution of JF525 signal across CCM replicates is morphologically consistent with neuronal Voltron expression. At 10x magnification (Figure 4, panels ii, iv), labeled structures exhibit a diffuse, process-spanning distribution spatially coincident with NeuN-positive soma and neurites. At 10x with 4x optical zoom (Figure 4, panels vi, viii), co-distribution of JF525 signal with individual NeuN-positive cells and their process arbors is apparent at the single-cell level, including a representative field in well A1 selected to illustrate this morphological correspondence. Labeled cells exhibit branched dendritic processes and soma

dimensions comparable to NeuN-positive neurons in the same field. These observations suggest that the JF525 channel signal reflects genuine Voltron expression in neurons rather than non-specific CY3 bleedthrough, given that bleedthrough would be expected to produce a more diffuse, cell-type-nonspecific pattern. This interpretation should nonetheless be treated as qualitative in the absence of spectral unmixing controls.

In NB4 replicates (A4-A5), NeuN labeling was present and morphologically appropriate (Figure 4, panels xi, xv). Signal in the JF525 channel was markedly weaker than in CCM wells across both magnifications, with limited, low-intensity puncta observed across the field (Figure 4, panels x, xiv). This reduction is visible in both the individual JF525 channel and the merged composite (Figure 4, panels xii, xvi), where the warm co-localization signal prominent in CCM merged panels is substantially attenuated. Given that the spectral overlap caveat applies equally to NB4 C1 wells, this reduced JF525 signal is most parsimoniously interpreted as reflecting lower Voltron expression under NB4 conditions, consistent with live imaging observations from Section 3.2, rather than a secondary effect of altered CY3 bleedthrough dynamics between conditions.

Figure 3.4.2

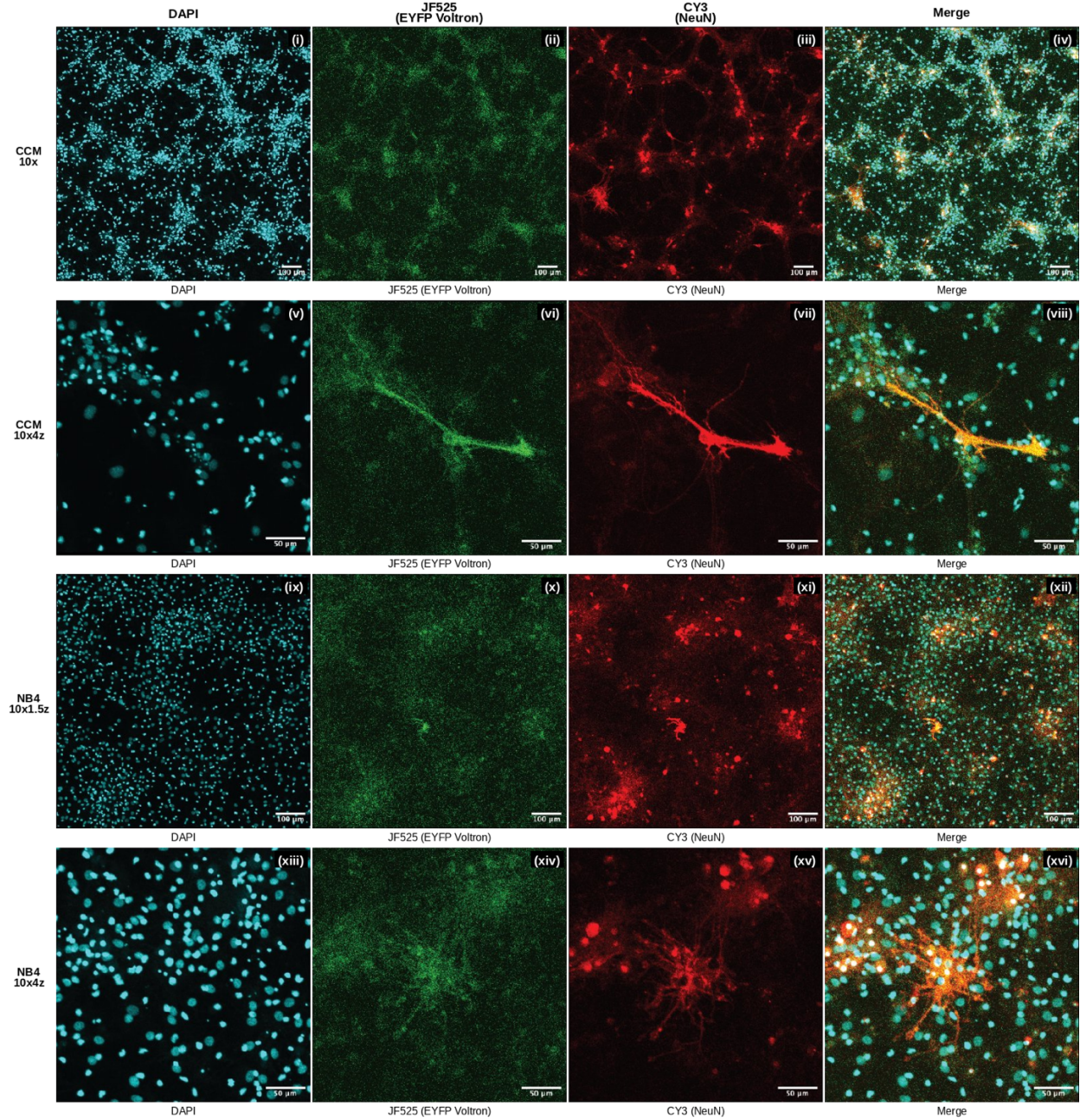


Figure 4. Immunohistochemical characterization of Voltron expression relative to NeuN-positive neurons across culture media conditions, representative fields (well A1, CCM; well A6, NB4; DIV 19; C1 antibody cocktail). Panels show (i–iv) CCM condition at 10x magnification, (v–viii) CCM condition at 10x with 4x optical zoom, (ix–xii) NB4 condition at 10x magnification, and (xiii–xvi) NB4 condition at 10x with 4x optical zoom. Within each row, columns display (left to right) DAPI nuclear counterstain, JF525-labeled EYFP Voltron signal, CY3-conjugated anti-NeuN immunolabel, and merged composite. In CCM wells, JF525 Voltron signal exhibits a diffuse, process-spanning

distribution that is spatially coincident with CY3-positive neuronal soma and neurites, providing morphological evidence for preferential Voltron expression in NeuN-positive neurons. High-magnification panels (v–viii) reveal co-distribution of JF525 signal with individual NeuN-positive cells and their processes at the single-cell level. In NB4 wells, JF525 Voltron signal is markedly reduced relative to CCM across both magnifications, while NeuN-positive populations remain morphologically intact, consistent with a reduction in transduction efficiency or transgene expression under NB4 conditions rather than loss of neuronal viability. Interpretation of Voltron neuronal specificity in C1 wells is qualitative and morphologically based; the CY3 secondary antibody emission peak (~570 nm) overlaps the JF608 detection window, rendering quantitative co-localization analysis unreliable in this cocktail without spectral unmixing. Scale bars: 100 μ m (rows 1, 3); 50 μ m (rows 2, 4).

3.4.2 — Microglial Characterization: Iba1 Labeling (Cocktail 2)

Iba1 IHC was performed to assess microglia within both culture conditions (Figure 5). C2 wells used the same goat anti-mouse CY3 secondary antibody as C1 and therefore carry an identical spectral overlap constraint of CY3 emission peak (~570 nm) that directly contaminates the JF608 detection channel, and the lower end of the CY3 excitation extends down toward the JF525 emission window, impeding independent interpretation of either Voltron channel in C2 wells without spectral unmixing. Examination of the CY3 channel across B wells reveals signal with neuronal rather than microglial process geometry, consistent with Voltron bleedthrough from the JF525 channel into the CY3 detection window rather than genuine Iba1 labeling of microglia (Figure 5, panels iii, vii, xi, xv). Given that the CY3 channel cannot be distinguished from Voltron signal in this cocktail, definitive assessment of Voltron co-localization with Iba1-positive microglia is not possible from these data. Microglial exclusion of Voltron expression therefore rests on the AF635-based C3 and C4 cocktail data presented in the following sections, where no spectral conflict with either Voltron channel exists.

Figure 3.4.3

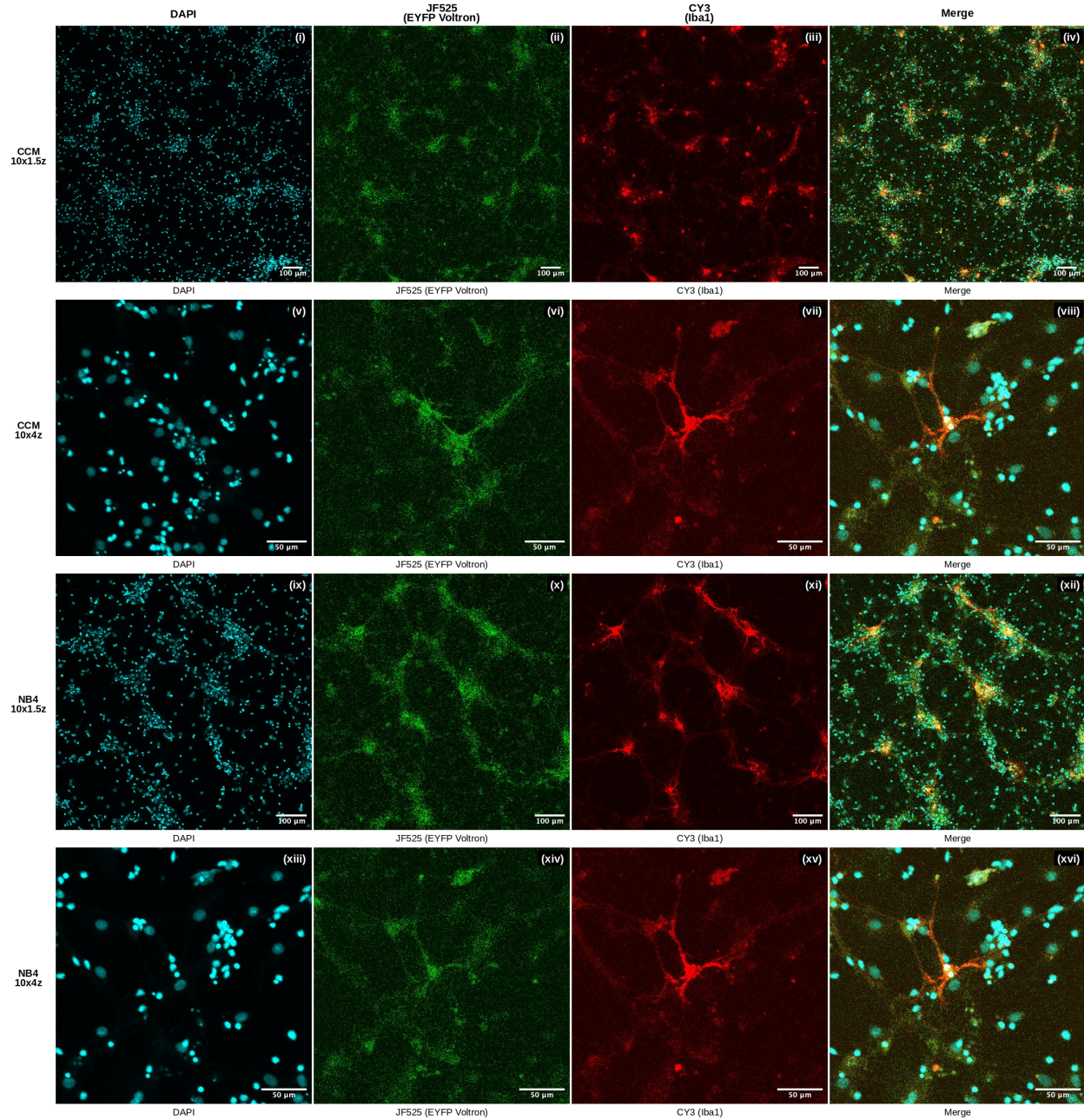


Figure 5. Immunohistochemical characterization of Iba1 labeling and Voltron signal in bulk cortical cultures across media conditions, representative fields (well B1, CCM; well B4, NB4; DIV 19; C2 antibody cocktail). Panels show (i--iv) CCM at 10x, (v--viii) CCM at 10x with 4x optical zoom, (ix--xii) NB4 at 10x, and (xiii--xvi) NB4 at 10x with 4x optical zoom. Within each row, columns display DAPI nuclear counterstain, JF525 EYFP Voltron, CY3 anti-Iba1, and merged composite. CY3 channel signal (panels iii, vii, xi, xv) displays neuronal process morphology inconsistent with microglial labeling, indicating Voltron bleedthrough rather than genuine Iba1 co-localization. These data are

presented for transparency; assessment of Voltron microglial specificity relies on the AF635-based C3 and C4 cocktails. Scale bars: 100 μ m (rows 1, 3); 50 μ m (rows 2, 4).

3.4.3 — Astrocyte Characterization: GFAP Labeling (Cocktail 3)

GFAP IHC was performed to characterize the astrocyte population within CCM cultures (Figure 6). The AF635 secondary antibody (excitation/emission 633/647 nm) used in Cocktail 3 does not overlap spectrally with either the JF525 or JF608 detection windows, making C3 wells the only cocktail in this study in which both Voltron channels were simultaneously interpretable alongside the cell-type marker without risk of secondary antibody contamination.

In CCM replicates (C1-C3), AF635 GFAP signal revealed a regular, extensively ramified astrocytic network spanning the imaging field, consistent across two independent representative fields from well C2 (Figure 6, panels iv, ix). Both JF525 EYFP Voltron and JF608 mCherry Voltron signals were present but spatially distinct from GFAP-positive structures across all fields (Figure 6, panels ii-iii, vii-viii), with no warm co-localization signal overlapping the AF635 network in the merged composites (Figure 6, panels v, x). At 10x with 4x optical zoom, a single stellate astrocyte with an elaborate, spiny process arbor is visible in the AF635 channel (Figure 6, panel xiv); neither Voltron channel co-localizes with this cell or its processes in the merged composite (Figure 6, panel xv), confirming spatial exclusion of Voltron expression from the astrocytic compartment at single-cell resolution.

The NB4 C3 replicate (well A6) was acquired but is not included in Figure 6. AF635 GFAP signal in A6 exhibited a markedly denser and more spatially uniform network morphology than observed in CCM C3 wells, which could reflect either reactive astrogliosis under NB4 conditions or a staining artifact from potential cross-contamination

from adjacent wells during the IHC protocol. As a single unreplicated well, A6 does not provide sufficient evidence to distinguish between these interpretations, and the non-neuronal exclusion of Voltron expression in the astrocyte compartment is therefore based on the CCM dataset alone.

