

NeuroBorealis

Live cell imaging of 3D cortical microtissue via AAV-induced fluorescence

By

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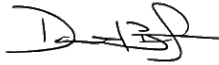
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Abstract

Over the last two decades there has been an overwhelming amount of scientific research aimed at furthering our understanding of the brain and neurodegenerative diseases. We know that neural function depends on intricate cascades of intercellular communication and molecular signaling across space and time, yet a comprehensive understanding of the subcellular organization underlying larger complex neural processes remains difficult to capture. One of the biggest hurdles in furthering our understanding of these complex cellular processes is a lack of visual access to the brain and development of imaging platforms capable of capturing dynamic system behavior at the cellular level. However, the development of live imaging techniques including high-resolution microscopy and advanced fluorescent probes has provided valuable information regarding highly complex and multidimensional cellular processes. The experiments outlined in this thesis work explore a proposed methodology which builds upon the advantage of high spatial resolution imaging and the effectiveness of fluorescent probes to better understand multicellular neural dynamics. **The central goal of this research is to develop a real-time imaging platform for longitudinal visualization of dynamic cellular morphology and cell-cell interactions amongst neurons, astrocytes, and microglia.** Using virally mediated expression of fluorescent reporter proteins in three key cell types (neurons, astrocytes and microglia), this thesis work presents an in vitro model for real time capture of dynamic cellular behavior in cultured three-dimensional primary cortical microtissues.

Background

In the last decade there has been an astounding surge in scientific research aimed at furthering our understanding of the brain and neurodegenerative disease. One of the biggest hurdles in understanding complex neural systems is an inability to easily probe and collect data from the brain. The design of effective tools and neurotechnologies for use in these investigations has become of critical importance. From capturing electrophysiological information via intracortical electrode arrays¹⁻⁴ to disrupting dysfunctional neural activity via deep brain stimulation,^{5,6} many technologies have been developed to physically interface and communicate with the neural networks. Additionally, advanced optical methods including high-resolution microscopy and fluorescent probes⁷⁻¹² provide less invasive mechanisms for interfacing with neural systems. As impactful research tools, these technologies can not only provide valuable information regarding cellular behavior, but they also create a unique vantage point for visualizing the spatial and temporal elements of dynamic neural systems that inherently add a great deal of depth and complexity to our understanding of the healthy and diseased brain.

Optical Methods

Cellular function depends on intricate cascades of molecular mechanisms in space and time, yet a comprehensive understanding of the subcellular organization underlying larger complex neural processes remains difficult to capture. The development of high-resolution fluorescence microscopy has given rise to advanced imaging methods capable of capturing such detailed cellular organization that often occurs on a scale that is beyond the resolving power of conventional microscopy. High-resolution imaging techniques, including stimulated emission depletion (STED),^{7,13-15} optical coherence tomography (OCT),^{16,17} and two-photon imaging,¹⁸ have dramatically impacted our understanding of dynamic processes in the brain at the single cell, as well as the systems level, by elucidating cellular structure and function at sub-micron scales with high spatial and temporal resolution.

For example, sub-diffraction imaging with stimulated emission depletion (STED) microscopy has led to the capture of activity-dependent changes in morphology,^{13,19} and synaptic vesicle recycling.^{13,19} Capture of these nanoscale processes highlights how high-resolution images can elucidate microscale cellular structures in spatial domains that were previously optically impenetrable. Similarly, the long wavelength light sources, microscale resolution, and volumetric data acquisition capabilities in OCT have led to non-invasive visualization of subcortical structures and microvasculature in rats fitted with a cranial window.^{20,21} Furthermore, recent developments in High-Speed OCT have revealed fast and small-scale cellular changes in retinal and corneal structures.^{22,23}

Additionally, two-photon microscopy has played an important role in the development of live cell imaging as it offers reduced cytotoxic and photobleaching effects and has improved three-dimensional data acquisition in deep tissue imaging.⁸ Like two-photon imaging, confocal-fluorescence microscopy can also provide three-dimensional sectioning in thicker specimens by using a pinhole to exclude background artifacts from the outside the focal plane. However, confocal-fluorescence imaging does rely on increased excitation for photon emission and therefore visible fluorescence in the specimen. On the other hand, two-photon imaging takes advantage of basic quantum optics concepts to increase imaging depth and reduce photobleaching effects. Two-photon microscopy relies on the absorption of two photons in a single quantized event at longer (less phototoxic) excitation wavelengths to achieve fluorophore excitation that is normally caused by one photon absorption at shorter (more cytotoxic) wavelengths. This phenomena generates high-image contrast and greatly reduces the risk of photobleaching and is therefore ideal for imaging live samples.⁸

Beyond improved microscopy methods, the development of functional fluorescent probes, including transgenic cell lines and animals, optogenetic actuators, calcium and voltage reporters, and viral vectors have allowed for live monitoring of cell-specific behaviors and interactions. Transgenic animal models offer a methodology for establishing stable fluorescence in various

neural cell phenotypes and can offer robust methods for in vivo imaging of cellular activity in the brain.^{24,25} Transgenic cell lines offer similar capabilities for long-term imaging in an in vitro setting as well.^{24,26} However, due to the limited number of available fluorophores to be selectively expressed, the potential for dynamic mixing of multiple fluorescent cell types in a single sample or animal becomes difficult. Further, while fluorescence imaging in transgenic animals is typically stable, some studies have demonstrated some challenges with long-term visualization through the cranial window in the in vivo setting.^{27,28}

In addition to transgenic animals, neuronal subpopulations can be selectively stimulated via optogenetic actuators such as channelrhodopsin, halorhodopsin, and archaerhodopsin which can be activated by an external fiber optic light sources.^{9,29,30} These optical probes are capable of providing spatiotemporal control of neural activity and function at the single cell level. Another method of live cell monitoring can be achieved through genetically encoded calcium (GECI) and voltage (GEVI) indicators which provide means of visualizing dynamic functional activities in targeted neuronal populations via transgene delivery of fluorescent GCaMP (GFP fusion protein and VSFP (voltage-sensitive fluorescent protein)).^{10–12,31} These optical probes enable fluorescence reporting of neuronal activity that is localized to electrophysiological signals on the single-cell scale. This level of spatiotemporal specificity in neural signals is typically impossible using EEG and ECoG systems as single cell signal capture is typically beyond the spatial resolution of less invasive non-implanted recording electrodes.

Most optical probes typically rely on viral vectors to genetically target specific cell populations for transgene delivery. Their application for induced cell-type specific fluorescence has been widely validated within in vitro and in vivo settings.^{32–35} Vector design and manipulation of the viral genome can have effects on transduction efficiency and cellular specificity. For this reason, viral vectors were carefully considered in their use as optical probes in the experiments outlined in this thesis. The transgene delivery mechanism and relevance of viral vectors is described in detail in the below section titled *Adeno Associated Viral Vectors*.

Each of these technologies has played a key role in effectively capturing dynamic neural processes in the brain. With the increased spatial and temporal resolution of innovative microscopy techniques, we are now capable of elucidating small-scale mechanisms and cellular interactions that underlie higher-order neural structure and function. Further, the development of stable fluorescent probes has provided real-time monitoring capabilities of unique cellular interactions and function in vivo and in vitro. Studies that combine high-resolution imaging techniques and advanced fluorescent probes can provide valuable information regarding highly-complex and multidimensional processes perhaps unveiling underlying neural mechanisms and functions that were previously out of reach using prior optical technologies. There are several examples of the benefits of such synergistic imaging approaches including the use of in vivo two-photon microscopy to characterize the progression of the biological response to implanted electrodes in transgenic mice fitted with cranial windows.^{27,36} These studies demonstrate that investigations of highly-dynamic and multi-cellular processes in the brain, such as the neuroinflammatory response to chronically implanted electrodes, may require these multifaceted high-resolution imaging platforms for complete characterization. The experiments outlined in this thesis work explore a proposed methodology which builds upon the advantage of high spatial resolution imaging and the effectiveness of fluorescent probes in order to better understand multicellular dynamics. **The central goal of this research is to develop a real-time imaging platform for longitudinal visualization of dynamic cellular morphology and cell-cell interactions amongst multiple cell types.**

Neuroinflammation

Neuroinflammation in the brain is a highly dynamic multicellular process that is not well understood. The neuroimmune responses has been shown to play a significant role in the degeneration of cellular and network function in brain injury and several neurodegenerative diseases.^{37–39}