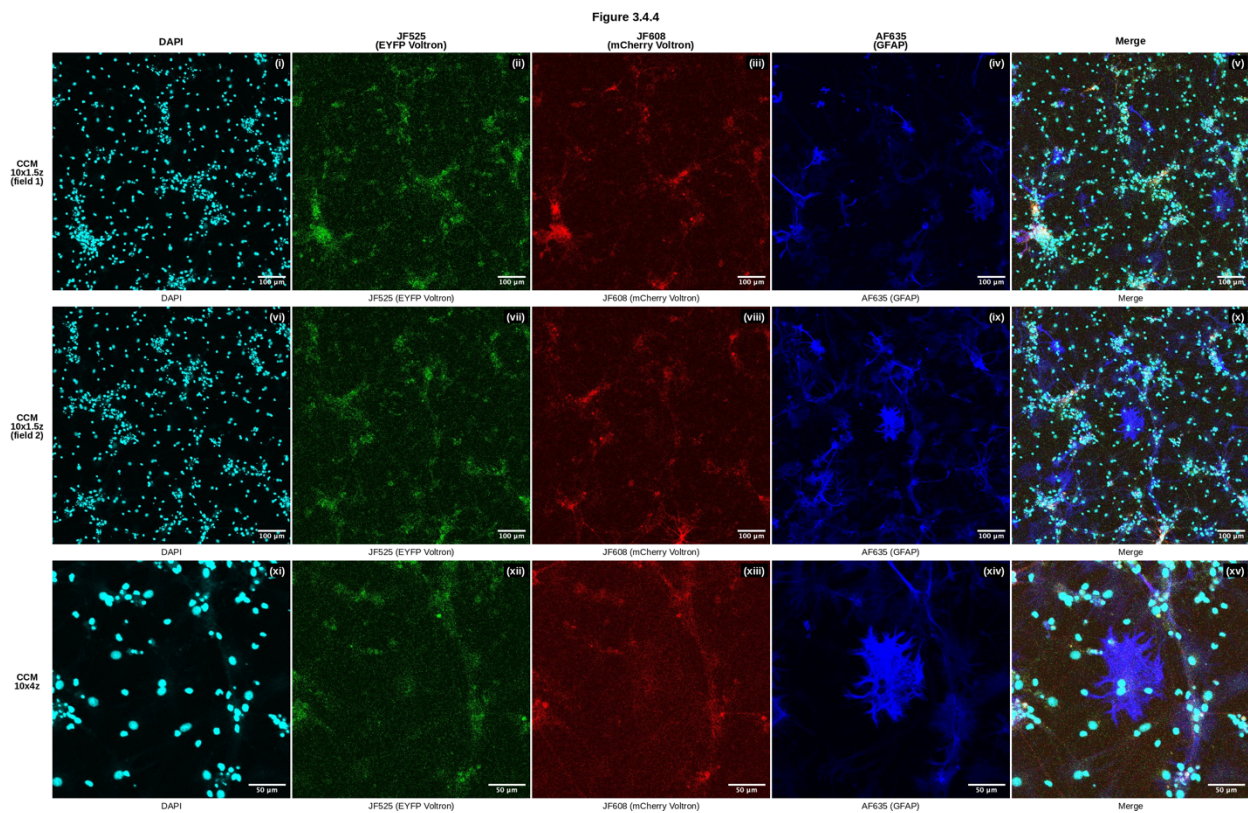


Figure 6. Immunohistochemical characterization of Voltron expression relative to GFAP-positive astrocytes, CCM condition, representative fields (well C2, DIV 19; C3 antibody cocktail). Panels show (i-v) CCM field 1 at 10x magnification, (vi-x) CCM field 2 at 10x magnification, and (xi-xv) CCM field 1 at 10x with 4x optical zoom. Within each row, columns display DAPI nuclear counterstain, JF525 EYFP Voltron, JF608 mCherry Voltron, AF635 GFAP, and merged composite. At 10x, AF635 signal reveals a regular, extensively ramified astrocytic network spanning the imaging field, consistent across both representative fields (panels iv, ix). At 4x optical zoom, a single stellate astrocyte with an elaborate, spiny process arbor is visible in the AF635 channel (panel xiv); neither JF525 nor JF608 Voltron signal co-localizes with this cell or its processes in the merged composite (panel xv), confirming spatial exclusion of Voltron expression from the astrocytic compartment at single-cell resolution. The AF635 secondary (633/647 nm) does not overlap spectrally with either Voltron channel, permitting independent interpretation of both channels. Scale bars: 100 µm (rows 1-2); 50 µm (row 3).

3.4.4 — Oligodendrocyte Precursor Cell Characterization: NG2 Labeling (Cocktail 4)

NG2 IHC was performed to assess the oligodendrocyte precursor cell (OPC) population within CCM and NB4 cultures. As with the C3 GFAP cocktail, the AF635 secondary antibody used for NG2 labeling does not overlap spectrally with either JF525 or JF608, permitting simultaneous interpretation of both Voltron channels alongside the cell-type marker.

In CCM replicates (D1-D3), AF635 NG2 signal was present across all three wells. At 10x magnification, NG2-positive cells displayed a characteristic OPC morphology bipolar and multipolar process extensions forming a distributed network throughout the field visible across two independent imaging fields from well D3 (Figure 7, panels iv, ix). This morphology is distinct from both the elaborate dendritic arbors of NeuN-positive neurons and the highly ramified astrocytic network seen in C3 wells, and is consistent with an OPC subpopulation expected in bulk cortical preparations. Both JF525 and JF608 Voltron signals were present but spatially distinct from AF635-positive OPCs across both overview fields (Figure 7, panels ii-iii, vii-viii), with no warm co-localization signal overlapping the NG2 network in the merged composites (Figure 7, panels v, x).

At 10x with 5x optical zoom (Figure 7, panels xi-xv), the spatial exclusion of Voltron from the OPC compartment is resolved at the single-cell level. A representative Voltron-expressing cell with warm orange co-localization in JF525 and JF608 channels (Figure 7, panel xiii-xiv) sits centrally within a field populated by NG2-positive processes; the merged composite (Figure 7, panel xv) shows clear segregation between this Voltron-expressing cell and the surrounding AF635 NG2 network, with no co-localization between the two signals.

The NB4 C4 replicate (well B6) was acquired but presented interpretive limitations due to probable cross-contamination from adjacent wells, as discussed in Section 3.4.1. The non-neuronal exclusion of Voltron expression in the OPC compartment therefore rests on the CCM D3 dataset, which provides two independent imaging fields with clean spectral separation across all channels.

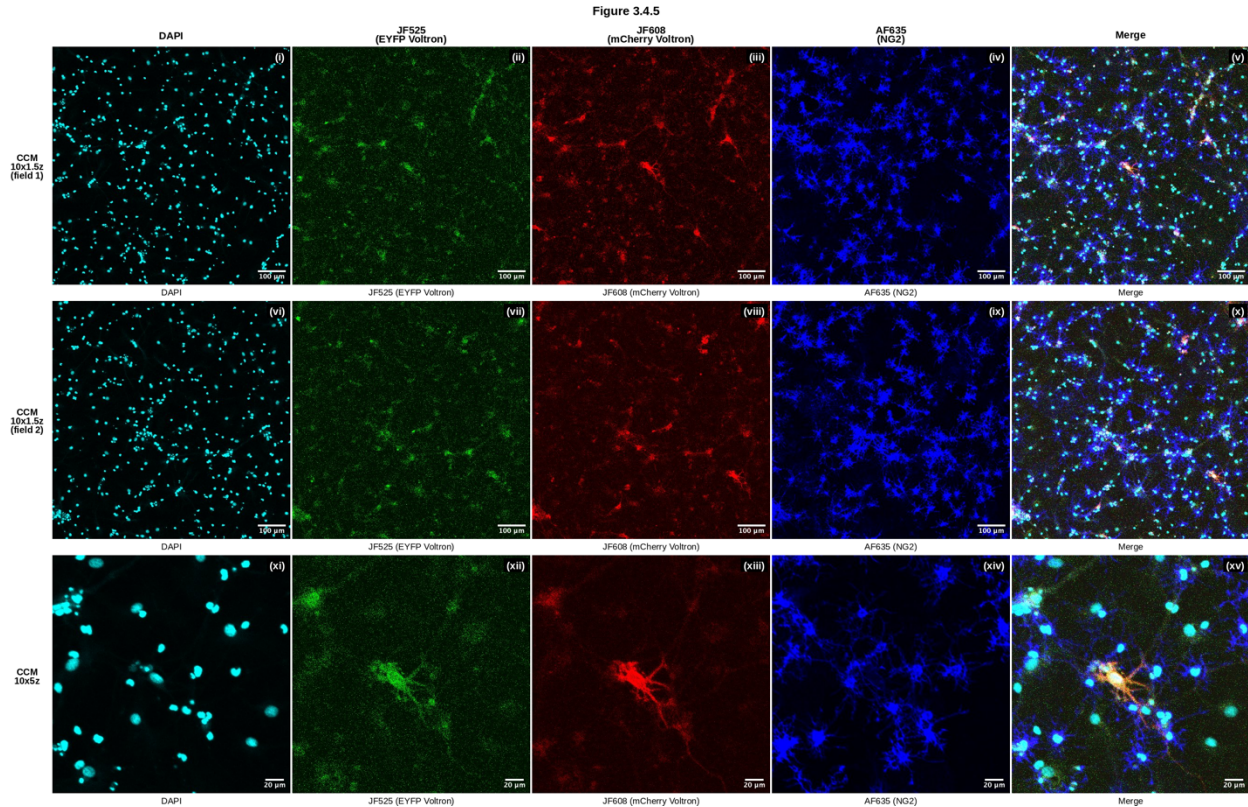


Figure 7. Immunohistochemical characterization of Voltron expression relative to NG2-positive oligodendrocyte precursor cells, CCM condition, representative fields (well D3, DIV 19; C4 antibody cocktail). Panels show (i--v) CCM field 1 at 10x magnification, (vi--x) CCM field 2 at 10x magnification, and (xi--xv) CCM field 1 at 10x with 5x optical zoom. Within each row, columns display DAPI nuclear counterstain, JF525 EYFP Voltron, JF608 mCherry Voltron, AF635 NG2, and merged composite. At 10x, AF635 signal reveals a distributed OPC population with characteristic bipolar and multipolar process morphology, consistent across both representative fields (panels iv, ix). Both Voltron channels are spatially distinct from NG2-positive cells across all fields. At 5x optical zoom, a single Voltron-expressing cell (panels xiii--xiv) sits within a field of NG2-positive processes with no co-localization in the merged composite (panel xv), confirming Voltron exclusion from the OPC compartment at single-cell resolution. The

AF635 secondary (633/647 nm) does not overlap spectrally with either Voltron channel, permitting independent interpretation of both channels. Scale bars: 100 μm (rows 1--2); 20 μm (row 3).

3.5 — Three-Dimensional Microtissue Results

3.5.1 — Initial Voltron AAV Titer Evaluation and Manual Stimulation

To determine a suitable viral titer for Voltron2 delivery into three-dimensional cortical microtissues, microtissues at DIV 01 were transduced with either a high-titer or low-titer dual-vector combination. The high-titer condition consisted of AAV1-hSyn-CRE-eGFP at 1×10^9 gc/mL paired with AAV1-hSyn-FLEX-Voltron2-ST at 1×10^{10} gc/mL. The low-titer condition used AAV1-hSyn-CRE-eGFP at 1×10^7 gc/mL paired with AAV1-hSyn-FLEX-Voltron2-ST at 1×10^8 gc/mL. Following transduction and JF585-HaloTag labeling, microtissues were imaged under electrical stimulation at 10 mA using the A-M Systems Model 4100 stimulator delivering asymmetric biphasic pulses applied manually throughout the recording duration.

Fluorescence images acquired from high-titer microtissues revealed a dense, nearly continuous labeling across the imaging field (Figure 8A(i), A(ii)). Voltron2-expressing processes took up the majority of the field of view. Individual soma and neurite boundaries were difficult to distinguish due to the high density of labeled cells. This expression pattern prevented reliable identification or separation of single-cell regions of interest, as fluorescence signal from surrounding labeled neurons exhibited substantial spatial overlap. Due to these limitations, no quantitative $\Delta F/F_0$ analysis was performed on high-titer recordings.

Low-titer transduction produced much sparser expression. At a 10 ms frame interval, a single resolvable cell was identified within the imaging field (Figure 8B(iii),

B(iv)). This cell had a baseline fluorescence of 382.49 arbitrary units and produced a mean stimulus-evoked $\Delta F/F_0$ of -0.71% , of negative polarity. The full $\Delta F/F_0$ trace for this cell is shown in Figure 9, where a progressive negative deflection is visible during the stimulation period.

At a 30 ms frame interval acquired from the same low-titer preparation, six individual cells with clear somata and neurite arbors were identified and isolated as regions of interest within the imaging field (Figure 8C(v), C(vi)). Baseline fluorescence values (F_0), computed by taking the mean intensity across a thirty-frame resting window, were 162.13, 231.20, 204.11, 237.64, 266.53, and 199.65 arbitrary units for Cells 1 through 6, respectively. Full $\Delta F/F_0$ traces across the recording duration for all six cells are shown in Figure 9.

Stimulus-evoked $\Delta F/F_0$ responses were computed by averaging across ten 10 Hz stimulus events for each cell. Peak $\Delta F/F_0$ values were -1.53% (Cell 1), -1.10% (Cell 2), -1.19% (Cell 3), -4.62% (Cell 4), -0.80% (Cell 5), and -1.18% (Cell 6), yielding a mean $\pm$ standard deviation of $-1.74 \pm 1.31\%$ across the six cells. Cell 4 exhibited a notably larger response magnitude relative to the remaining five cells, which clustered between -0.80% and -1.53% . All six cells produced responses of negative polarity, consistent with the voltage-dependent fluorescence decrease characteristic of Voltron2 upon membrane depolarization.

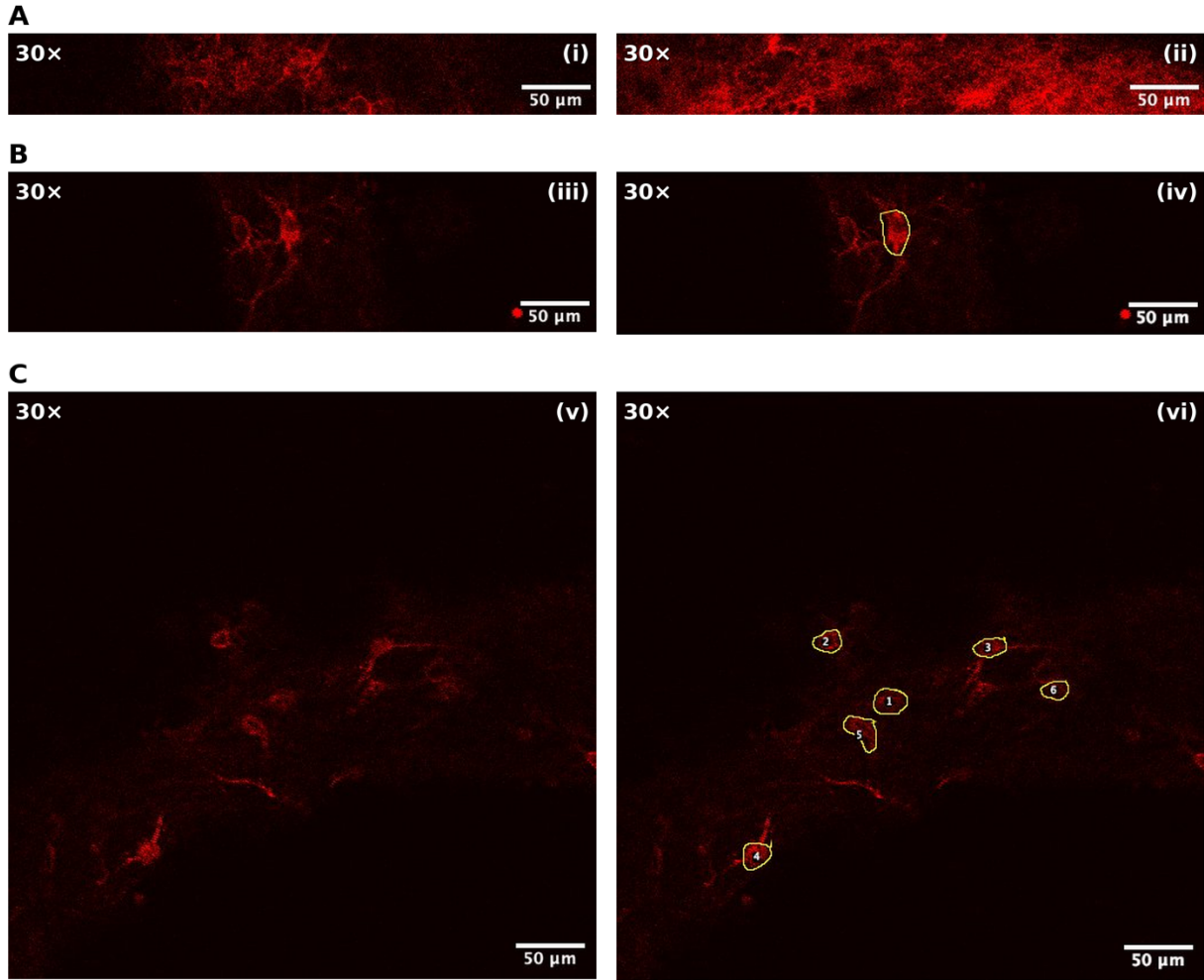


Figure 8. Voltron2 expression patterns across high- and low-titer AAV transduction conditions in three-dimensional cortical microtissues. Representative fluorescence images acquired at day in vitro twenty-eight from microtissues transduced with high-titer or low-titer dual-vector AAV combinations and labeled with JF585-HaloTag dye. Scale bars, 50 μm . (A) High-titer transduction produced dense, continuous Voltron2-ST fluorescence across the imaging field, with individual somatic and neuritic boundaries unresolvable in both fields of view (i, ii). (B) Low-titer transduction imaged at a 10 ms frame interval; (iii) raw fluorescence image and (iv) corresponding ROI overlay showing the single resolvable cell. (C) Low-titer transduction imaged at a 30 ms frame interval; (v) raw fluorescence image and (vi) corresponding ROI overlays identifying six individually resolved cells.

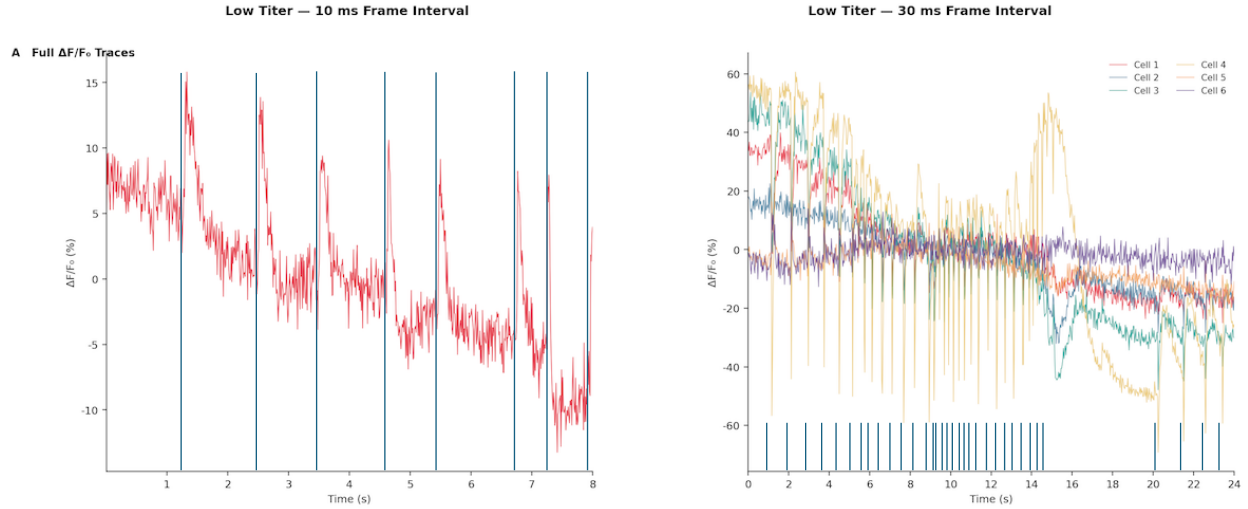


Figure 9. Full $\Delta F/F_0$ traces for low-titer Voltron2 transduction conditions. (Left) Single-cell $\Delta F/F_0$ trace recorded at a 10 ms frame interval, showing progressive negative deflection during the stimulation epochs. (Right) Six-cell $\Delta F/F_0$ traces recorded at a 30 ms frame interval across six individually resolved neurons. Cell colors correspond to ROI identities shown in Figure 8C(vi). All traces normalized to a thirty-frame resting baseline (F_0).

3.5.2 — Three-Dimensional Programmed Stimulation of Microtissues

Additional microtissues were transduced using the high-titer dual-vector combination described in Section 3.5.1 and labeled with JF585-HaloTag dye prior to imaging. Recordings were acquired at a 19.14 ms frame interval over a total duration of 7,713 ms (403 frames) using the Olympus FV3000 resonant scanning confocal system at 30x magnification. Electrical stimulation was delivered using the A-M Systems Model 4100 stimulator via a computer-controlled asymmetric biphasic pulse protocol with a current amplitude of 100 mA, a phase duration of 180 μ s, an inter-phase delay of 10 ms, and a stimulus period of 1,000 ms.

Six individual cells with resolvable somatic boundaries were identified and segmented as regions of interest within the imaging field (Figure 10). Cell boundaries were initially delineated manually in Fiji and subsequently retraced from the mean

reference frame using a combination of Otsu thresholding and watershed segmentation to enable motion-corrected fluorescence extraction, as described below. Baseline fluorescence values, computed as the mean intensity across a 31-frame resting window, were 460.35, 389.52, 337.74, 383.30, 364.07, and 331.04 arbitrary units for Cells 1 through 6, respectively. Six stimulus events were detected across the recording duration from the fluorescence data, with onset times identified as frames exhibiting peak absolute $\Delta F/F_0$ deflections across the cell population.

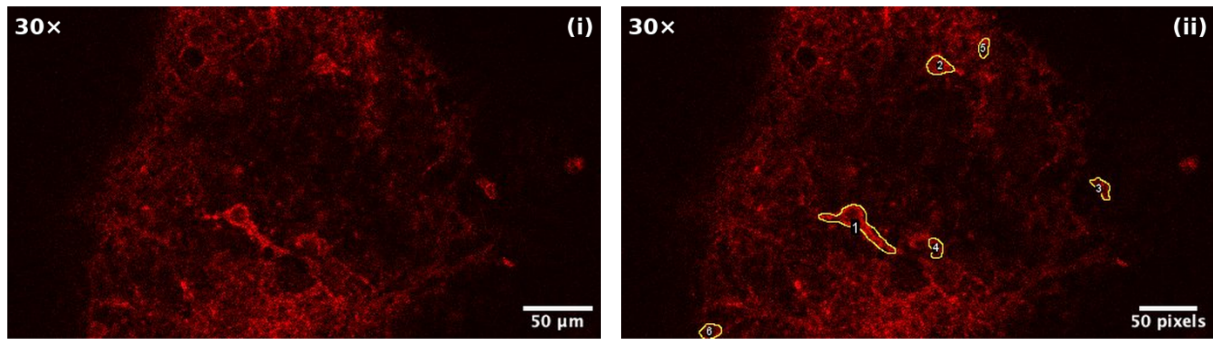


Figure 10. Voltron2-ST expression and region of interest selection in a high-titer three-dimensional cortical microtissue at day in vitro thirty-eight. (i) Raw fluorescence image showing JF585-labeled Voltron2 signal across the microtissue. (ii) Corresponding ROI overlay identifying six individually resolved cells used for $\Delta F/F_0$ analysis. Scale bars, 50 μm . Magnification 30x.

Upon electrical stimulation, three cells exhibited stimulus-locked negative $\Delta F/F_0$ deflections consistent with the voltage-dependent fluorescence decrease of Voltron2 upon membrane depolarization (Figure 13). Cells 3, 4, and 6 displayed peak $\Delta F/F_0$ values of -54.12%, -37.11%, and -47.34%, respectively, yielding a mean $\pm$ standard deviation of $-46.19 \pm 6.99\%$ across this subpopulation. Cross-event summary statistics, Figure 11, and per-event peak $\Delta F/F_0$ values, Figure 12, for all six recorded cells are shown below. Negative deflections were observed in all three cells at each of the six detected stimulus events, with response onset occurring within one frame (19.14 ms) of stimulus delivery in

most events (Figure 15). Response magnitudes varied across stimulus events, ranging from -19.62% to -54.12% (Cell 3), -18.33% to -37.11% (Cell 4), and -19.49% to -47.34% (Cell 6) across the recording duration. Pre-stimulus baseline fluorescence in this subpopulation exhibited a small amount of frame-to-frame variability, with standard deviations of 3.80% , 2.97% , and 3.80% for Cells 3, 4, and 6, respectively.

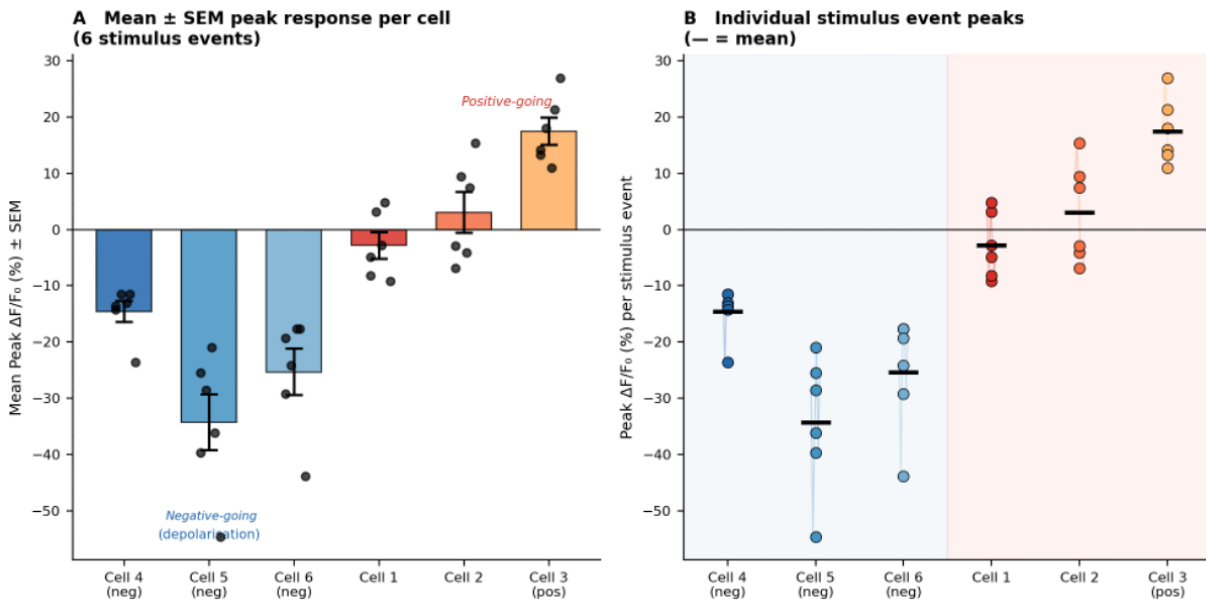


Figure 11. Per-cell summary of stimulus-evoked peak $\Delta F/F_0$ across six stimulus events. (A) Mean $\pm$ SEM peak $\Delta F/F_0$ per cell with individual event values overlaid. Blue and red bars indicate negative- and positive-going cells, respectively. (B) Individual per-event peak values with cross-event means indicated by horizontal bars. Peak $\Delta F/F_0$ extracted as maximum absolute deflection within a ± 15 -frame window around each stimulus. $\Delta F/F_0$ from uncorrected raw traces, rolling 8th-percentile baseline correction.