Although the underlying causes of neurodegenerative diseases are not fully understood, neuroinflammation has widely been recognized as a potential mechanism in multiple disease pathophysiologies.^{40–42,43,40–42} Neuroinflammation is typically associated with central nervous system (CNS) specific signaling cascades, resulting in the activation of microglia and astrocytes activation of microglia and astrocytes. Activation of these local immune cells triggers a cascade of intercellular communication and signaling including the production of antigens and release of proinflammatory cytokines, such as, interleukin-1 beta and -6 (IL-1 β and IL-6), and tumor necrosis factor-alpha (TNF- α). The continued upregulation of pro-inflammatory cytokines, causes changes in cellular interactions amongst microglia, astrocytes and neurons and a prolonged inflammatory response which ultimately leads to degeneration of healthy neurons and dysfunction of.^{44–46}

In addition to neuroinflammation associated with neurodegenerative diseases, the inflammatory response to implantable devices, such as, recording electrodes can have confounding effects on the stability of the device-tissue interface in the brain.^{47–50} During implantation, the initial injury and disruption of the blood brain barrier activates microglia which release proinflammatory cytokines that subsequently cause an influx and activation of astrocytes and monocytes to the site of implantation.^{51,52,53,54,28,55} The initial response and recruitment of glial cells leads to glial scarring, a hallmark sign of neuroinflammatory effects on implanted devices.^{49,56–58} The glial scarring has been associated with implant material degradation^{59–63} and overall tissue-interface instability over time.^{28,55,64} It has been hypothesized that neuroinflammatory effects on the device-tissue interface and glial scarring can be mitigated with avoidance of vascular damage upon implantation.⁶⁵ However, several studies have also shown that even with avoidance of vasculature there is still unavoidable capillary damage that will cause similar downstream cellular reactions.^{28,65,66}

Device instability and material degradation caused by the acute inflammatory response has been widely studied. However, the majority of these analyses were completed from an ‘after the fact’ histological analysis. With the exception of some two-photon imaging studies capturing

the immediate cellular reaction during implantation,^{36,27,51} there has yet to be a platform with which this dynamic process can be characterized in real time over an extended chronic timeline. Variability in outcomes for implanted devices suggests that the mechanism and progression of the multi-cellular interactions in device failure is intrinsically complex. Understanding the cause of these failures at the device-tissue interface is limited by a lack of dynamic visualization of the three-dimensional neural infrastructure and inter-cellular responses around implanted devices over time. Limited visualization and monitoring of such a dynamic multi-cellular process may be solved by multi-cell type fluorescent imaging. Due to the fact that in vivo capture of such processes requires a cranial window that can affect the inflammatory response itself,^{27,28,36} an engineering three-dimensional neural cell culture model containing stable fluorescent probes in multiple cells types was considered for the development of the NeuroBorealis platform.

Three Dimensional Cell Culture

Traditional monolayer in vitro models and engineered cultures can provide repeatable, well-controlled, and environmentally isolated experimentation for effective molecular and cellular investigations of neural function.⁶⁷⁻⁷³ With fewer cells and reduced spatial dimension, these two-dimensional in vitro models of the brain offer reduced complexity and the ability to visualize isolated cellular activity which have been successful in assessing toxicity and efficacy of therapeutics at the single cell level. However, a growing body of evidence suggests that the two-dimensional monolayer cultures are inadequate in modeling essential cellular functions that are seen in vivo.⁷⁴ It has been demonstrated that the limited physical dimensions of monolayer cultures lack biomechanical and biochemical signaling which can influence various cellular processes such as migration, proliferation, and extracellular signalling.⁷⁵⁻⁷⁸ For this reason, three dimensional in-vitro models with increased physiological relevance and the ability to recapitulate in vivo-like cellular interactions and morphology have piqued scientific interest across the field.⁷⁹⁻

Regardless of the platform, culturing neural cells is itself a very sensitive process. Typical primary culture processes involve collection of a tissue sample from a host subject followed by tissue dissociation into a cellular suspension by means of enzymatic and mechanical manipulation. Younger cells, having gone through fewer differentiated states, are more likely to survive in culture and therefore dissociation of tissue collections from embryonic or neonatal subjects is recommended.⁸² For primary monolayer cultures, dissociated tissues are processed into single cell suspensions and seeded onto flat culture plates where they attach to the two-dimensional surface. However, when dissociated cells are left in suspension, they reaggregate and reassemble into three-dimensional structures.⁸³ Without specific selection for a unique cell type, dissociated cells will form heterogeneous neural cell cultures. Furthermore, studies have shown that neurons can remodel within the three-dimensional aggregates and form axonal networks and fully developed synapses.^{84–86}

Unlike the two-dimensional cultures where the cells adhere to a plate surface causing flattened, atypical morphologies,^{87,88} the aggregates allow cells to assemble in three-dimension and maintain their normal physiological morphologies.^{88,89} In addition to having physiologically relevant morphologies, the three-dimensional cultures can form an endogenous extracellular matrix (ECM) without the need of exogenous scaffolding.^{77,78} Cells are highly sensitive to the stimuli provided by a surrounding matrix^{87,90–92} as well as the mechanical properties of the extracellular environment.⁹³ Therefore, the presence of an endogenous ECM in three-dimensional cultures provides in vivo like cell-cell and cell-ECM interactions that are essential to neural cell health and function.

Moreover, it is evident that three-dimensional cell cultures are an excellent model for application and testing of neurotechnologies as they provide superior physiological relevance to two-dimensional cultures, with cytoarchitecture and function that is similar to the native in vivo environment^{94,95} while providing better optical access compared to tradition in vivo models. In this thesis work, we utilize three-dimension neural microtissues that self-assemble following

dissociation of cortical tissue to create an engineered in vitro platform with long-term live imaging compatibility. Such a platform could provide a controllable environment from which highly-complex and dynamic processes, such as neuroinflammation, could be characterized. Additionally, an engineered culture platform with versatile and multicellular fluorescent probing via viral vectors is designed to be translated to other in vitro and in vivo models of the healthy and diseased brain including pluripotent stem cell-derived organoids.

Adeno Associated Viral Vectors

In order to facilitate the investigation of complex neural processes like neuroinflammation, we aim to explore a methodology to induce multicell fluorescence that could be applied across multiple in vitro and in vivo models of the brain. To achieve this, we have chosen adeno-associated viral (AAV) vectors for transgene delivery of fluorescent reporters to multiple cell types in a single sample. Viral vectors have been utilized as fluorescent reporters in monitoring cellular behaviors but the effective mechanism of transgene delivery via adeno associated viral vectors has also been used in treatment of neurodegenerative disease^{96–100,–104,101,102,103} There are several benefits to using AAVs as a mechanism of transgene delivery in the brain including reduced immunogenicity, long-term gene expression, and cell-specific integration^{103, 101}

The parental AAV virus has a small genome (5 Kbp) and contains the two inverted terminal repeats (ITRs), the rep and cap encoding replicase and structural proteins along with p5,19, and 40 promoters (**Fig 1**). However, the viral vector particles (like those used in this thesis work) only contain a recombinant transgene ssDNA, eukaryotic promoters (EP), the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE), and the human growth hormone (hGH) polyadenylation signal (polyA), which are all flanked by the two ITRs. (**Fig 1A**) The polyA hGH and the WRPE, when transcribed, create tertiary structures that are known to enhance expression in the CNS.^{105–108} The ITRs are the only elements from the wildtype AAV genome that remain in the packaged vector particles. Without the rep and cap genes which are necessary for viral

genome replication, the AAV vectors remain primarily episomal and have a low frequency (if any) of stable integration into the host cell genome.