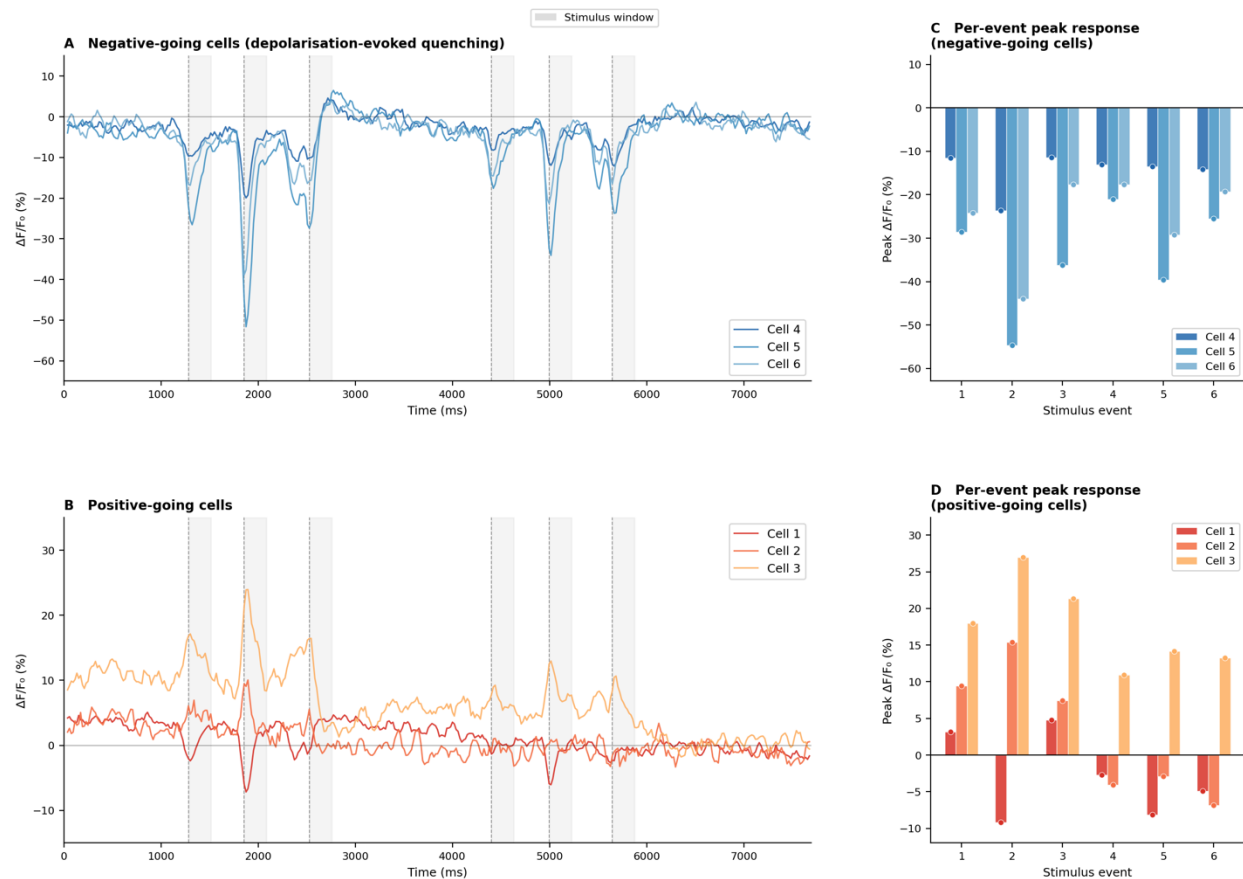


Figure 12. Stimulus-evoked Voltron2 $\Delta F/F_0$ responses in three-dimensional primary cortical microtissue. (A, B) Smoothed $\Delta F/F_0$ traces for negative-going (A) and positive going (B) cells across the full 403-frame recording. Grey shading and dashed lines indicate stimulus delivery windows and onset, respectively. (C, D) Per-event peak $\Delta F/F_0$ for each cell group across six stimulus events with individual event values overlaid. Traces smoothed with a 4-frame running average; $\Delta F/F_0$ computed using a rolling 8th-percentile baseline correction.

The remaining three cells exhibited positive $\Delta F/F_0$ deflections coincident with stimulus delivery (Figure 14). Cells 1, 2, and 5 displayed peak $\Delta F/F_0$ values of 8.05%, 31.19%, and 50.27%, respectively, across the six stimulus events. Response onset occurred within one frame of stimulus delivery in the majority of events for Cells 2 and 5. Cell 1 displayed markedly smaller deflections relative to its pre-stimulus baseline, with per-event peaks ranging from 3.36% to 8.05%, representing a substantially attenuated

response compared to the 15.31% to 31.19% and 23.05% to 50.27% ranges observed in Cells 2 and 5, respectively.

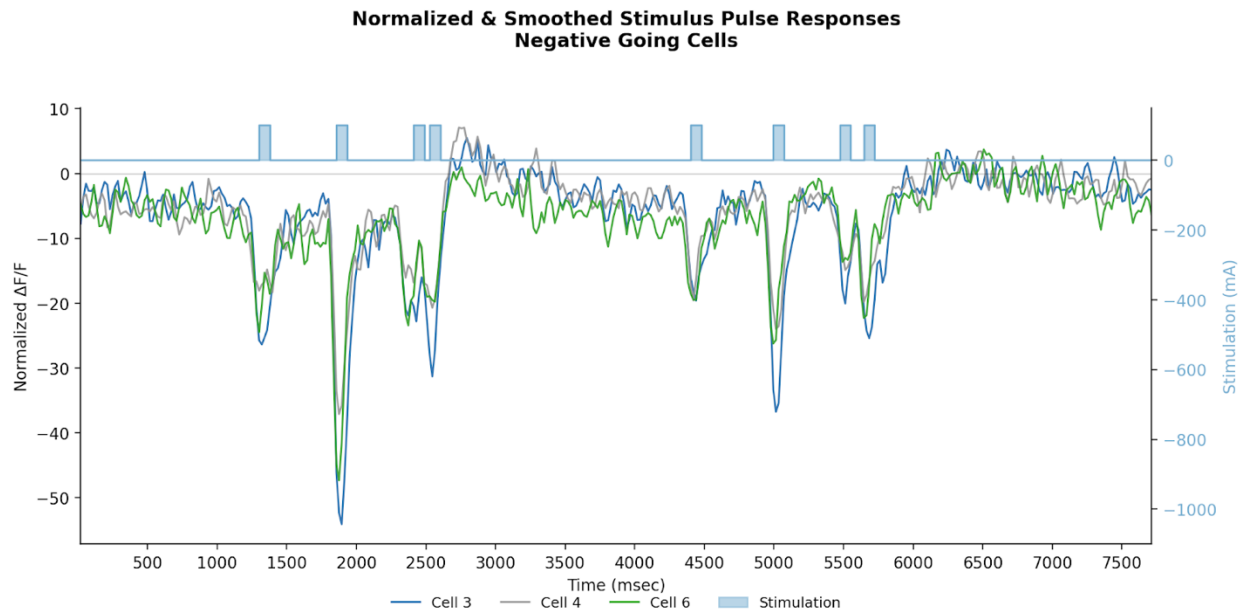


Figure 13. Normalized and smoothed stimulus-evoked $\Delta F/F_0$ responses in negative-going cells. Smoothed $\Delta F/F_0$ traces for Cells 3, 4, and 6, which exhibited stimulus-locked negative deflections consistent with Voltron2 voltage-dependent fluorescence decrease upon membrane depolarization. The stimulation waveform (blue, right axis) indicates the timing and amplitude of each delivered asymmetric biphasic pulse. Traces smoothed using a Savitzky-Golay filter (window = 5 frames, polynomial order = 2). Time axis in milliseconds; frame interval 19.14 ms.

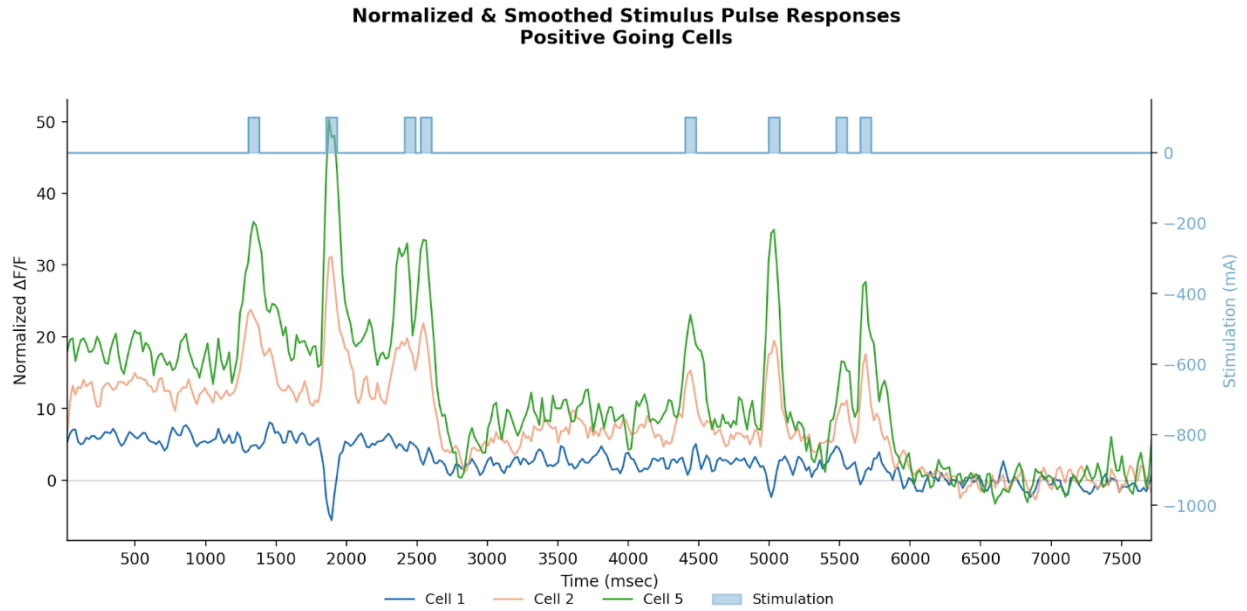


Figure 14. Normalized and smoothed stimulus-evoked $\Delta F/F_0$ responses in positive-going cells. Smoothed $\Delta F/F_0$ traces for Cells 1, 2, and 5, which exhibited stimulus-locked positive deflections. The stimulation waveform (blue, right axis) indicates the timing and amplitude of each delivered asymmetric biphasic pulse. Traces smoothed using a Savitzky-Golay filter (window = 5 frames, polynomial order = 2). Time axis in milliseconds; frame interval 19.14 ms.

To assess the contribution of lateral tissue motion to these stimulus-evoked responses, Farnebäck dense optical flow was applied to the raw image stack frame-by-frame across the full 403-frame recording. Per-frame XY displacement fields were used to warp each frame to a 50-frame mean reference prior to fluorescence extraction, and $\Delta F/F_0$ was recomputed from the corrected traces using the same rolling 8th-percentile baseline correction described in Section 2.15. The mean stimulus-induced fold-change in lateral displacement across all six events was 1.03x, indicating minimal bulk tissue motion at stimulus onset. Full-trace Pearson correlations between displacement magnitude and $\Delta F/F_0$ were negative for the negative-going cells ($r = -0.43$ to -0.49 , $p < 0.0001$) and variable for the positive-going cells ($r = -0.28$ to $+0.29$). Motion contamination scores, computed as the ratio of peak displacement to peak absolute $\Delta F/F_0$ at each stimulus

event, were substantially lower for the negative-going cells (0.024–0.055) than for Cells 1 and 2 (0.132–0.166), with Cell 3 intermediate (0.047). Following XY correction, peak response magnitudes in the negative-going subpopulation were modestly attenuated while response polarity and stimulus-locking were preserved across all six detected stimulus events. Several cells in the positive-going subpopulation exhibited increased inter-event variance and polarity reversals following correction, with Cell 3 showing the largest change in mean peak $\Delta F/F_0$ relative to the uncorrected values (–11.32%). Motion-corrected per-event peak summary statistics for all six cells are presented in Figures 15 and 16.

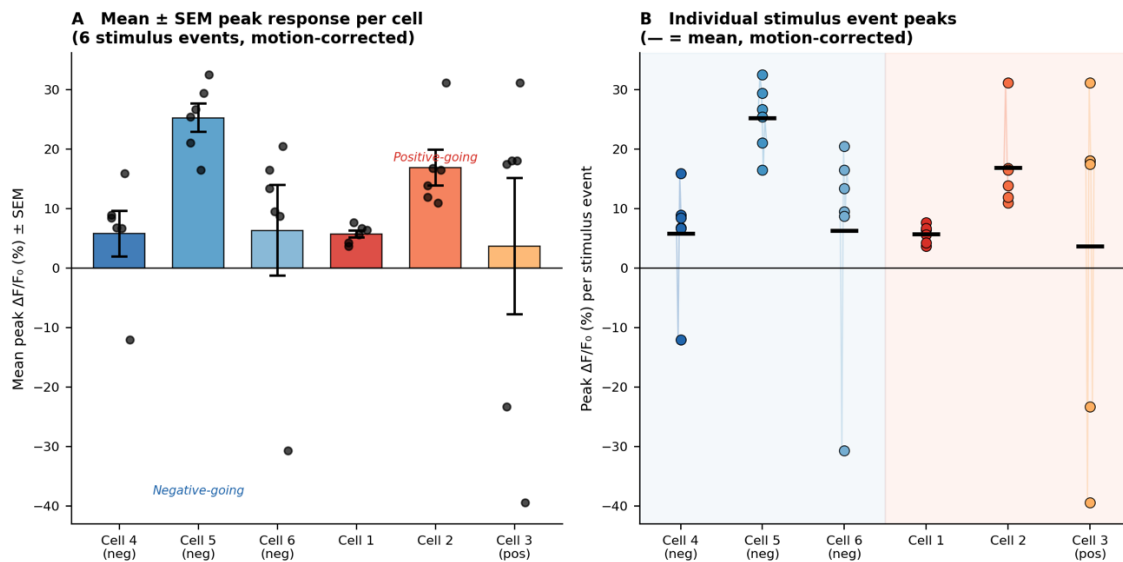


Figure 15. Per-cell summary of stimulus-evoked peak $\Delta F/F_0$ following Farnebäck optical flow XY motion correction, across six stimulus events. (A) Mean $\pm$ SEM peak $\Delta F/F_0$ per cell with individual event values overlaid. High inter-event variance and polarity reversals in several cells are consistent with residual z-axis focal plane drift not addressable by XY correction. (B) Individual per-event peaks with cross-event means indicated by horizontal bars. Corrected values represent a lower bound estimate of true voltage signal magnitude given the potential for partial overcorrection of genuine fluorescence changes.

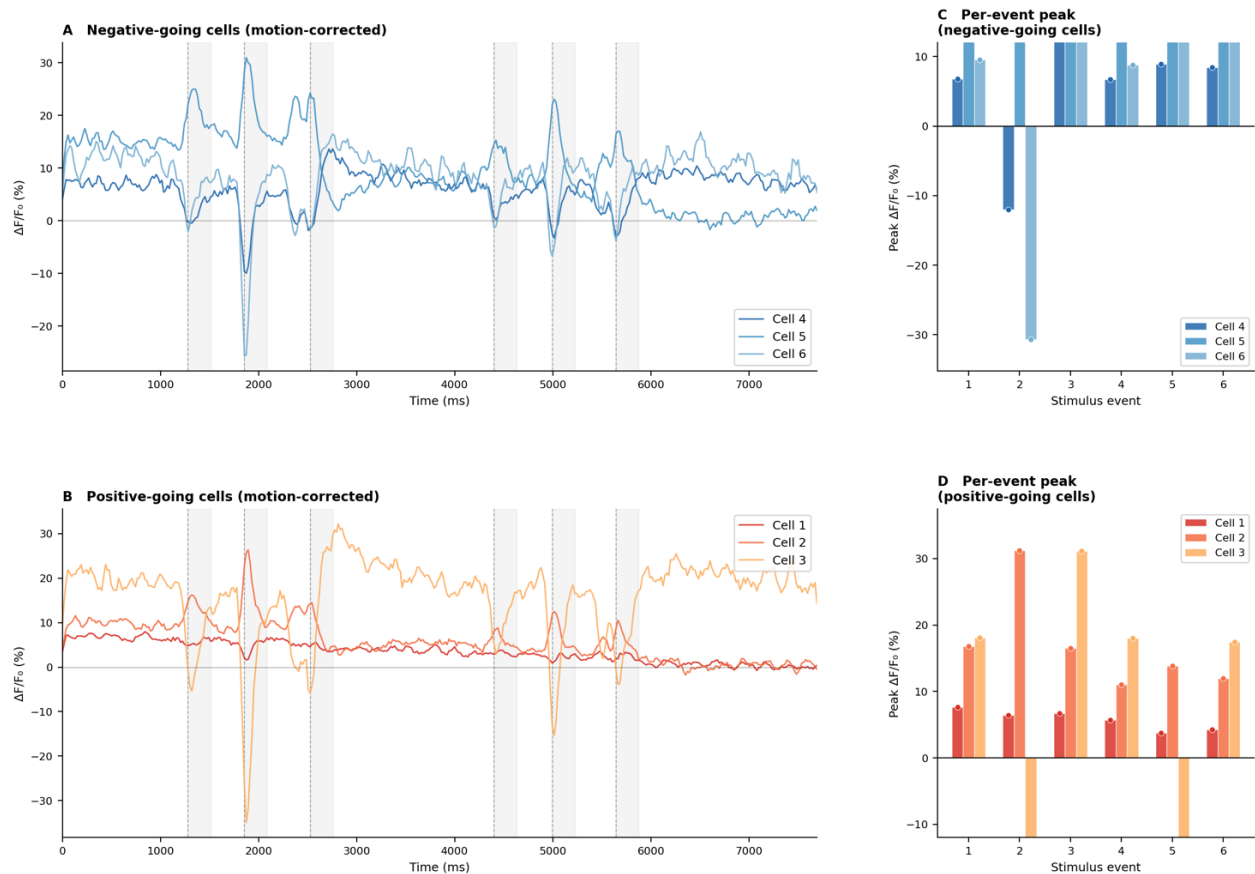


Figure 16. Stimulus-evoked Voltron2 $\Delta F/F_0$ responses following Farnebäck dense optical flow XY motion correction. (A, B) Motion-corrected $\Delta F/F_0$ traces for negative-going (A) and positive-going (B) cells across the full 403-frame recording. Grey shading and dashed lines indicate stimulus windows and onset. (C, D) Per-event peak motion-corrected $\Delta F/F_0$ for each cell group across six stimulus events. Each frame warped to a 50-frame mean reference prior to fluorescence extraction; rolling 8th-percentile baseline correction applied post-warp.

3.6 — Stimulation Plate Cover Impedance Characterization and Operating Window Validation

Before electrical stimulation could be applied reproducibly across microtissue preparations, the frequency-dependent behavior and spatial uniformity of the custom stimulation plate cover required empirical characterization. To characterize the frequency-dependent electrical behavior of the custom stimulation plate and identify an optimal operating window for microtissue stimulation, impedance measurements were performed

across eight electrode sites (four per row, designated A1 through A4 and B1 through B4) using a 100 mVpp sinusoidal input signal swept from 10 Hz to 100 kHz in CCM bath solution. Bipolar stimulation was delivered via 22 AWG solid wire electrodes. Output voltage was measured at each frequency across all eight sites, and gain was computed as the ratio of output to input voltage amplitude.

The frequency-dependent gain profile revealed a clear resonant peak in the 1 kHz range (Figure 17A). Mean output across the eight electrode sites increased progressively from 35.50 ± 2.79 mVpp at 10 Hz to a maximum of 125.50 ± 1.73 mVpp at 1 kHz, corresponding to a mean gain of 1.26 ± 0.02 . Output declined modestly at frequencies above 1 kHz, reaching 116.25 ± 2.05 mVpp at 3 kHz (gain 1.16 ± 0.02), and approached unity gain between 10 kHz and 100 kHz, where mean output ranged from 96.75 ± 2.74 to 102.00 ± 1.73 mVpp across the remaining frequency points.

Inter-electrode consistency, quantified as the coefficient of variation (CV) across the eight sites, was highest at low frequencies and decreased markedly at mid-range frequencies (Figure 17B). CV values were 7.86% and 8.27% at 10 Hz and 30 Hz, respectively, reflecting substantial variability in low-frequency output across electrode sites. CV decreased progressively with increasing frequency, reaching a minimum of 1.38% at 1 kHz and remaining below 2% through 10 kHz (1.70%). At frequencies above 10 kHz, CV remained low, ranging from 2.32% at 30 kHz to 2.83% at 100 kHz.

Based on the combination of peak gain and minimal inter-electrode variability, the 1 to 3 kHz range was identified as the optimal operating window for microtissue electrical stimulation. Within this window, gain exceeded unity at both frequencies (1.26 ± 0.02 at 1 kHz and 1.16 ± 0.02 at 3 kHz) and inter-electrode CV remained below 2%, indicating

consistent and amplified signal delivery across all eight electrode sites. This frequency range was therefore selected to define the stimulation parameters used in subsequent microtissue recordings.

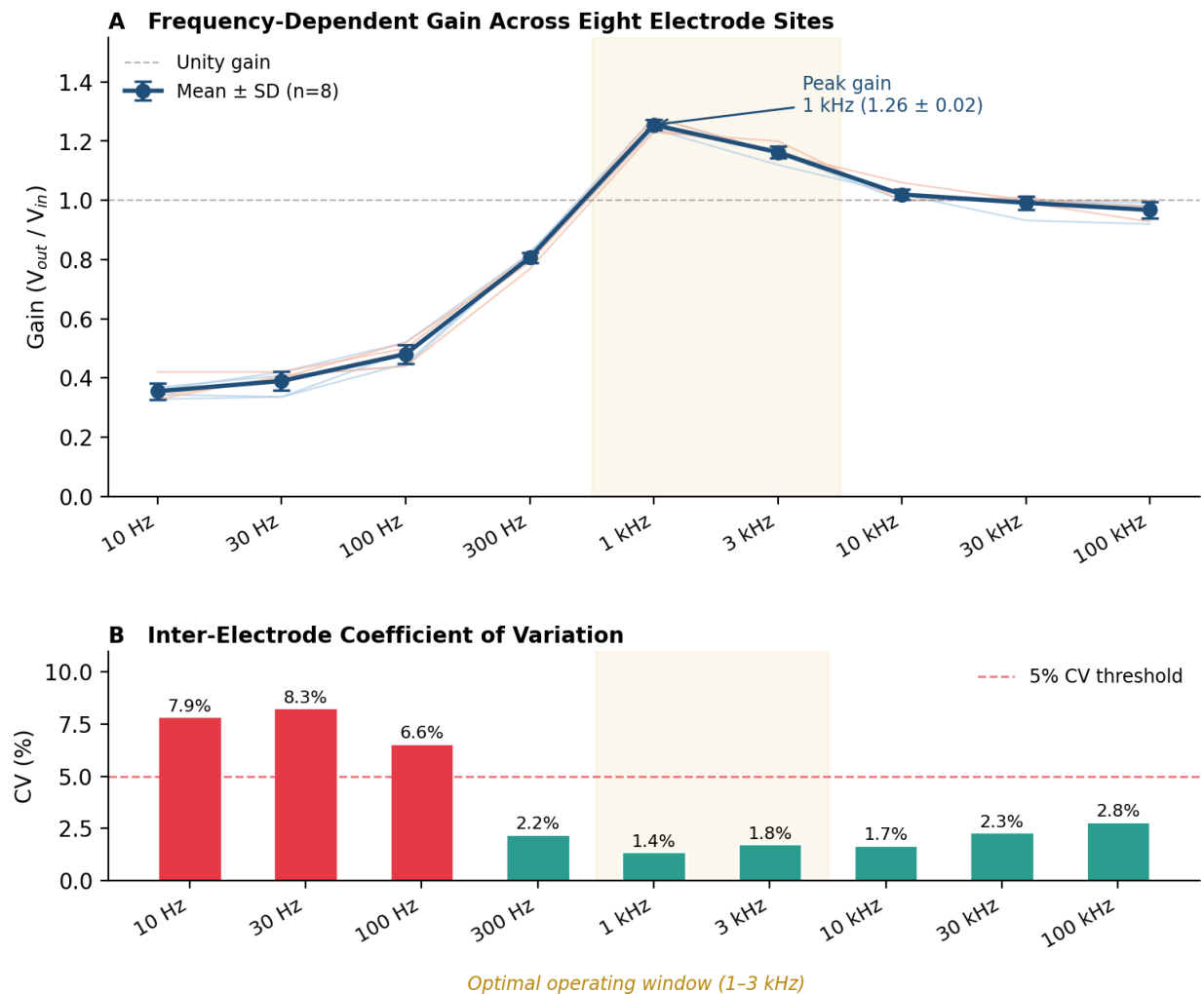


Figure 17. Frequency-dependent gain and inter-electrode consistency of the custom stimulation plate. (A) Mean gain (V_{out}/V_{in}) across eight electrode sites (A1 through A4, B1 through B4) as a function of stimulus frequency, with individual electrode traces shown in light color and mean $\pm$ SD overlaid. Dashed horizontal line indicates unity gain. (B) Coefficient of variation (CV) across the eight electrode sites at each frequency. Red bars indicate CV above 5%; teal bars indicate CV at or below 5%. Shaded region in both panels denotes the optimal operating window (1 to 3 kHz). Input signal: 100 mVpp sinusoidal waveform; bath solution: CCM; electrodes: 22 AWG solid wire bipolar configuration; n = 8 electrode sites.

Ch. 4 — Discussion

4.1 — Voltron2 as a Viable GEVI for Engineered Cortical Tissue: Expression, Labeling, and Signal Detection

The results presented in Chapter 3 support the feasibility of Voltron2 as a genetically encoded voltage indicator for optical electrophysiology in engineered three-dimensional cortical microtissues. Successful AAV transduction of Voltron2 via a dual-vector hSyn-driven strategy, effective JF585-HaloTag dye labeling, and the detection of stimulus-evoked fluorescence deflections across individual neurons demonstrate that the foundational requirements for voltage imaging in this platform have been met. At the low-titer condition, individual cell bodies and neurite arbors were visually separable with baseline fluorescence values ranging from 162 to 382 arbitrary units, and membrane-localized JF585 signal was consistent with the soma-targeted localization of Voltron2. High-titer transduction prevented single-cell ROI selection entirely in the initial titer evaluation, as overlapping signal from densely labeled neurons could not be distinguished in the three-dimensional tissue volume, establishing viral titer as a crucial factor of optical separability in this platform.

Stimulus-evoked voltage responses were detected in six individually resolved neurons in the programmed stimulation recording. Three cells produced negative $\Delta F/F_0$ deflections with peak values of -54.12%, -37.11%, and -47.34%, with response onset within one frame (19.14 ms) of stimulus delivery. The negative polarity of these responses is consistent with the voltage-dependent fluorescence decrease of Voltron2 at membrane depolarization, in which the JF dye fluorescence is quenched as membrane potential shifts toward depolarized values. The remaining three cells exhibited positive $\Delta F/F_0$ deflections with peak values of 8.05%, 31.19%, and 50.27%, which are not consistent

with canonical Voltron2 behavior. This is likely attributed to stimulation-induced microtissue motion, causing cells to shift out of the excitation focal plane. Additional contributing factors may include electrode proximity artifacts. Further validation of electrode stimulation in three-dimensional microtissue is required to clarify this observation.

Acquisition rate was a significant constraint on signal detection throughout this work. The 19.14 ms frame interval used in the programmed stimulation experiment represented a practical compromise between temporal resolution and signal-to-noise ratio for this preparation. Voltron2 has been shown to resolve individual action potentials at frame rates exceeding 1 kHz in optimized preparations, but achieving comparable performance in a three-dimensional microtissue would likely require higher expression, increased excitation power, and imaging approaches better suited to scattering tissue. Two-photon microscopy may improve optical sectioning and imaging depth in this context, although high-speed volumetric voltage imaging would remain technically challenging.

All together, these results establish foundational proof of concept for Voltron2-based voltage imaging in engineered cortical microtissues, extending the demonstrated utility of this indicator beyond dissociated cultures and acute slice preparations to a three-dimensional engineered tissue architecture. The ability to detect stimulus-evoked fluorescence responses in individually neurons represents a meaningful advance over calcium imaging approaches, which cannot resolve subthreshold membrane dynamics or rapid voltage transients. The constraints identified here, including titer-dependency, frame rate limitations, and the presence of positive deflections in a subset of cells, outline the technical parameters that must be refined before this platform can be used for quantitative

single-cell voltage imaging. The optimization pathway toward that goal is addressed in Section 4.7.

4.2 — Acquisition Parameter Optimization for Voltage Imaging in Three-Dimensional Microtissues

Voltage imaging with Voltron2 imposes competing demands on acquisition parameters that do not arise in slower modalities such as calcium imaging. Detecting the small, rapid fluorescence changes associated with membrane depolarization requires both sufficient temporal resolution to capture transient events and enough photon collection per frame to distinguish those changes above the noise floor. In the resonant scanning confocal system used here, these requirements are in direct competition: increasing frame rate by reducing the scanned ROI or eliminating line averaging reduces the per-pixel photon collection, while maintaining averaging or a large field of view constrains achievable frame rates. The acquisition parameter choices made across the 2D and 3D recordings in this study reflect the iterative navigation of this tradeoff.

The initial 2D live recording attempt at approximately 282 Hz with no line averaging points out the consequences of prioritizing frame rate at the expense of signal quality. Without line averaging, each pixel was sampled once per line pass, leaving shot noise at its maximum and placing the expected Voltron2 $\Delta F/F_0$ signal of 2 to 8% per action potential below the detectable threshold in the recorded traces. Furthermore, the 7.34x digital zoom reduced the physical field of view and shortened laser dwell time per pixel, further attenuating per-pixel photon yield. The use of roundtrip scanning mode introduced an additional source of signal degradation. Without precise alignment of the bidirectional scan offset, alternating scan lines were spatially displaced, producing a comb artifact that degraded both spatial resolution and per-pixel signal fidelity. Together, these parameter

choices prevented detection of spontaneous voltage activity in the 2D preparation, not because signal was absent, as spontaneous activity has been identified in other studies at DIV 14, but because the acquisition conditions were insufficient to resolve it above noise.

In the three-dimensional microtissue recordings, frame interval selection had a measurable impact on the magnitude of detectable fluorescence responses. At a 30 ms frame interval in the low-titer preparation, six cells yielded quantifiable $\Delta F/F_0$ responses with a mean peak of $-1.74 \pm 1.31\%$. At a 10 ms frame interval from the same preparation, the single identified cell produced a mean peak $\Delta F/F_0$ of -0.71% , consistent with lower per-frame photon collection compressing the detectable signal envelope at faster acquisition speeds. The 19.14 ms frame interval used in the programmed stimulation experiment, selected to balance temporal resolution with signal quality in the high-titer preparation, yielded the largest stimulus-evoked deflections observed across all recordings, with negative-going cells reaching peak $\Delta F/F_0$ values of up to -54.12% .