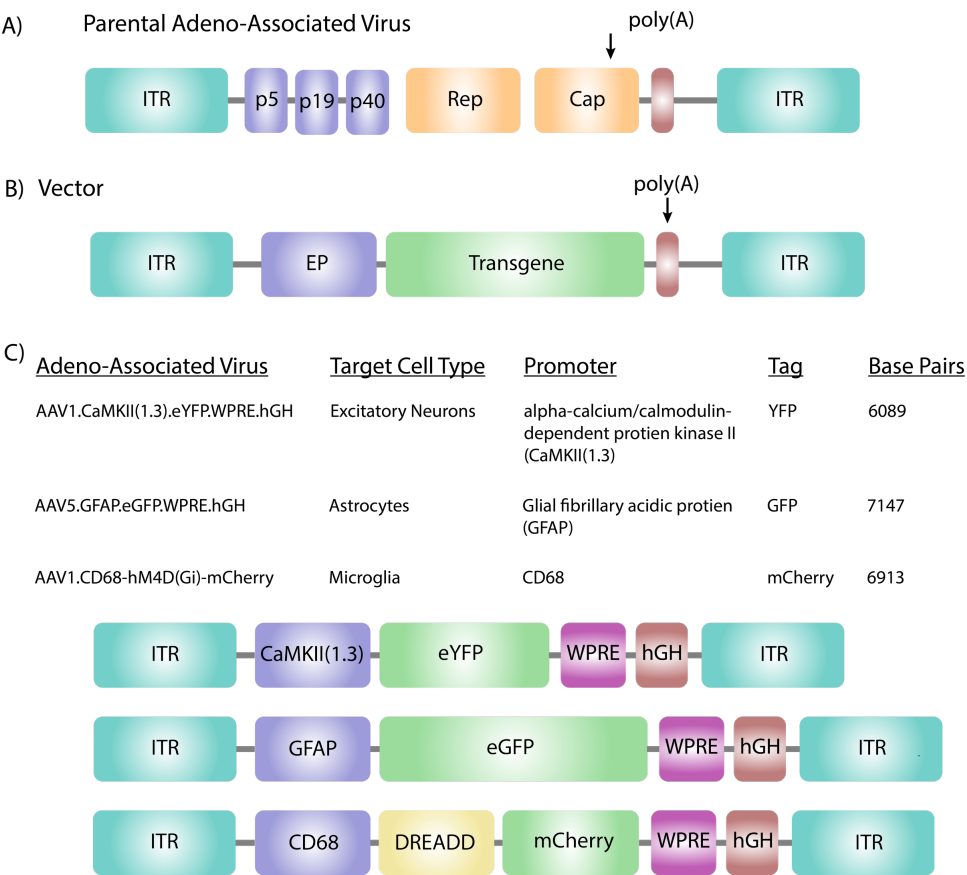


Figure 1: AAV Vector Construction
 Genomic organization of adeno-associated virus (AAV) vector derived from parental wildtype AAV. Vector organization and genomic mapping for neuron-targeting AAV1, astrocyte-targeting AAV5, and microglia-targeting AAV1 used in the experiments outlined in this thesis work. (modified from Vannucci et al 2013¹⁰⁰)

Although AAV viral vector productions are somewhat simplistic, they are not without limitations. With less than 5 kilobase pairs, the AAV genome is considered relatively small, which can make it less effective in clinical gene therapy as most therapeutic genes exceed the bounds of the AAV cloning capacity. Some studies have reported use of specific serotypes with larger cloning capacity (Alloca et al) while others have shown that the full-length genomes were not completely packaged suggesting a <5kb cutoff.^{109–111} An attempted solution to the issue of limited cloning capacity has been transgene splicing and/or recombination into separate viral vectors.¹¹²

Additionally, AAVs have been argued to have expression timelines that are ineffective or “too slow” for use of gene therapy in vivo.¹¹³ Original AAVs only package single-stranded DNA and thus require a ‘time-consuming’ second-strand synthesis to achieve expression. However, self-complementary adeno-associated viruses (scAAVs) have been developed to allow for faster expression in the host cell. The scAAVs package two strands that can self-anneal to double-stranded DNA. Without the omission of a second-strand synthesis step, scAAVs can achieve expression on a fast time scale making them a desirable tool in gene therapy.¹¹⁴ There are remaining challenges surrounding the use of AAV vectors for transgene delivery in vivo and in vitro including cellular specificity as well as delayed onset and reduced efficiency of expression.

Typical in vivo expression results 3-21 days following transduction whereas it can occur as early as 1-2 days post transduction days in vitro.^{115,116} Previous studies have demonstrated successful AAV expression in vitro where viral transductions were performed on two-dimensional cell cultures or organotypic slices.^{34,116} Additional studies have demonstrated successful but varied AAV expression in three-dimensional cell culture models (others^{35,117} including cortical neural spheroids generated from human induced pluripotent stem cells.¹¹⁸

The tropism of AAVs differs drastically between various vector serotypes. However, the AAV1, 2, 5, 8, & 9 serotypes have been known to successfully infect cells of the CNS^{115,119}. Studies have shown a high degree of variability in cellular selectivity of AAVs to neurons and astrocytes^{120,121} leading to inconclusive results as to which serotype may be ideal for in vitro transduction of neurons and astrocytes. Similarly, achieving AAV expression in microglia has been met with many challenges¹²² one of which may be the serotype selectivity.³³ However, increased transgene expression in microglia has been achieved with specialized modification to the AAV6 capsid variant containing a self-complementary genome and microglia promoters (CD68 and F4/80).³²

For this thesis work AAV1 serotype was selected for expression trials in microglia based on a previous study indicating 20% efficiency with the CD68 promoter in vivo.^{123,124} Additionally,

AAV1 and AAV5 were selected for expression trials in neurons and astrocytes respectively based on the increased selectivity and expression observed in vivo.¹¹⁵ To date, we are unaware of any study using transduction of three viral vectors in a single sample for the purpose of monitoring simultaneous multi-cellular activities in real time.

Materials and Methods

Primary Cortical Tissue Dissociation

The tissue dissociation protocol was adapted from previous studies⁸⁰ and all animal handling and procedures were carried out in accordance with approved Brown University IACUC protocols. Postnatal day 1 (P1) Sprague Dawley rats were anesthetized by hypothermia and whole brains were dissected and placed in cold Hibernate-A solution (BrainBits LLC, HA) with added B-27 (Fisher Scientific, 17504044) supplements (HA-B27). From the whole brain tissue, cortices were carefully dissected and the dura was removed. Papain (BrainBits LLC, PAP) is a proteolytic enzyme used for breakdown of proteins and tissue while preserving cell health. 6 mg of lyophilized papain was reconstituted in 3 mL Hibernate-A minus Calcium (HA-Ca, Brainbits LLC, HA-Ca), and placed in 30°C water bath for 10 min. Dissected cortices were cut into smaller pieces and pooled together and placed in 15 mL conical. The 3 mL of warmed papain was added and cells were left to dissociate for 30 min 30°C water bath with inversion mixing every 5 min. Following the 30 min papain incubation, the supernatant was gently removed and a fresh 2 mL of warmed HA-B27 was added. The dissociated tissue was resuspended in 2 mL of HA-B27 and triturated approximated 20 times with a 1000 µl pipette until cells were dispersed in solution and no obvious tissue sections remained. The cell suspension was then dripped through a filter (40µm) and an additional 3 mL of warmed HA-B27 was used to wash over the filter. The filtered 5 mL suspension was then centrifuged at 150 g for 5 min in a 15 mL conical tube. The supernatant was carefully aspirated to keep the cell pellet intact. A fresh 2 mL of warmed complete cortical media (CCM) (50 mL solution containing 48.4 mL Neurobasal-A culture medium (Fisher

Scientific, 12-349-015), 1 mL B27 (Fisher Scientific, 17504044), 0.5 mL penicillin-streptomycin, (Sigma-Aldrich, P4333) and 0.125 mL Glutamax (ThermoFisher Scientific, 35050061) was added to the pellet and gently resuspended. The suspension was added dropwise to a 40µm filter, washed over with 3 mL of warmed CCM, and centrifuged at 150 g two more times. A final supernatant removal, resuspension and centrifugation was completed before final resuspension in 5 mL of warmed CCM. A small aliquot from the final suspension was taken and diluted 1:5 in CCM and then 1:1 in Trypan Blue (Sigma-Aldrich, T8154). A live/dead cell count was determined by hemocytometer and a cell viability was assessed. If cells were <90% viability they were not used for seeding.