These observations point to several acquisition parameter requirements for reliable voltage detection in this platform. Line averaging is a prerequisite for spontaneous activity recording in low-expression preparations, as the per-pixel fluorescence at the titer levels required for distinction is insufficient to resolve small changes in $\Delta F/F_0$ without signal integration. Frame intervals in the 19 to 30 ms range represent a practical operating window for the current system and preparation, providing adequate SNR for stimulus-evoked responses while also maintaining sufficient temporal resolution to observe inter-epoch signal dynamics. Reducing frame interval below 10 ms without a corresponding increase in expression level or laser power is not likely to yield additional resolvable cells

and may further weaken already small signals. Future work employing two-photon excitation, which provides essential optical sectioning and higher per-pulse photon yield in scattering tissue, could substantially impact these constraints and enable higher frame rate recordings with an improved single-cell isolation in the three-dimensional tissue system.

4.3 — Media Formulation Effects on Transduction and Imaging Quality

Both CCM and NbActiv4 media conditions supported Voltron2 transduction, transgene expression, and JF585-HaloTag dye labeling in two-dimensional cortical cultures, as confirmed by fluorescence imaging at DIV 14 to DIV 16. Membrane-localized signal was observed in neurons from both conditions, and the density of Voltron2-expressing cells was consistent across CCM and NbActiv4 wells, with neither condition producing signal crowding or a deficit in the number of recordable neurons per field. These results established that the CCM formulation is permissive for Voltron2 delivery and dye conjugation, providing the two-dimensional baseline required before scaling to three-dimensional microtissues. The primary differentiating observation between conditions was not a failure of transduction under either formulation but rather a difference in neuronal morphology and non-neuronal cell density.

Under brightfield imaging at DIV 14, CCM wells exhibited lower overall cell density with individually resolved neuronal somata and clearly extended processes, consistent with the established role of serum-free Neurobasal A/B-27 formulations in suppressing glial proliferation. NbActiv4 wells showed a higher and more confluent cellular background, with a denser overall culture apparent throughout the culture period. In live fluorescence imaging, CCM neurons exhibited bright, soma-concentrated dual-channel

labeling with well-resolved dendritic arbors in both the JF-525 and JF-608 channels. NbActiv4 neurons showed a more diffuse labeling pattern with neurite-dominant fluorescence distribution, though somatic signal intensity was comparable across conditions. The expression density comparison did not reveal a meaningful difference in transduction efficiency attributable to media composition alone.

Immunohistochemical characterization confirmed that Voltron2 expression was restricted to the neuronal compartment in CCM cultures. Voltron2 signal was not present in GFAP-positive astrocytes nor NG2-positive oligodendrocyte precursor cells using AF635 secondary antibodies and did not overlap with either Voltron2 detection channel. In contrast, NeuN and Iba1 cocktails were paired with a CY3 secondary antibody whose emission overlapped the JF608 detection window, preventing quantitative co-localization analysis in those wells. Despite this limitation, the distribution of JF525 signal in NeuN-stained samples was morphologically consistent with neuronal Voltron2 expression at the single-cell level. Collectively, these results support the conclusion that the hSyn-driven dual-vector AAV strategy successfully restricted Voltron2 expression to neurons under CCM conditions.

CCM was selected as the standard formulation for the three-dimensional microtissue experiments due to its serum-free, chemically defined composition, reduced non-neuronal background enabling improved single-cell optical resolution, and established use with the Borton Lab microtissue platform. NbActiv4 has been reported to increase spontaneous spike rates and synaptophysin-positive puncta density relative to standard Neurobasal/B-27 in dissociated hippocampal cultures, suggesting a potential benefit for enhancing spontaneous voltage activity detection. However, the increased glial

density observed in NbActiv4 cultures in this study introduces a significant imaging tradeoff. A controlled, replicated comparison with standardized serum exposure and matched viral titers would be necessary to determine whether any electrophysiological advantages of NbActiv4 outweigh its reduced optical clarity in this cortical system.

4.4 — Electrical Stimulation and Functional Activity in Cortical Microtissues

Electrical stimulation in the initial titer evaluation was delivered manually using the Abdelfattah laboratory custom electrode, with individual pulses applied by hand during simultaneous confocal acquisition. Stimulus-evoked $\Delta F/F_0$ deflections of polarity were detectable in all six individually resolved cells at the 30 ms frame interval, with a visible temporal delay between stimulus delivery and the onset of fluorescence response apparent in the full traces shown in Figure 9. The precise duration of this delay cannot be quantified from the current dataset because of the lag between the operator-initiated pulse and the corresponding imaging frame, as there is no synchronized trigger between the stimulator and the acquisition clock. Nonetheless, the consistent negative polarity and the visual correspondence between stimulus events and fluorescence deflections across ten averaged events confirm that the recorded responses reflect genuine stimulus-evoked membrane potential changes rather than noise or motion artifact. These results established the functional viability of the platform at DIV 28 under low-titer transduction conditions prior to the development of the computer-controlled protocol.

The transition to computer-controlled asymmetric biphasic pulse delivery in the programmed stimulation experiment was motivated by the need for consistent, repeatable stimulus timing that would support cross-event averaging and reduce operator-dependent variability. Stimulus-evoked $\Delta F/F_0$ deflections were detected in the high-titer preparation

at DIV 38, with three cells producing negative deflections and three producing positive deflections across six detected stimulus events. The programmed protocol produced qualitatively similar response characteristics relative to the manual approach, reflecting the fact that the limiting factors in both cases were shared: the frame rate of the acquisition and the expression-level-dependent signal-to-noise of Voltron2. Manual ROI delineation introduces subjectivity into the boundary of the sampled region, and because $\Delta F/F_0$ is computed from the mean fluorescence across all pixels within the ROI, inclusion of out-of-focus neuritic processes, adjacent cell bodies, or background pixels will attenuate the true somatic voltage signal. This source of variability applies equally to both stimulation methods and represents a methodological constraint that was partially addressed in the programmed stimulation experiment through automated ROI retracing for motion-corrected fluorescence extraction, as described in Section 2.17.

The negative-going responses in Cells 3, 4, and 6 are interpreted as genuine Voltron2 depolarization-evoked fluorescence quenching since two converging lines of post-hoc evidence. First, Farnebäck dense optical flow analysis of the raw image stack revealed a stimulus-induced fold-change in mean lateral displacement of only 1.03x, establishing that bulk tissue motion at stimulus onset was negligible. The displacement magnitude trace, stimulus-locked average, and per-cell correlation results are shown in Figure 18. Second, full-trace Pearson correlations between per-frame displacement magnitude and $\Delta F/F_0$ were negative for all three cells ($r = -0.43$ to -0.49 , $p < 0.0001$), indicating that fluorescence decreases when tissue motion increases, the opposite of what a motion artifact producing positive deflections would produce. Motion contamination scores, defined as the ratio of peak displacement to peak absolute $\Delta F/F_0$

at each stimulus event, were consistently low for these cells (0.024–0.055), confirming that lateral displacement accounted for only a small fraction of the response magnitude. Following XY motion correction via per-frame Farnebäck warping to a common reference frame, peak response magnitudes in the negative-going subpopulation were modestly attenuated while response polarity and stimulus-locking were preserved across all six events, with per-cell corrections less than 1.4%. Taken together, these findings support the interpretation that the negative-going responses reflect genuine cathode-proximal membrane depolarization rather than optical artifact.

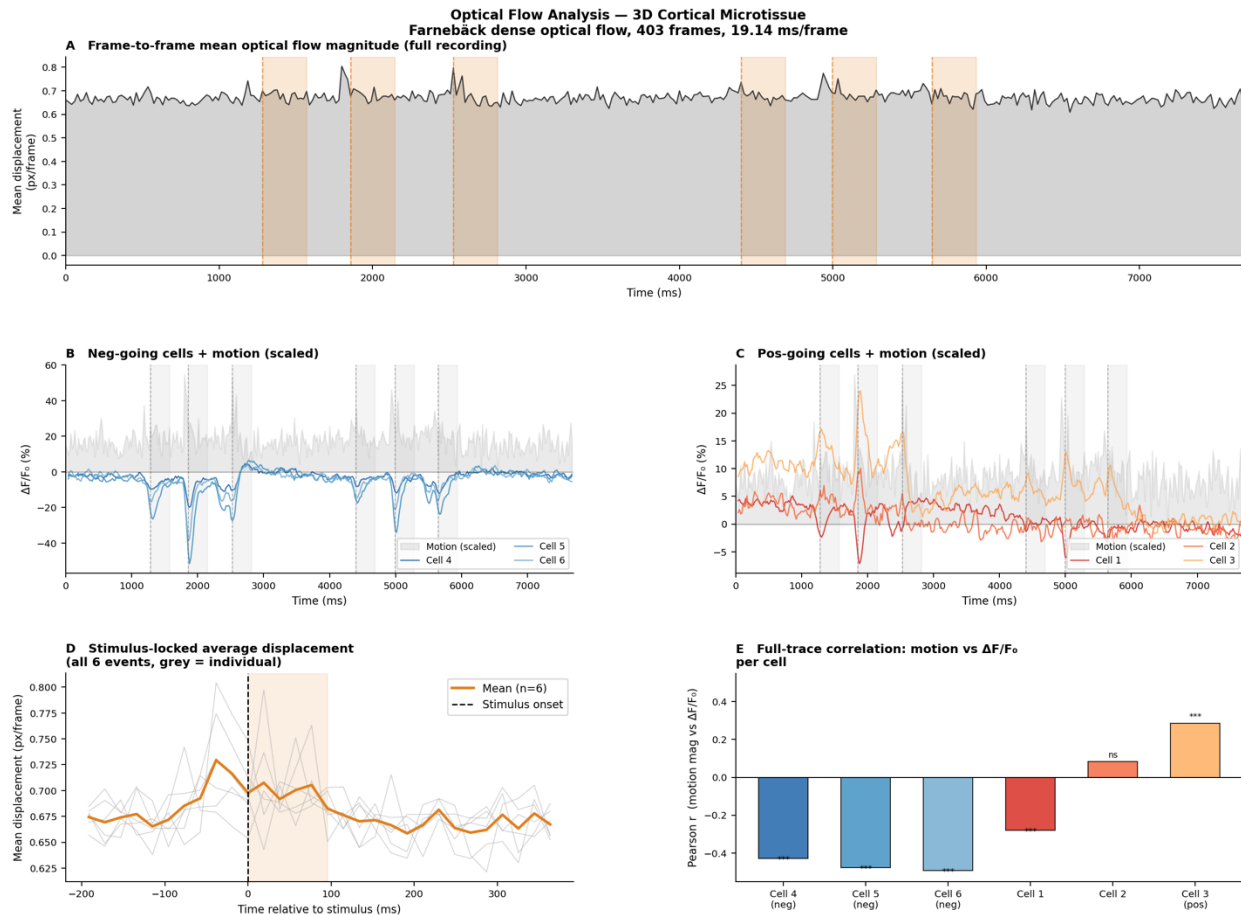


Figure 18. Optical flow analysis of lateral tissue motion across the full recording. (A) Frame-to-frame mean displacement magnitude with stimulus onsets indicated by orange dashed lines. (B, C) $\Delta F/F_0$ traces for negative-going (B) and positive-going (C)

cells overlaid with scaled motion magnitude (grey fill). (D) Stimulus-locked mean displacement averaged across six events (orange), with individual events in grey. (E) Full-trace Pearson correlation between displacement magnitude and $\Delta F/F_0$ per cell. Significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant.

The positive-going $\Delta F/F_0$ deflections observed in Cells 1, 2, and 5 are less readily attributed to a single mechanism and are most likely the product of two contributing factors acting in combination. The first is the position- and orientation-dependent effect of field stimulation on neuronal membrane potential. In bipolar bath field stimulation, the applied electric field establishes a directional gradient across the tissue where neurons near the cathode experience net depolarization while neurons near the anode experience net hyperpolarization. This is consistent with established cable theory models of extracellular field effects on neurons and confirmed by direct voltage imaging studies in field-stimulated neuronal preparations. Cells 1, 2, and 5 may therefore reflect genuine anodal hyperpolarization, which in Voltron2 would produce a fluorescence increase via reduced eFRET efficiency as membrane potential shifts away from depolarized values. Because the precise spatial relationship between each ROI and the electrode orientation was not mapped during the experiment, this interpretation cannot be confirmed from the existing data but represents a directly testable scenario through co-registration of cell position with electrode geometry in future experiments.

The second contributing factor is z-axis focal plane drift potentially driven by electrolytic gas evolution at the electrode surface. At the current amplitude of 100 mA delivered through 22 AWG wire electrodes in saline bath, bubble nucleation at the electrode surface is physically expected and would propagate pressure waves through the bath medium. The trampoline geometry of the microtissue platform constrains lateral movement via the agarose pegs but leaves the tissue free to oscillate along the z-axis,

making vertical displacement the preferential axis of stimulus-induced mechanical disturbance. Spatial displacement maps comparing baseline and stimulus-epoch mean flow are shown in Figure 19. Cells positioned near the edge of the confocal depth of field would be disproportionately sensitive to such displacement, producing stimulus-locked fluorescence changes whose polarity and magnitude vary stochastically across events depending on the direction and extent of each focal plane shift. This mechanism is supported by the observation that motion contamination scores were substantially higher for Cells 1 and 2 (0.132–0.166) than for the negative-going subpopulation, and that several cells exhibited increased inter-event variance and polarity reversals following XY motion correction, a pattern consistent with residual z-artifact that lateral correction cannot address. Per-cell motion contamination scores and the relationship between peak displacement and peak $\Delta F/F_0$ across stimulus events are shown in Figure 20. Physical movement of the stimulating electrode in the absence of current delivery did not reproduce the observed deflection pattern, ruling out static mechanical contact as a causal mechanism. The corrected $\Delta F/F_0$ values for the positive-going subpopulation are best interpreted as a lower bound on any genuine voltage signal present, given that optical flow-based XY correction carries a risk of partial overcorrection of genuine fluorescence changes when signal and displacement are spatially coincident within soma-targeted ROIs, and given that z-axis drift remains uncharacterized. Definitive separation of genuine hyperpolarization from residual z-artifact in this subpopulation would require pharmacological validation to confirm sodium channel dependence, active z-tracking during stimulation, or two-photon excitation, which reduces sensitivity to focal plane drift by confining fluorescence generation to the diffraction-limited focal volume.