Formation of Three-dimensional Microtissue

Agarose gel microfeatures were molded into the bottom of wells on a non-treated 24-well glass bottom plate (CellVis P241.5HN) using a stainless steel injection mold. 2% (w/v) hot agarose was made by adding 50 ml of sterile PBS to 1 gram of autoclaved ultra pure agarose (ThermoFisher 16-500-100) and heating in a microwave in 30 second increments until boiling point and agarose powder is fully dissolved. 1 mL of hot agarose was injected with a luer-locking syringe through the custom-machined stainless steel mold into the bottom of each well and then allowed to cool on ice until solidified. The steel injection mold was carefully removed leaving a molded microfeature within 200µm of the bottom of the plate with 6 intact agarose pegs. (See Figure 8 for reference) 1 mL of CCM was added to each molded well to allow for medium diffusion into agarose. Molded plates were kept in the humidified incubator until seeding and 2x1 mL media changes were completed over two days prior to the seeding. Just before seeding, the full 1mL of media was removed from each well. Cells were seeded into agarose-molded cell culture plates (Fig 2) at 2.7×10^8 cells/mL in 10 µl volumes. Following careful seeding, the plates were placed in the incubator for 25 minutes followed by the addition of 1 mL of CCM added dropwise to each well. At 24 hours post seeding successful self-assembled microtissues formed, marked by the

distinct inward tissue contraction around the agarose pegs (Fig 2D) A full 1 mL media change was completed every other day following seeding.

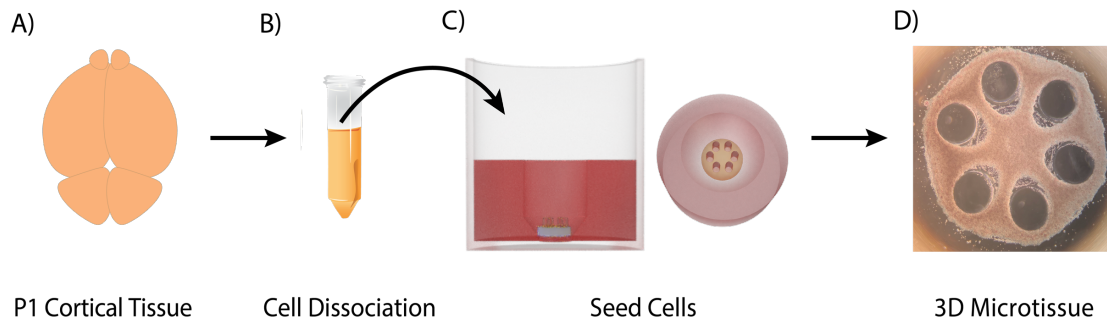


Figure 2: Three-dimensional Microtissue Formation

Cortical tissue collection from P1 rats and dissociated into cell suspension for seeding into agarose microfeature which was previously molded into the cell-culture plate well. Self-assembled 3D microtissue formation at 24 hours following cell seeding.

Viral Transduction

All AAV-related procedures were carried out in accordance with 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 and proper personal protective equipment was worn at all times. All pipette tips, serological pipettes, 15 mL conicals, and microcentrifuge tubes used during the transduction experiments outlined in the work of this thesis research, were subsequently soaked in 10% bleach solution for a minimum of 10 minutes following use. Additionally, the biosafety hood was sprayed with 10% bleach and wiped down 10 min later at the beginning and end of any virus handling followed by a 15 minute exposure to UV light. Details regarding viral titers and dilutions carried out during experimentation are the outlined results of this thesis work.

AAV viral vectors were purchased from Addgene and aliquots were stored at -80C. For a detailed list of the recombinant components and mappings of the three AAV vectors used please reference **Figure 1B & 1C**. It is important to note that the AAV1.CaMKII(1.3).eYFP.WPRE.hGH and AAV5.GFAP.eGFP.WPRE.hGH vectors were designed as control vectors, meaning they do

not encode for any additional transgene other than the yellow and green fluorescent proteins (YFP and GFP). The microglia-targeting AAV1.CD68.hM4D(Gi).mCherry vector was not designed as a control vector and does contain the designer receptor exclusively activated by designer drugs (DREADD) hM4D. The hM4D is a modified human muscarinic receptor that is activated by the Gi signaling pathway. The receptor is only activated in the presence of the designer drug clozapine-N-oxide (CNO). For the purposes of these experiments, the AAV1.CD68.hM4D.mCherry vector was effectively used as a control virus for fluorescence labelling with no activation of the DREADD.

Live Cell Imaging

An Olympus Confocal Laser Scanning Microscope (Olympus VF3000) was used in the real-time image capture described in this research. The confocal microscope offers 4-laser multichannel imaging capabilities using 405nm, 488nm, 561, and 640nm lasers. Using multichannel excitation, high-speed resonant scans of the microtissues were collected using the 10x and oil-immersion 30x objectives. Additionally, the confocal microscope has the capability of three-dimensional image acquisition by collecting images along the z-axis.

The microtissues in agarose-molded cell culture plates were carefully transported in warmed Styrofoam chambers from the incubator to the Brown University Leduc imaging facility. To limit cells exposure outside the temperature and CO₂ controlled incubator, each plate containing microtissues was only imaged for 1 hour at a time. Details regarding the unique acquisition parameters which were developed for these experiments, including spectral deconvolution, 30x image stitching, and imaging timelines are outlined in the results of this thesis work.

Fixing and Removal of Agarose

On predetermined experimental termination days, microtissues were fixed in cold solution (4% (w/v) paraformaldehyde and 8% (w/v) sucrose solution) for 2 hours followed by 3 subsequent PBS washes. After 3 PBS washes, agarose molds holding the microtissues were carefully

extracted from each well of the plate. Using a razor blade, the excess agarose was cut away from around the microtissue being careful not to cut too close to the tissue itself. Then the remaining piece of the mold containing the sample was placed in PBS and heated on a 200C hot plate. Samples were allowed to melt for approximately 10 minutes with frequent agitation until the residual agarose and pegs have melted away. Following complete agarose removal, the free-floating microtissues were then transferred to a 48-well plate and washed twice more with hot PBS.

Immunohistochemistry

Following fixation and agarose removal, the microtissues were immunostained for cell-specific antigens. 300 µl of block solution (10 mL solution consisting of 1 mL normal goat serum, 8.9 mL 1xPBS, 0.1mL TritonX-100, and 0.4 g of Bovine serum albumin) was added to each 48-well containing a microtissue and placed on a shaker with gentle agitation for 2 hours. Following the 2 hour block, solution was removed and 300 µl of primary antibody diluted in blocking solution was added to each well and placed back on the shaker for room temperature incubation overnight. The neuron-targeting AAV1.CaMKII(1.3).eYFP positive samples were immunostained with neuronal nuclei marker NeuN (mouse anti-NeuN, Milipore MAB377 1:500). The astrocyte-targeting AAV5.GFAP.eGFP positive samples were immunostained with glial fibrillary acidic protein (GFAP) (Chicken anti-GFAP, Milipore AB5541 1:200). The microglia-targeting AAV1.CD68.hM4D.mCherry positive samples were immunostained with microglial marker, ionized calcium-binding adapter molecule-1 (Rabbit anti-Iba, WAKO 019-19741 1:200). On the following day, primary antibody solutions were removed from each well and microtissues were subsequently washed twice for 2 hours with PBT solution (50 mL solution consisting of (49.9 mL of 1x PBS, 0.1mL of Triton X-100). 300 µl of blocking solution was again added to each well and left to shake for 2 hrs. Following block, 300 µl of secondary antibody diluted at 1:800 in blocking solution were added to their corresponding wells and left to shake at room temperature overnight. For mouse anti-NeuN labeled microtissues (Goat anti-mouse CY3, JacksonImmuno 115-165-

068, 1:800). For chicken anti-GFAP labeled microtissues, (Goat anti-chicken Alexa Fluor 488, Invitrogen A11039, 1:800). For rabbit anti-Iba1 labeled microtissues (Goat anti-rabbit Alexa Fluor 635 Invitrogen A31577, 1:800). On the third day, secondary antibody solution was removed and microtissues were washed with PBT four times over four hours. Following the PBT washes, the dsDNA fluorescent probe DAPI (ThermoFisher D1306 1:1000) was diluted in PBT 1:1000 and added to microtissues and left to incubate for one hour. DAPI solution was removed and each well was replaced with 500 μ L of PBS. Microtissues were transferred to 10mm glass bottom petri dishes for confocal imaging or placed at 4C for storage.

Results

Optimization of Viral Transduction in Microtissues

Introducing excitatory neuron-targeting AAV (pAAV1.CamKII(1.3).eYFP.WPRE.hGH) into the microtissue on day 7 in vitro by direct incubation (adding viral particles in solution dropwise to the microfeature surface followed by addition of culture medium 1 hour later) (**Fig 3A Method 1**) did not result in visible fluorescence when imaged at subsequent days 3(DIV10), 5(DIV12), and 7(DIV14) following transduction. A secondary approach was performed in which the total well medium volume was replaced on DIV7 with 1 mL of AAV+culture medium cocktail (dilute viral particles directly into 1 mL of cell culture medium) followed by a 1 day (**Fig 3A Method 2.1**) and a 3 day (**Fig 3A Method 2.2**) incubation before subsequent medium change and imaging at the following days post-transduction (DPT) 3, 5, and 7. The 3 day AAV+culture medium cocktail incubation (**Fig 3A Method 2.2**) resulted in stable fluorescence in neurons by day 7(DIV14) following transduction (**Fig 3B**). For Method 1, 6 μ L of AAV particles at 1.0e10 vg/mL (vector genomes per milliliter) were added dropwise to individual microtissues. Following the addition of the 1 mL of culture medium, the estimated final titers were approximately 1.0e8 vg/mL and 1.0e4 vg/mL respectively.

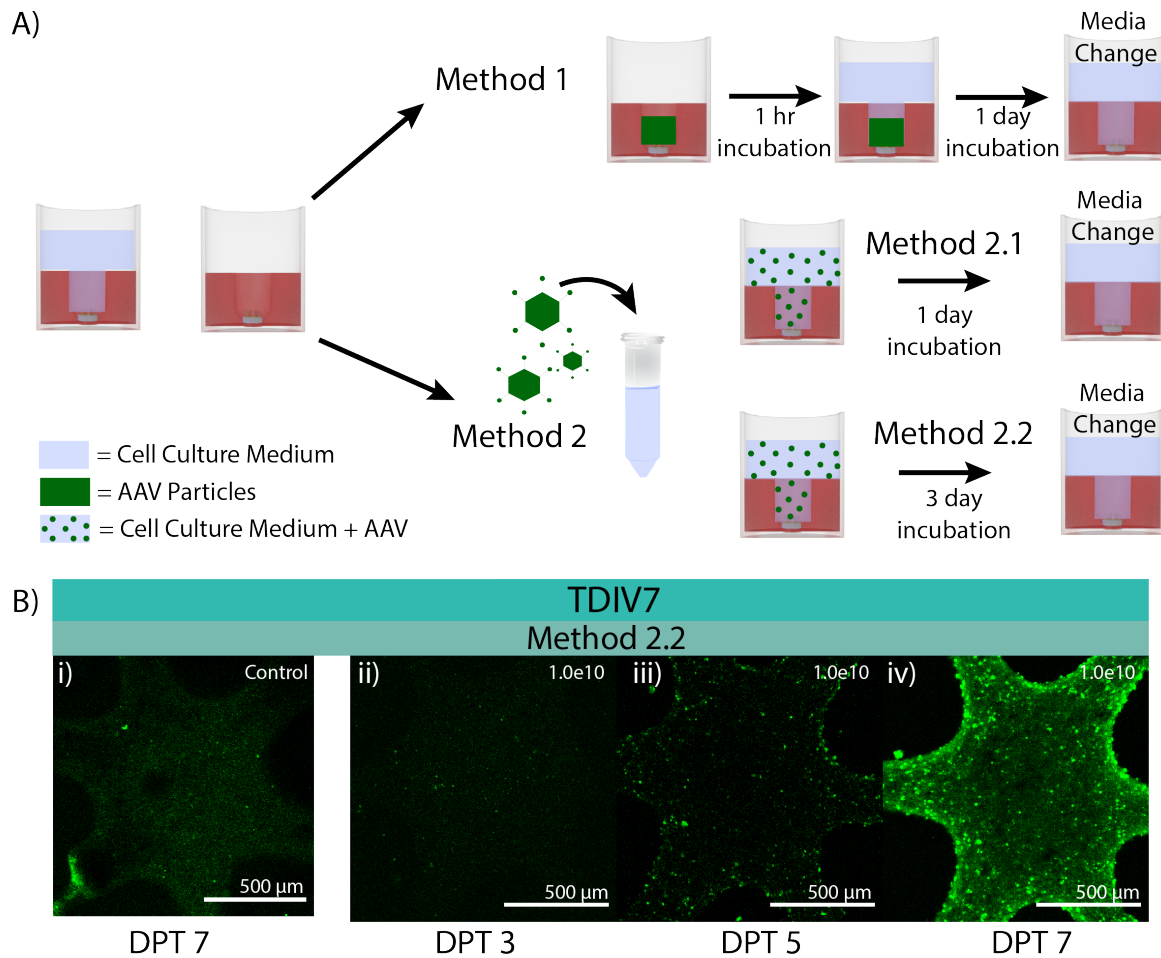


Figure 3: Transduction Method Validation

Direct AAV particle incubation described by *Method 1* versus cell culture medium+AAV cocktail incubation described by *Method 2* (Fig3A). Fluorescence onset results following day in vitro 7 transduction (TDIV7) and subsequent imaging at 3, 5, and 7 days post-transduction (DPT) (Fig3B, i-iv)

Once a successful transduction technique was established, the neuron-targeting AAV (pAAV1.CamKII(1.3).eYFP.WPRE.hGH) was optimized by assessing the following parameters: transduction titers, addition of serum to the transduction cocktail, and day of transduction. Using transduction method 2.2, a titer analysis was performed. Low (1.52×10^5 vg/ml), medium (3.8×10^{10} vg/mL), and high (7.6×10^{10} vg/mL) titers were tested. Transduction was performed on DIV7 and following the three day incubation with AAV+medium cocktail, samples were imaged at day 7 post-transduction (DPT7) (**Fig 4A**). The low titer samples showed very low visible fluorescence (**Fig 4A,ii**). The medium titer samples had obvious and stable fluorescence in neuron cell bodies

as well as extensions (**Fig 4A,iii**). The high titer samples (**Fig 4A,iv**) displayed signs of toxicity and cell death with visible “holing” in the microtissue (**Fig 4A,v**). The addition of DRAQ7, a far-red non-toxic dye labeling nuclei in dead and permeabilized cells, indicated acute cell death in the holes of the microtissue subjected to high titer doses (**Fig 4A,vi**). The addition of 10% horse serum to the AAV+medium cocktail enhanced viral fluorescence when imaged on DPT7 following a DIV7 transduction (**Fig 4A,vii**) compared to serum negative samples (**Fig 4A,Viii**). Additionally, a DIV1 transduction was performed to determine if an earlier expression was possible. After a DIV1 transduction with a titer of 3.8×10^{10} vg/mL followed by a three day incubation microtissues were imaged at DPT3, 5, & 7 (**Fig 4B,i-iv**) and stable fluorescence was observed at DIV7 (**Fig 4B,iv**). The samples were subsequently fixed and immunostained with NeuN and again imaged for quantitative analysis of transduction efficiency (**Fig 4C, i-vii**). Histology analysis using NeuN (**Fig 4C, i & iv**) which labels neuronal nuclei and AAV induced YFP (**Fig 4C, ii & v**) were performed. Obvious co-expression of NeuN and YFP was observed (**Fig 4C, iii & vi**) NeuN+ cells were counted and compared to YFP+ counted cells from three separate volumetric cubes imaged from the center of the microtissue. Transduction efficiency was determined to be 54.2%. (**Fig 4C, vii**)

Using the previously described AAV+medium cocktail transduction method 2.2 expression of the astrocyte-targeting AAV (AAV5.GFAP.GFP.WPRE.hGH) was optimized. A low, medium, and high titer analysis was performed: low (1.04×10^5 vg/ml), medium (2.6×10^{10} vg/mL), and high (5.2×10^{10} vg/mL). Following the DIV7 transfection and a three day incubation with AAV+medium cocktail, samples were imaged on DPT7 (**Fig 5A, i-iv**). The low titer samples showed very low visible fluorescence (**Fig 5A, ii**). The medium titer samples did not display the same obvious and stable fluorescence that was seen in neuron-targeting AAV (**Fig 5A, iii**). However, the high titer samples did show stable fluorescence at DPT7 (**Fig 5A, iv**) but unlike the neuron-targeting AAV samples they did not show signs of severe toxicity and cell death with no visible “holing” in the microtissue with the addition DRAQ7 (**Fig 5A, v-vi**). The addition of 10% horse serum to the