Spatial Optical Flow Maps — Stimulus-Induced Tissue Motion
3D Cortical Microtissue, Farnebäck dense flow, averaged over 6 stimulus events

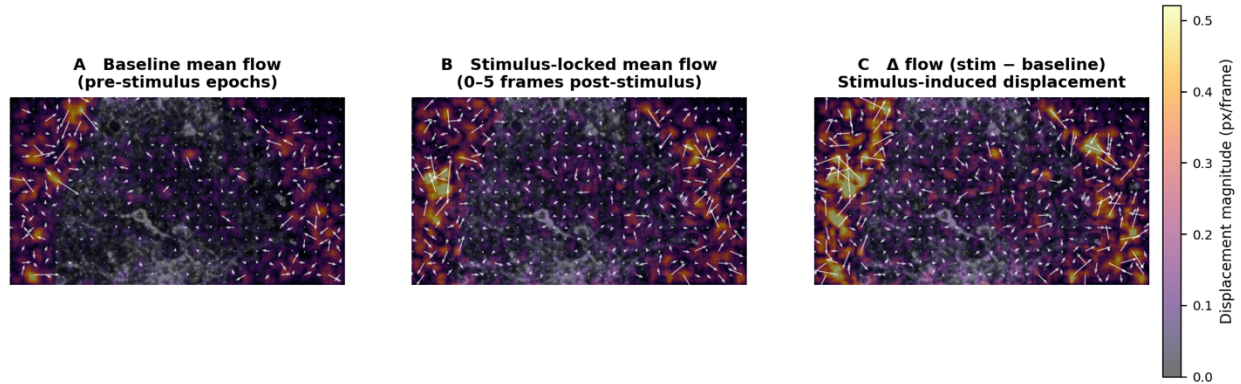


Figure 19. Spatial distribution of stimulus-induced lateral tissue displacement. (A) Baseline mean flow. (B) Stimulus-locked mean flow (0-5 frames post-onset). (C) Stimulus minus baseline difference, representing net stimulus-induced displacement. Inferno heatmap overlaid on mean reference frame; white arrows indicate displacement direction and magnitude. Shared color bar applies to all panels. Farnebäck dense optical flow averaged across six stimulus events.

Motion Contamination Assessment — Separating Voltage Signal from Motion Artifact
3D Cortical Microtissue, 6 stimulus events

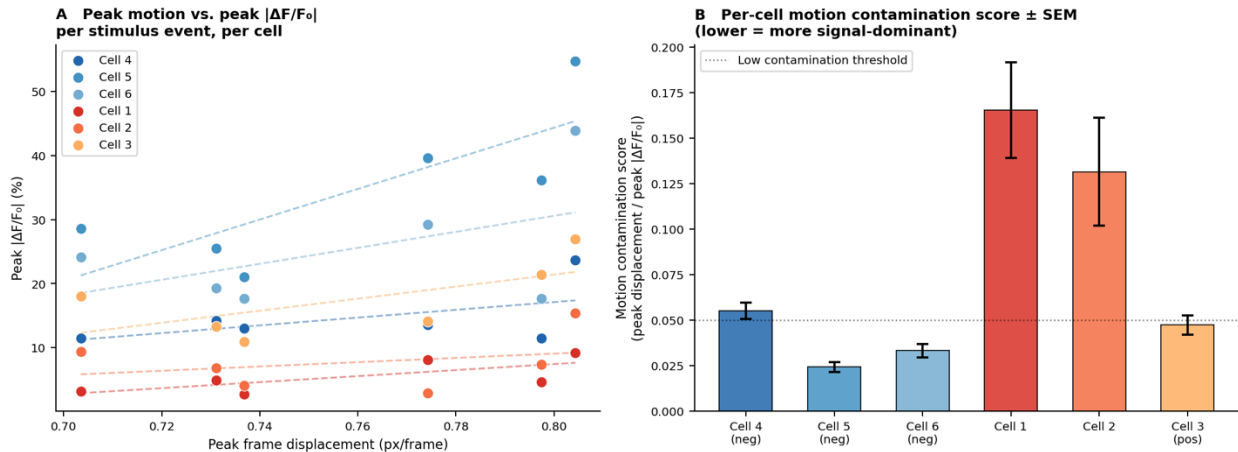


Figure 20. Per-cell motion contamination assessment across six stimulus events. (A) Peak displacement versus peak absolute $\Delta F/F_0$ per event per cell, with per-cell linear fits. (B) Mean contamination score $\pm$ SEM per cell, defined as peak displacement divided by peak absolute $\Delta F/F_0$. Lower scores indicate signal-dominant responses. Blue and red shading indicate negative- and positive-going cell groups.

The existence of a functional recording window is consistent with broader observations in primary cortical microtissue literature, where network activity and synchrony typically emerge between DIV 14 and DIV 21, mature through weeks four to six, and can plateau or decline at later timepoints depending on culture conditions, media composition, and the accumulation of metabolic waste over extended culture periods. The DIV 38 timepoint at which evoked responses were successfully recorded falls within the expected window of peak functional maturity for this platform. These observations collectively suggest that voltage imaging experiments in this system should be prioritized within the three-to-four-week DIV window, and that systematic confirmation of stimulus responsiveness should be treated as an experimental inclusion criterion rather than assumed from indicator expression alone.

4.5 — Stimulation Plate Design and Impedance Characterization as Prerequisites for Reproducible Recordings

The development of a custom stimulation plate cover capable of delivering reproducible electrical stimulation to three-dimensional cortical microtissues during simultaneous confocal imaging addresses a practical gap not covered by current stimulation approaches for dissociated cultures. Planar MEA and bath electrode systems designed for two-dimensional models do not accommodate the complex geometry of microtissues formed within an agarose microfeature, and commercially available stimulation chambers are not compatible with the optical path requirements of the inverted Olympus FV3000 confocal system. The custom 3D-printed cover with integrated 22 AWG solid core wire bipolar electrodes per well provided a geometry-fixed, repeatable solution in which electrode-to-tissue spacing was consistent across wells and experiments. Impedance characterization of this platform prior to biological experiments was necessary

to establish whether stimulus parameters would produce equivalent current delivery across the eight electrode sites, as inter-electrode variability in transfer function would otherwise compromise comparisons of stimulation responses across recording sessions.

The frequency sweep from 10 Hz to 100 kHz revealed a frequency-dependent gain profile with a clear resonant peak near 1 kHz, where mean output across all eight electrode sites reached 125.50 ± 1.73 mVpp against a 100 mVpp sinusoidal input, corresponding to a mean gain of 1.26 ± 0.02 . This behavior reflects the reactive impedance characteristics of the electrode-electrolyte interface in CCM bath solution, where capacitive coupling at the electrode surface produces attenuation at low frequencies and the system approaches resistor-like behavior at higher frequencies. Output declined above 1 kHz and approached unity gain between 10 and 100 kHz. The 1 to 3 kHz range was identified as the optimal operating window for two independent reasons: gain exceeded unity at both 1 kHz and 3 kHz, and the inter-electrode coefficient of variation reached its minimum within this window at 1.38% at 1 kHz, remaining below 2% through 10 kHz. Below 300 Hz, the coefficient of variation (CV) exceeded 5%, reaching 7.86% and 8.27% at 10 Hz and 30 Hz respectively, indicating that low-frequency stimulation would produce meaningfully unequal current delivery across electrode sites within the same preparation.

The consequence of higher inter-electrode CV at low frequencies is that the stimulus experienced by a microtissue at one electrode pair could differ substantially from that at any adjacent site under the same parameters. For voltage imaging experiments where $\Delta F/F_0$ response is the primary dependent variable, this degree of variability would complicate cross-site and cross-session comparisons. The consistency achieved within

the 1 to 3 kHz window supports the interpretation that electrical stimulus delivery would be effectively equivalent across the eight electrode sites when used in a programmed stimulation recording session.

The impedance characterization was performed using sinusoidal inputs rather than the asymmetric biphasic pulses used experimentally, meaning the measured transfer function provides an approximation of electrode behavior rather than a direct characterization under the experimental waveform. Biphasic pulses have broad frequency content determined by their phase duration and inter-phase delay, and the actual current delivered at each phase will be shaped by electrode impedance across that spectral range. A more complete characterization would include direct measurement of delivered charge per phase under experimental waveform conditions. Nonetheless, the sinusoidal sweep is sufficient to identify the operating window and confirm inter-electrode consistency for the purposes of this study. The 22 AWG solid wire electrode geometry produces a relatively large contact areas to distribute current broadly across the preparation. Future work around the stimulation platform is discussed in section 4.7.

An important consideration arising from the programmed stimulation results concerns the current amplitude delivered via the Abdelfattah laboratory custom electrode. At 100 mA through electrodes in media, electrolytic gas evolution at the electrode surface is physically expected and represents a likely contributor to z-axis focal plane drift not addressable by lateral motion correction alone. Future integration of the stimulation plate cover into biological recordings should incorporate platinum or platinum-iridium electrodes to minimize gas evolution, evaluate reduced current amplitudes through

decreased inter-electrode spacing, and include impedance characterization under experimental waveform conditions rather than sinusoidal approximation.

4.6 — Limitations of the Current Study

Several sources of variability and interpretive constraint apply to the findings presented here. Manual stimulation in the titer evaluation was not synchronized with the acquisition clock, preventing precise quantification of stimulus-response latency. The positive-going $\Delta F/F_0$ deflections in the programmed stimulation recording were partially but not fully resolved by optical flow analysis and XY motion correction, with z-axis focal plane drift remaining uncharacterized. Manual ROI delineation introduces operator subjectivity into fluorescence extraction, photobleaching of JF585 produced baseline drift across recordings, and the 19.14 ms frame interval is insufficient to resolve individual action potential waveforms. No spontaneous voltage activity was detected, limiting the dataset to stimulus-evoked responses only. Within the two-dimensional immunohistochemistry experiment, CY3 spectral overlap with the JF608 detection channel precluded quantitative co-localization analysis in the NeuN and Iba1 cocktails. All experiments used neonatal rat primary cortical preparations, and translation to human iPSC-derived tissue would require independent re-optimization of transduction, labeling, and stimulation parameters.

4.7 — Future Directions

The most immediate priority is conducting additional experiments for more biological replication across independently prepared microtissue cohorts to establish the reproducibility of the stimulus-evoked $\Delta F/F_0$ responses reported here. Replicated experiments should aim to include co-registration of ROI position with electrode geometry

to test the field orientation hypothesis for the mixed-polarity responses. Replacement of manual ROI sectioning with automated soma segmentation, using either morphological image analysis or the soma-targeting signal of Voltron2 itself, would remove a significant source of operator-dependent variability from the signal extraction workflow. Synchronization of the stimulation trigger with the confocal acquisition clock would enable precise stimulus related averaging and quantification of stimulus response delays.

Integration of the custom stimulation plate cover into biological experiments represents a natural next step. Use of the plate cover would standardize electrode-to-tissue geometry across wells and experiments, reducing the inter-session variability introduced by manual electrode placement. On the acquisition side, the transition from the current single-photon resonant-scanner confocal configuration to a widefield scientific CMOS camera system would enable higher frame rates across a larger field of view, increasing the number of simultaneously recordable cells and improving the temporal resolution of stimulus-evoked response detection. For deeper tissue imaging, two-photon excitation of Voltron2 using near-infrared wavelengths would improve optical sectioning and likely reduce out-of-focus background in the three-dimensional tissue volume. However, this would require validation of Voltron2 voltage sensitivity under two-photon excitation conditions.

Longitudinal voltage imaging across a defined range of culture ages, from DIV 14 through DIV 45, would systematically characterize the functional recording window and identify the timepoints at which stimulus-evoked responses are most reliable and largest in magnitude. This would complement existing calcium imaging studies from the Borton lab by adding a membrane potential readout to the maturation timeline. Combining

voltage imaging with simultaneous calcium imaging using spectrally compatible indicators would allow direct comparison of the two modalities in the same preparation, quantifying the relationship between calcium transient amplitude and $\Delta F/F_0$ deflection magnitude for the same stimulus. Longer term, extension of the platform to human iPSC-derived cortical microtissues would enable disease modeling applications in which electrophysiological phenotypes associated with channelopathies or neuroinflammatory conditions could be characterized at the membrane potential level and provide a readout that calcium imaging cannot. The combination of Voltron2-based voltage imaging with the established neuroinflammation and device-tissue interface modeling applications of the primary microtissue platform would position this system as a broadly applicable tool for mechanistic studies of cortical network dysfunction where direct membrane potential measurement is essential for examining the cellular basis of functional changes.

Ch. 5 — Conclusion

This thesis investigated the integration of Voltron2, a chemigenetic genetically encoded voltage indicator, into an established three-dimensional primary cortical microtissue platform to enable optical electrophysiology at the level of individual membrane potential. The work addressed four interconnected aims: validating Voltron2 expression and JF dye labeling under the Borton Lab culture conditions, characterizing stimulus-evoked voltage responses in three-dimensional microtissues following dual-vector AAV transduction, identifying acquisition parameter requirements for reliable signal detection, and developing and characterizing an electrical stimulation platform for reproducible field stimulation of microtissues during confocal imaging.

Across these aims, the principal findings are as follows. Both CCM and NbActiv4 media conditions supported Voltron2 transduction and JF585 dye labeling, with CCM selected as the standard formulation on the basis of lower non-neuronal background and established compatibility with the microtissue platform. Immunohistochemical characterization confirmed that Voltron2 expression was restricted to the neuronal compartment in CCM cultures, with no co-localization observed with GFAP-positive astrocytes or NG2-positive oligodendrocyte precursor cells. Viral titer was identified as a critical determinant of optical separability, with high-titer transduction producing densely labeled fields incompatible with single-cell ROI selection and low-titer conditions enabling the distinction of individual neurons. Stimulus-evoked $\Delta F/F_0$ deflections of negative polarity consistent with Voltron2 voltage-dependent fluorescence behavior were detected in individual neurons at DIV 28 and DIV 38, with peak responses reaching $-46.19 \pm 6.99\%$ in the programmed stimulation experiment. Post-hoc optical flow analysis and XY motion

correction confirmed that lateral tissue displacement was negligible at stimulus onset and that the negative-going responses were preserved following correction, supporting their interpretation as genuine depolarization-evoked fluorescence quenching. Residual inter-event variance in a subset of cells was attributed to z-axis focal plane drift. Impedance characterization of the custom stimulation plate cover identified 1 to 3 kHz as the optimal operating window for reproducible field stimulation, with inter-electrode coefficient of variation below 2% across eight electrode sites within this range.

Collectively, these results establish a foundational proof of concept for Voltron2-based optical electrophysiology in engineered three-dimensional cortical microtissues. The ability to detect stimulus-evoked membrane potential changes in individually resolved neurons within a self-assembled primary cortical tissue represents a meaningful advance over calcium imaging approaches, which cannot resolve subthreshold dynamics or rapid voltage transients, and over extracellular MEA. The three-dimensional microtissue platform occupies a valuable position in the landscape of in vitro neural models, offering greater physiological relevance than two-dimensional cultures while maintaining the reproducibility and experimental accessibility that iPSC-derived organoids currently lack. Adding membrane potential measurement to this platform opens capabilities for mechanistic studies of cortical network dysfunction and electrophysiological phenotyping in disease models that were previously inaccessible without invasive electrode-based recording. The technical foundation established here, and the analytical framework developed to interrogate its limitations, define a clear development pathway toward routine, quantitative voltage imaging in three-dimensional cortical tissue.