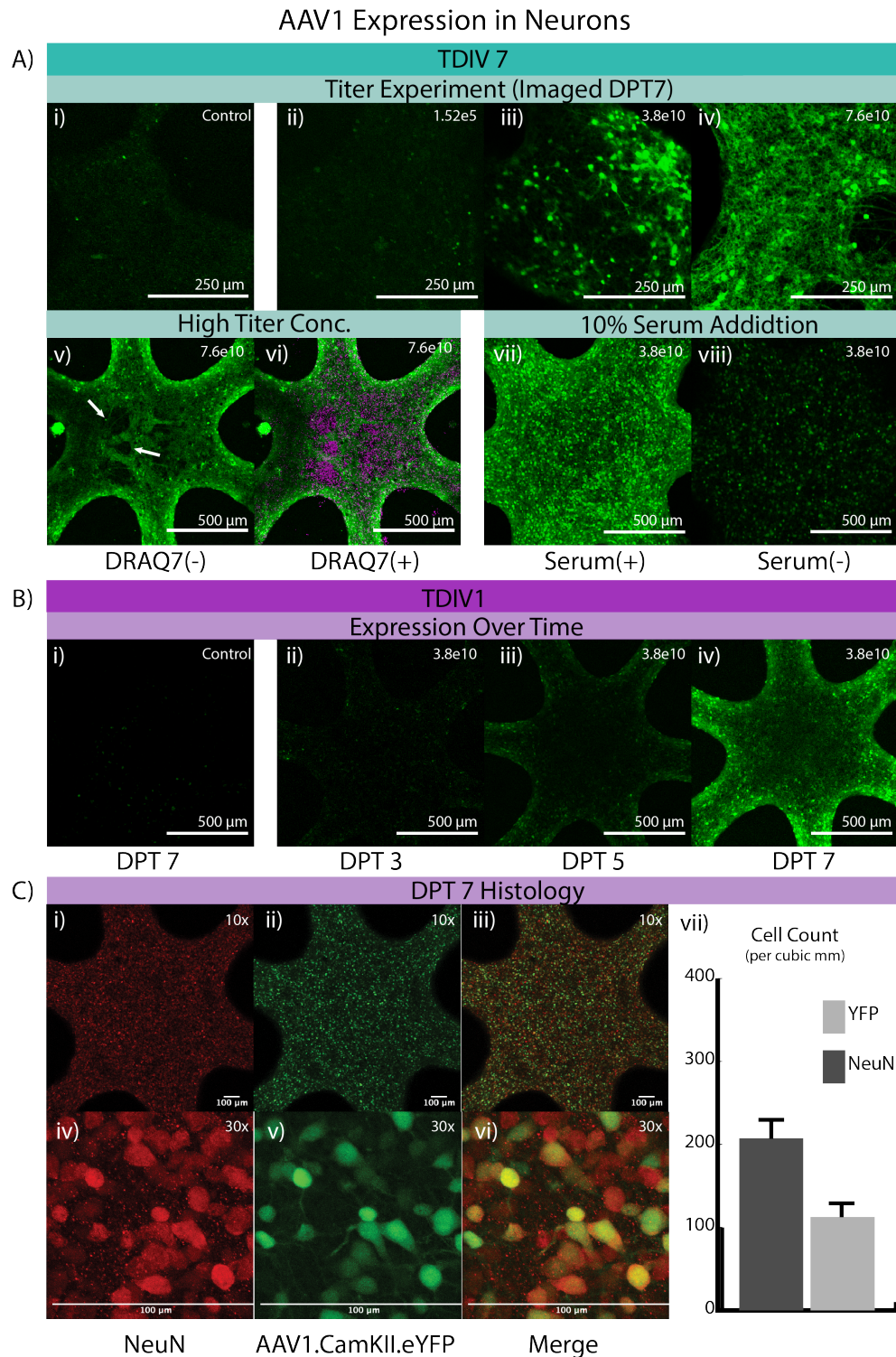


Figure 4: Neuron-targeting AAV1 Expression

Neuron targeting AAV1.CaMKII(1.3).eYFP expression results from transduction at day in vitro 7 (TDIV7) and subsequently imaged at 7 days post transduction (DPT7) (Fig4A) compared to results from transduction at day in vitro 1 (TDIV1) and subsequently imaged at several days post transduction (DPT3-7) (Fig4B). Immunohistochemistry (Fig4C, i-vi) and transduction efficiency results (Fig4C, vii) from transduction day in vitro 1 (TDIV1) and subsequent fixing at 7 days post transduction (DPT7).

AAV+medium cocktail enhanced viral fluorescence when imaged on DPT7 (**Fig 5A,vii**) when compared to the serum negative samples (**Fig 5A, viii**). Similar to the optimization of the neuron-targeting AAV, an additional DIV1 transduction was performed to determine whether or not an earlier transduction day was possible. After a DIV1 transduction with a titer of 5.2×10^{10} vg/mL followed by a three day incubation, microtissues were imaged at DPT3, 5, & 7 (**Fig 5B,i-iv**) and stable fluorescence was observed at DPT7 (**Fig 5B, iv**). The samples were subsequently fixed and immunostained with GFAP and again imaged for quantitative analysis of transduction efficiency (**Fig 5C, i-vii**). Histology analysis using GFAP (**Fig 5C, i & iv**) which labels the glial fibrillary acidic protein in astrocytes and AAV induced GFP (**Fig 5C, ii & v**) were performed. Obvious co-expression of GFAP and GFP was observed (**Fig 5C, iii & vi**) GFAP+ cells were counted and compared to GFP+ counted cells from three separate volumetric cubes imaged from the center of the microtissue. Transduction efficiency was determined to be 63.9% (**Fig 5C, vii**)

Using the previously described AAV+medium cocktail transduction method 2.2 expression of the microglia-targeting AAV (AAV1.CD68.hM4D(Gi).mCherry) was assessed. Initially, DIV1 transductions were performed with titer assessments at a low (1.2×10^5 vg/mL), medium (2.3×10^{10} vg/mL), and high (4.6×10^{10} vg/mL) titer dosing. Only very minimal fluorescence was observed with the medium and high titers when imaged at DPT7 (**Fig 6A, iii-iv**). However, qualitative assessment shows that the observed fluorescent signal was not significantly different from the controls (**Fig 6A,i**). A subsequent DIV7 transduction experiment was completed using the same low, medium, and high titer doses. Interestingly, we did not see any stable fluorescence at the expected DPT7 (**Fig 6B, i-iv**). The addition of 10% horse serum to the AAV+medium cocktail only appeared to moderately increase the fluorescent signal in samples transduced at DIV7 with 4.6×10^{10} vg/mL of AAV (**Fig 6B, vii-viii**). The minimally fluorescent images appear to have low levels AAV related fluorescence in the 10x objective (**Fig 6B, v**). However, with additional optical zoom, microtissues with minimal fluorescence revealed AAV related mCherry expression in what appeared to be globular morphologies (**Fig 6B,vi**). We predict that this could be indicative of AAV

AAV5 Expression in Astrocytes

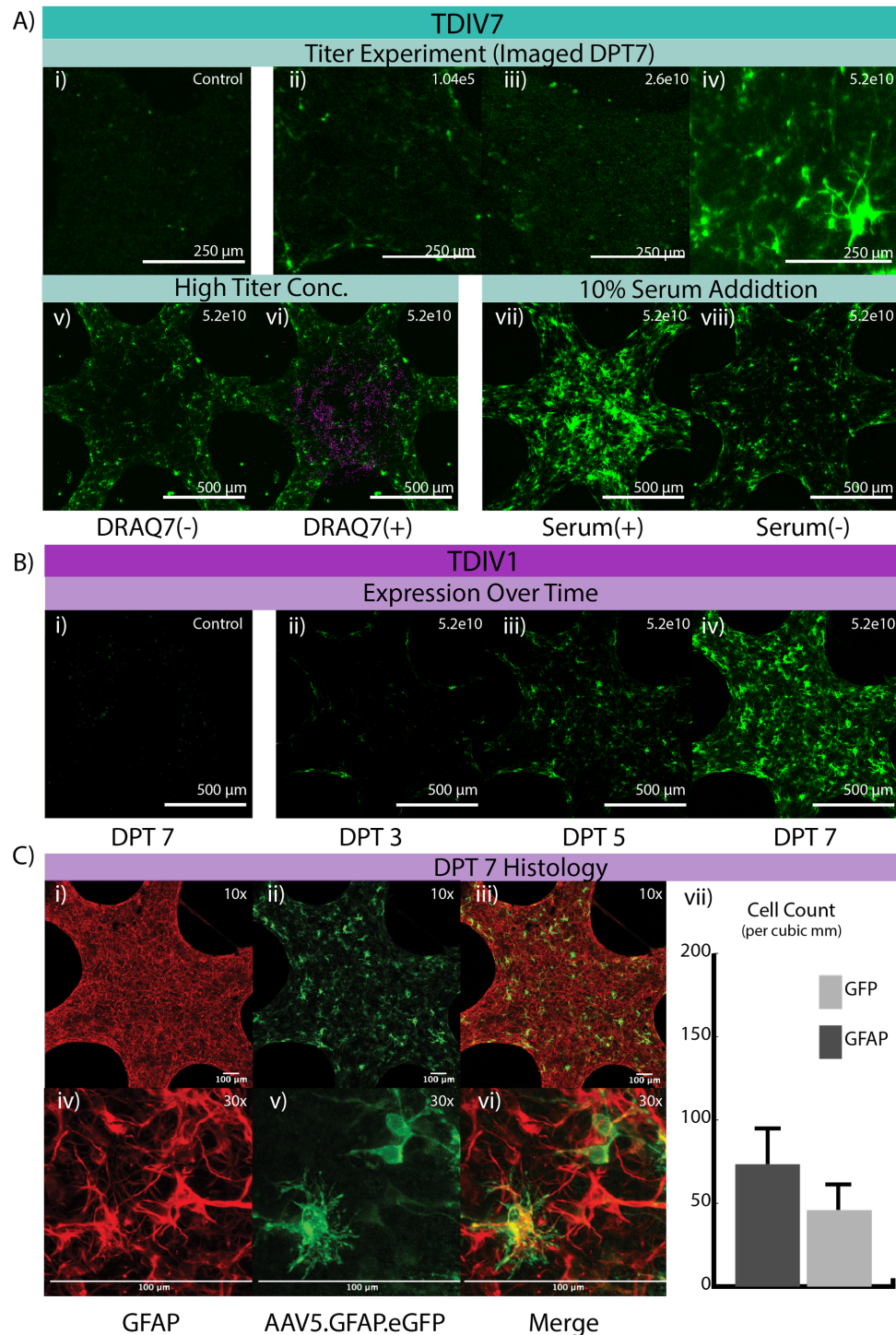


Figure 5: Astrocyte-targeting AAV5 Expression

Astrocyte-targeting AAV5.GFAP.eGFP expression results from transduction at day in vitro 7 (TDIV7) and subsequently imaged at 7 days post transduction (DPT7) (Fig5A) compared to results from transduction at day in vitro 1 (TDIV1) and subsequently imaged at several days post transduction (DPT3-7) (Fig5B). Immunohistochemistry (Fig5C, 1-vi) and transduction efficiency results (Fig5C, vii) from transduction day in vitro 1 (TIDV1) and subsequent fixing at 7 days post transduction (DPT7).

expression in reactive microglia as the activated microglia phenotype is typically marked by a more round and globular morphology^{125,126}. A final experiment using the medium transduction titer of 4.6e10 vg/mL was completed where samples were transduced at DIV7 and imaged at the later time points of DPT13,15, and 17 (**Fig 6C, i-iv**). Fluorescence onset was seen starting at DPT15 (**Fig 6C, iii**) and stable fluorescence was seen at DPT17 (**Fig 6C, iv**). The DIV7 transduction samples were subsequently fixed and immunostained with Iba1 and again imaged for quantitative analysis of transduction efficiency (**Fig 6D, i-vi**). Histology analysis using Iba1 (**Fig 6D, i & iv**) which labels the Ionized calcium binding adaptor molecule 1 in microglia and AAV induced mCherry (**Fig 6D, ii & v**) were performed. Obvious co-expression of Iba1 and mCherry was observed (**Fig 6D, iii & vi**). Iba1+ cells were counted and compared to mCherry+ counted cells from three separate volumetric cubes imaged from the center of the microtissue. Transduction efficiency was determined to be 65.9% (**Fig 6D, vii**). Note that the transduction efficiencies were calculated from samples transduced at DIV7 and fixed at DPT7. At the time of this thesis submission we were unable to report on the transduction efficiency at DPT17 where stable fluorescence was observed due to campus-wide regulations restricting laboratory work for COVID-19 related reasons. However, we expect to see increased transduction efficiency at DPT17 due to the fact that we see an increased number of fluorescent microglia at those later time points.

Optimization of Triple AAV Transduction Timeline

Simultaneous monitoring of multiple cell types in real-time across a longitudinal study of a single microtissue required the develop a detailed experimental timeline and protocol for triple transduction. By analyzing the ratio of signal to noise (SNR), fluorescent expression profiles were created for each virus. The SNR was selected as a metric with which we could quantify the onset of stable fluorescence that was previously only qualitatively assessed by eye during the live imaging sessions. A threshold indicating stable fluorescence was determined by averaging the fluorescence in the pixels of a selected ROI and dividing by the averaged fluorescence of a

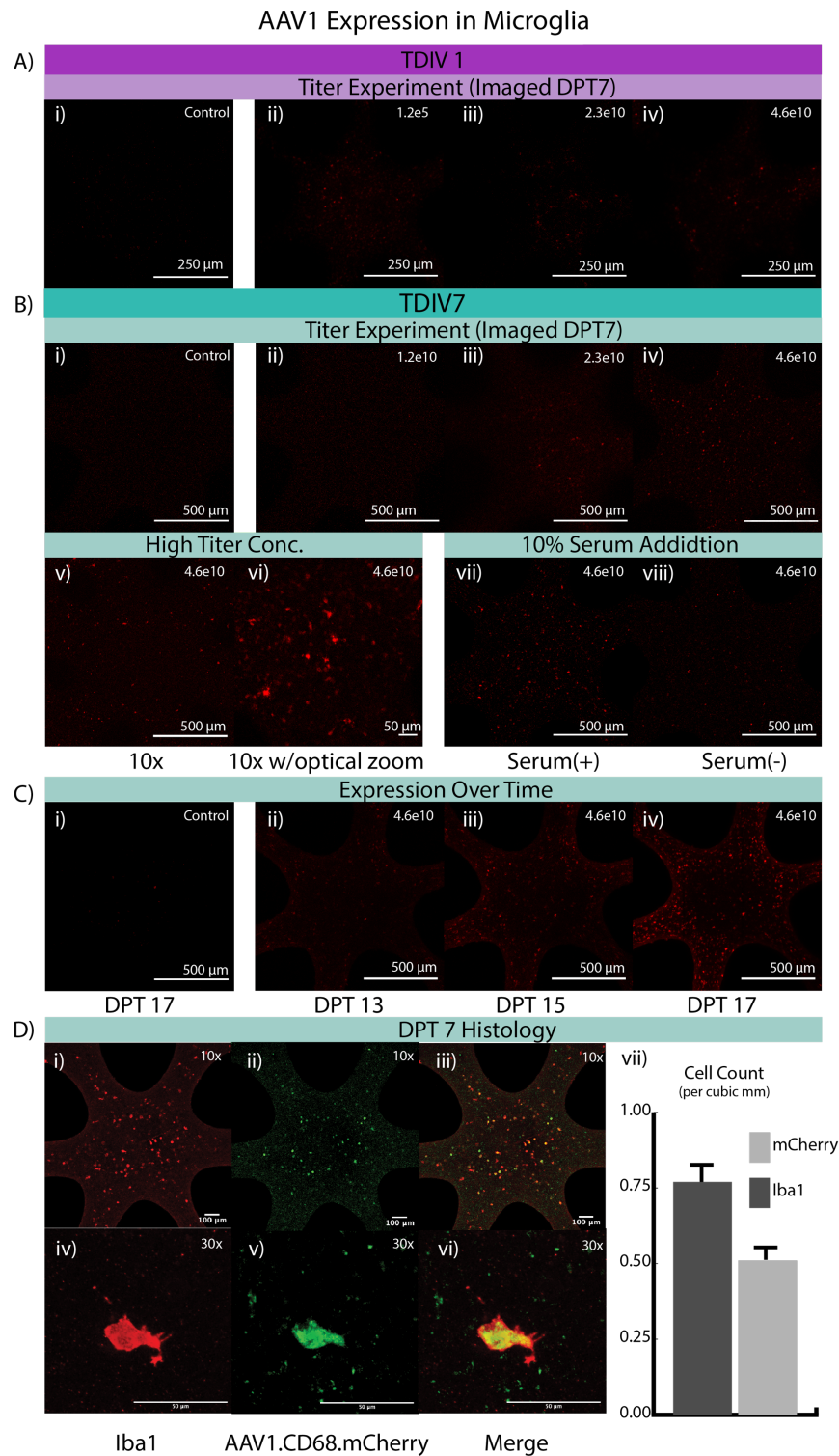


Figure 6: Microglia-targeting AAV1 Expression

Microglia-targeting AAV1.CD68.hM4D(Gi).mCherry expression results from transduction at day in vitro 1 (TDIV1) and subsequently imaged at 7 days post transduction (DPT7) (Fig6A) compared to results from transduction at day in vitro 7 (TDIV7) and subsequently imaged at several days post transduction (DPT7-17) (Fig6B). Immunohistochemistry (Fig6C, 1-vi) and transduction efficiency results (Fig6C, vii) from transduction day in vitro 7 (TIDV7) and subsequent fixing at 7 days post transduction (DPT7).