References

1. Bean BP. The action potential in mammalian central neurons. *Nat Rev Neurosci.* 2007;8(6):451-465. doi:10.1038/nrn2148
2. Knöpfel T, Song C. Optical voltage imaging in neurons: moving from technology development to practical tool. *Nat Rev Neurosci.* 2019;20(12):719-727. doi:10.1038/s41583-019-0231-4
3. Gidon A, Zolnik TA, Fidzinski P, et al. Dendritic action potentials and computation in human layer 2/3 cortical neurons. *Science.* 2020;367(6473):83-87. doi:10.1126/science.aax6239
4. Antic SD, Empson RM, Knöpfel T. Voltage imaging to understand connections and functions of neuronal circuits. *J Neurophysiol.* 2016;116(1):135-152. doi:10.1152/jn.00226.2016
5. Buzsáki G, Draguhn A. Neuronal Oscillations in Cortical Networks. *Science.* 2004;304(5679):1926-1929. doi:10.1126/science.1099745
6. Lin MZ, Schnitzer MJ. Genetically encoded indicators of neuronal activity. *Nat Neurosci.* 2016;19(9):1142-1153. doi:10.1038/nn.4359
7. Trujillo CA, Gao R, Negraes PD, et al. Complex Oscillatory Waves Emerging from Cortical Organoids Model Early Human Brain Network Development. *Cell Stem Cell.* 2019;25(4):558-569.e7. doi:10.1016/j.stem.2019.08.002
8. Tasnim K, Liu J. Emerging Bioelectronics for Brain Organoid Electrophysiology. *Journal of Molecular Biology.* 2022;434(3):167165. doi:10.1016/j.jmb.2021.167165
9. Abdelfattah AS, Kawashima T, Singh A, et al. Bright and photostable chemigenetic indicators for extended in vivo voltage imaging. *Science.* 2019;365(6454):699-704. doi:10.1126/science.aav6416
10. Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Arch.* 1981;391(2):85-100. doi:10.1007/BF00656997
11. Anecchino LA, Schultz SR. Progress in automating patch clamp cellular physiology. *Brain and Neuroscience Advances.* 2018;2:2398212818776561. doi:10.1177/2398212818776561
12. Kodandaramaiah SB, Franzesi GT, Chow BY, Boyden ES, Forest CR. Automated whole-cell patch-clamp electrophysiology of neurons in vivo. *Nat Methods.* 2012;9(6):585-587. doi:10.1038/nmeth.1993
13. Obergrussberger A, Friis S, Brüggemann A, Fertig N. Automated patch clamp in drug discovery: major breakthroughs and innovation in the last decade. *Expert Opinion on Drug Discovery.* 2021;16(1):1-5. doi:10.1080/17460441.2020.1791079

14. Buzsáki G. Large-scale recording of neuronal ensembles. *Nat Neurosci*. 2004;7(5):446-451. doi:10.1038/nn1233
15. Steinmetz NA, Aydin C, Lebedeva A, et al. Neuropixels 2.0: A miniaturized high-density probe for stable, long-term brain recordings. *Science*. 2021;372(6539):eabf4588. doi:10.1126/science.abf4588
16. Obien MEJ, Deligkaris K, Bullmann T, Bakkum DJ, Frey U. Revealing neuronal function through microelectrode array recordings. *Front Neurosci*. 2015;8. doi:10.3389/fnins.2014.00423
17. Soscia DA, Lam D, Tooker AC, et al. A flexible 3-dimensional microelectrode array for in vitro brain models. *Lab Chip*. 2020;20(5):901-911. doi:10.1039/C9LC01148J
18. Spira ME, Shmuel N, Huang SHM, Erez H. Multisite Attenuated Intracellular Recordings by Extracellular Multielectrode Arrays, a Perspective. *Front Neurosci*. 2018;12. doi:10.3389/fnins.2018.00212
19. Kagan BJ, Kitchen AC, Tran NT, et al. *In vitro* neurons learn and exhibit sentience when embodied in a simulated game-world. *Neuron*. 2022;110(23):3952-3969.e8. doi:10.1016/j.neuron.2022.09.001
20. Huang Q, Tang B, Romero JC, et al. Shell microelectrode arrays (MEAs) for brain organoids. *Sci Adv*. 2022;8(33):eabq5031. doi:10.1126/sciadv.abq5031
21. Peterka DS, Takahashi H, Yuste R. Imaging Voltage in Neurons. *Neuron*. 2011;69(1):9-21. doi:10.1016/j.neuron.2010.12.010
22. Grinvald A, Hildesheim R. VSDI: a new era in functional imaging of cortical dynamics. *Nat Rev Neurosci*. 2004;5(11):874-885. doi:10.1038/nrn1536
23. Bando Y, Sakamoto M, Kim S, Ayzenshtat I, Yuste R. Comparative Evaluation of Genetically Encoded Voltage Indicators. *Cell Reports*. 2019;26(3):802-813.e4. doi:10.1016/j.celrep.2018.12.088
24. Chen TW, Wardill TJ, Sun Y, et al. Ultrasensitive fluorescent proteins for imaging neuronal activity. *Nature*. 2013;499(7458):295-300. doi:10.1038/nature12354
25. Xu Y, Zou P, Cohen AE. Voltage imaging with genetically encoded indicators. *Current Opinion in Chemical Biology*. 2017;39:1-10. doi:10.1016/j.cbpa.2017.04.005
26. Siegel MS, Isacoff EY. A Genetically Encoded Optical Probe of Membrane Voltage. *Neuron*. 1997;19(4):735-741. doi:10.1016/S0896-6273(00)80955-1
27. Gong Y, Huang C, Li JZ, et al. High-speed recording of neural spikes in awake mice and flies with a fluorescent voltage sensor. *Science*. 2015;350(6266):1361-1366. doi:10.1126/science.aab0810

28. Adam Y, Kim JJ, Lou S, et al. Voltage imaging and optogenetics reveal behaviour-dependent changes in hippocampal dynamics. *Nature*. 2019;569(7756):413-417. doi:10.1038/s41586-019-1166-7
29. Villette V, Chavarha M, Dimov IK, et al. Ultrafast Two-Photon Imaging of a High-Gain Voltage Indicator in Awake Behaving Mice. *Cell*. 2019;179(7):1590-1608.e23. doi:10.1016/j.cell.2019.11.004
30. Yang HH, St-Pierre F. Genetically Encoded Voltage Indicators: Opportunities and Challenges. *J Neurosci*. 2016;36(39):9977-9989. doi:10.1523/JNEUROSCI.1095-16.2016
31. St-Pierre F, Marshall JD, Yang Y, Gong Y, Schnitzer MJ, Lin MZ. High-fidelity optical reporting of neuronal electrical activity with an ultrafast fluorescent voltage sensor. *Nat Neurosci*. 2014;17(6):884-889. doi:10.1038/nn.3709
32. Chavarha M, Villette V, Dimov IK, et al. Fast two-photon volumetric imaging of an improved voltage indicator reveals electrical activity in deeply located neurons in the awake brain. *bioRxiv*. Preprint posted online October 17, 2018. doi:10.1101/445064
33. Evans SW, Shi DQ, Chavarha M, et al. A positively tuned voltage indicator for extended electrical recordings in the brain. *Nat Methods*. 2023;20(7):1104-1113. doi:10.1038/s41592-023-01913-z
34. Hochbaum DR, Zhao Y, Farhi SL, et al. All-optical electrophysiology in mammalian neurons using engineered microbial rhodopsins. *Nat Methods*. 2014;11(8):825-833. doi:10.1038/nmeth.3000
35. Piatkevich KD, Jung EE, Straub C, et al. A robotic multidimensional directed evolution approach applied to fluorescent voltage reporters. *Nat Chem Biol*. 2018;14(4):352-360. doi:10.1038/s41589-018-0004-9
36. Kannan M, Vasan G, Huang C, et al. Fast, in vivo voltage imaging using a red fluorescent indicator. *Nat Methods*. 2018;15(12):1108-1116. doi:10.1038/s41592-018-0188-7
37. Grimm JB, English BP, Choi H, et al. Bright photoactivatable fluorophores for single-molecule imaging. *Nat Methods*. 2016;13(12):985-988. doi:10.1038/nmeth.4034
38. Grimm JB, Muthusamy AK, Liang Y, et al. A general method to fine-tune fluorophores for live-cell and in vivo imaging. *Nat Methods*. 2017;14(10):987-994. doi:10.1038/nmeth.4403
39. Grimm JB, Brown TA, English BP, Lionnet T, Lavis LD. Synthesis of Janelia Fluor HaloTag and SNAP-Tag Ligands and Their Use in Cellular Imaging Experiments. In: Erfle H, ed. *Super-Resolution Microscopy: Methods and Protocols*. Springer; 2017:179-188. doi:10.1007/978-1-4939-7265-4_15

40. Abdelfattah AS, Zheng J, Singh A, et al. Sensitivity optimization of a rhodopsin-based fluorescent voltage indicator. *Neuron*. 2023;111(10):1547-1563.e9. doi:10.1016/j.neuron.2023.03.009
41. Abdelfattah AS, Valenti R, Zheng J, et al. A general approach to engineer positive-going eFRET voltage indicators. *Nat Commun*. 2020;11(1):3444. doi:10.1038/s41467-020-17322-1
42. Lim ST, Antonucci DE, Scannevin RH, Trimmer JS. A Novel Targeting Signal for Proximal Clustering of the Kv2.1 K⁺ Channel in Hippocampal Neurons. *Neuron*. 2000;25(2):385-397. doi:10.1016/S0896-6273(00)80902-2
43. Lam D, Enright HA, Cadena J, et al. Tissue-specific extracellular matrix accelerates the formation of neural networks and communities in a neuron-glia co-culture on a multi-electrode array. *Sci Rep*. 2019;9(1):4159. doi:10.1038/s41598-019-40128-1
44. Raimondi I, Tunesi M, Forloni G, Albani D, Giordano C. 3D brain tissue physiological model with co-cultured primary neurons and glial cells in hydrogels. *J Tissue Eng*. 2020;11:2041731420963981. doi:10.1177/2041731420963981
45. Janzen D, Bakirci E, Wieland A, Martin C, Dalton PD, Villmann C. Cortical Neurons form a Functional Neuronal Network in a 3D Printed Reinforced Matrix. *Adv Healthcare Materials*. 2020;9(9):1901630. doi:10.1002/adhm.201901630
46. Dingle YTL, Liaudanskaya V, Finnegan LT, et al. Functional Characterization of Three-Dimensional Cortical Cultures for *In Vitro* Modeling of Brain Networks. *iScience*. 2020;23(8):101434. doi:10.1016/j.isci.2020.101434
47. Callegari F, Brofiga M, Tedesco M, Massobrio P. Electrophysiological features of cortical 3D networks are deeply modulated by scaffold properties. *APL Bioeng*. 2024;8(3):036112. doi:10.1063/5.0214745
48. Atherton E, Brown S, Papiez E, Restrepo MI, Borton DA. Lipopolysaccharide-induced neuroinflammation disrupts functional connectivity and community structure in primary cortical microtissues. *Sci Rep*. 2021;11:22303. doi:10.1038/s41598-021-01616-5
49. Atherton E, Hu Y, Brown S, et al. A 3D *in vitro* model of the device-tissue interface: functional and structural symptoms of innate neuroinflammation are mitigated by antioxidant ceria nanoparticles. *J Neural Eng*. 2022;19(3):036004. doi:10.1088/1741-2552/ac6908
50. Brown S, Atherton E, Borton DA. A Three-Dimensional Primary Cortical Culture System Compatible with Transgenic Disease Models, Virally Mediated Fluorescence, and Live Microscopy. In: Huang YWA, Pak C, eds. *Stem Cell-Based Neural Model Systems for Brain Disorders*. Springer US; 2023:153-167. doi:10.1007/978-1-0716-3287-1_12

51. Dingle YTL, Boutin ME, Chirila AM, et al. Three-Dimensional Neural Spheroid Culture: An In Vitro Model for Cortical Studies. *Tissue Eng Part C Methods*. 2015;21(12):1274-1283. doi:10.1089/ten.tec.2015.0135
52. Helmchen F, Denk W. Deep tissue two-photon microscopy. *Nat Methods*. 2005;2(12):932-940. doi:10.1038/nmeth818
53. Ji N, Freeman J, Smith SL. Technologies for imaging neural activity in large volumes. *Nat Neurosci*. 2016;19(9):1154-1164. doi:10.1038/nn.4358
54. Wilt BA, Fitzgerald JE, Schnitzer MJ. Photon Shot Noise Limits on Optical Detection of Neuronal Spikes and Estimation of Spike Timing. *Biophys J*. 2013;104(1):51-62. doi:10.1016/j.bpj.2012.07.058
55. Brooks FP, Davis HC, Wong-Campos JD, Cohen AE. Optical constraints on two-photon voltage imaging. *bioRxiv*. Published online May 17, 2024:2023.11.18.567441. doi:10.1101/2023.11.18.567441
56. Xiao S, Cunningham WJ, Kondabolu K, et al. Large-scale deep tissue voltage imaging with targeted-illumination confocal microscopy. *Nat Methods*. 2024;21(6):1094-1102. doi:10.1038/s41592-024-02275-w
57. Weber TD, Moya MV, Kılıç K, Mertz J, Economo MN. High-speed multiplane confocal microscopy for voltage imaging in densely labeled neuronal populations. *Nat Neurosci*. 2023;26(9):1642-1650. doi:10.1038/s41593-023-01408-2
58. Wang M, Sun J, Crosby A, et al. Direct interaction of human serum proteins with AAV virions to enhance AAV transduction: immediate impact on clinical applications. *Gene Ther*. 2017;24(1):49-59. doi:10.1038/gt.2016.75
59. Lin P, Lin Y, Lennon DP, Correa D, Schluchter M, Caplan AI. Efficient Lentiviral Transduction of Human Mesenchymal Stem Cells That Preserves Proliferation and Differentiation Capabilities. *Stem Cells Transl Med*. 2012;1(12):886-897. doi:10.5966/sctm.2012-0086
60. Najafi S, Rahimpour A, Ahmadi H, et al. The significance of chemical transfection/transduction enhancers in promoting the viral vectors-assisted gene delivery approaches: A focus on potentials for inherited retinal diseases. *Electronic Journal of Biotechnology*. 2024;72:29-40. doi:10.1016/j.ejbt.2024.07.005
61. Brewer GJ, Boehler MD, Jones TT, Wheeler BC. NbActiv4 medium improvement to Neurobasal/B27 increases neuron synapse densities and network spike rates on multielectrode arrays. *Journal of Neuroscience Methods*. 2008;170(2):181-187. doi:10.1016/j.jneumeth.2008.01.009
62. Abera AS, Miles MW, Hoppa MB. Subthreshold electric fields bidirectionally modulate neurotransmitter release through axon polarization. *bioRxiv*. Preprint posted online February 22, 2025. doi:10.1101/2025.02.22.639625

63. Berzhanskaya J, Chernyy N, Gluckman BJ, Schiff SJ, Ascoli GA. Modulation of hippocampal rhythms by subthreshold electric fields and network topology. *J Comput Neurosci*. 2013;34(3):369-389. doi:10.1007/s10827-012-0426-4