background ROI. The selected ROI was drawn around a cell that showed visibly detectable fluorescent signal. The SNR values at each day were determined by averaging the fluorescent signal in 5 cellular ROIs and dividing by the averaged fluorescent signal of 5 background ROIs. Figure **7A and 7B** shows the plots of SNR normalized to the stable fluorescence threshold for the neuron-targeting AAV1 and the Astrocyte-targeting AAV5 over the course of 8 days following transduction on DIV1. TDIV1 transduction of the neuron-targeting AAV1.CaMKII(1.3).eYFP virus showed stable fluorescence by DPT7 (**Fig 7A**) with SNR breaking threshold between DPT6 and DPT7. TDIV1 transduction of the astrocyte-targeting AAV5.GFPA.eGFP virus showed stable fluorescence by DPT7 (**Fig 7B**) with SNR breaking threshold just before DPT7.

Based on the expression profiles that are unique to each of the three AAVs previously investigated, a timeline for triple transduction was developed. Due to the fact that stable fluorescence in microglia could only be achieved when transduced at DIV7 with the addition of 10% horse serum and then subsequently imaged at DPT17 (corresponding DIV24), the triple transduction timeline needed to be structured around this virus. Since the neuron-targeting AAV1 and the astrocyte-targeting AAV5 did not appear to have altered expression profiles upon shifted transduction days, we hypothesized that a forward shift would similarly not induce changes in expression. The specific transduction days were chosen in such a way that fluorescence in each cell type would be visibly stable by DIV24, the day at which the microglia fluorescence stabilizes (**Fig 7C**).

Based on the newly selected transduction days, the expression profiles were compared to those previously calculated based on the DIV1 transductions. A DIV12 transduction of neuron-targeting AAV1 was assessed by imaging at DPT 6, 8, and 10 to monitor fluorescence onset (**Fig 7D, i-iii**). Stable fluorescence onset was again calculated by analyzing the signal to noise ratios during the fluorescence onset over time. As expected, the SNR threshold was broken by DPT7 (**Fig 7E**). A DIV17 transduction of astrocyte-targeting AAV5 was assessed by imaging at DPT 5, 7, and 9 to monitor fluorescence onset (**Fig 7F, i-iii**). Stable fluorescence onset was again

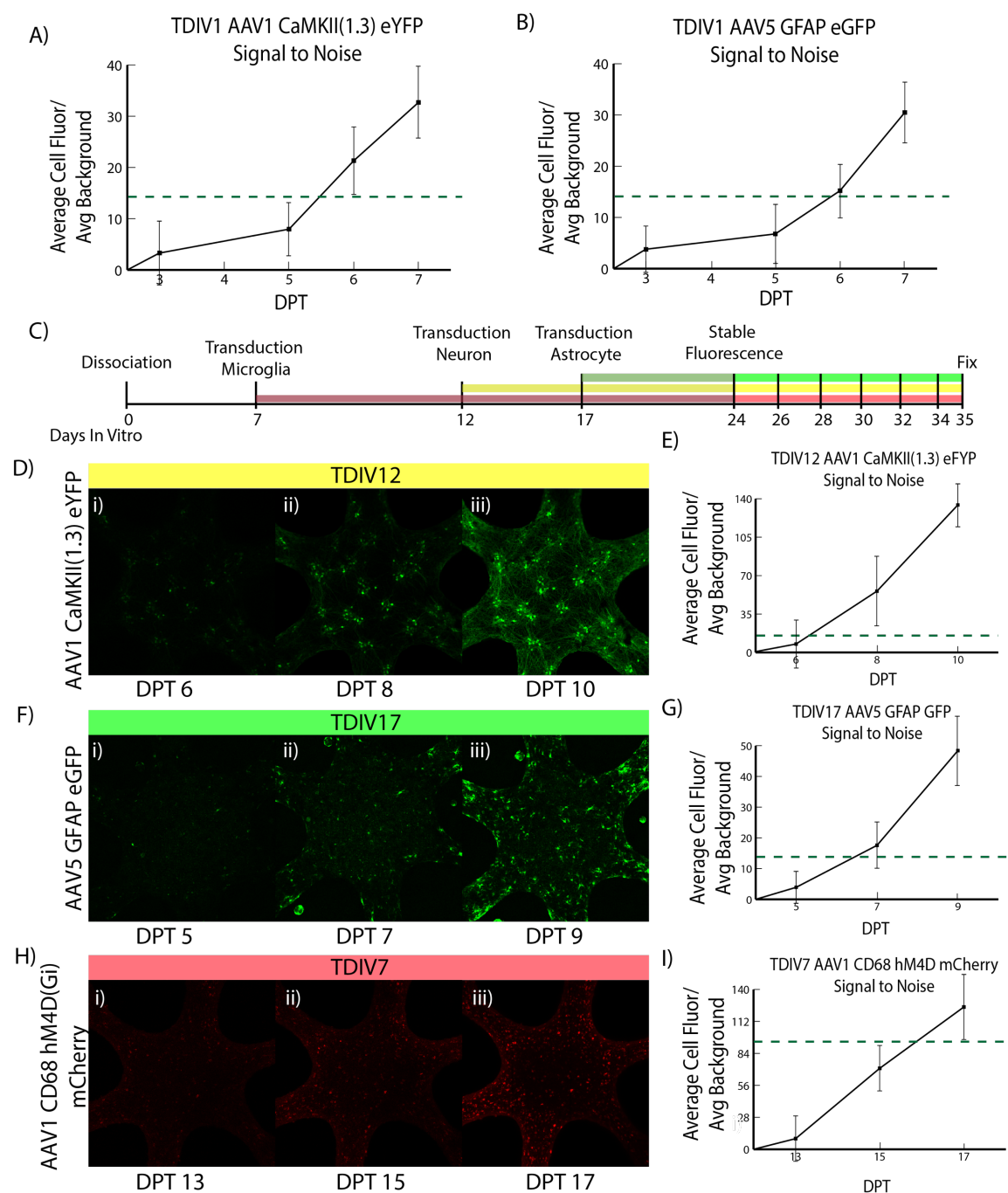


Figure 7: Triple Transduction Timeline

The ratio of signal to noise was measured during fluorescence onset for neuron-targeting AAV1 (Fig7A) and for astrocyte-targeting AAV5 (Fig7B) following transduction at day in vitro 1(TDIV1). Triple AAV experimental timeline for multiple transductions in a single microtissue (Fig7C). Monitoring of fluorescence onset over time in of all three AAVs following transductions at days determined by triple AAV experimental timeline (FigD, F, & H) and the corresponding signal to noise results for assessment of stable fluorescence onset (Fig E, G, & I). Error bars = standard deviation. Unpaired t-test comparing expression profiles for Fig 7A & 7E produced p value of $p=0.3098$. Unpaired t-test comparing expression profiles for Fig 7B & 7G produced p value of $p=0.5710$.

calculated by analyzing the signal to noise ratios during the fluorescence onset over time. As expected, the SNR threshold was broken just prior to DPT7 (**Fig 7G**). An unpaired t-test indicated that no statistically significant difference was observed between the shifted expression profiles and the previously calculated TDIV1 profiles for both the neuron and the astrocyte viruses. The DIV7 transduction of microglia-targeting AAV1 was assessed by imaging at DPT 13, 15, and 17 to monitor fluorescence onset (**Fig 7H, i-iii**). Stable fluorescence onset was calculated by analyzing the signal to noise ratios during the fluorescence onset over time. The SNR threshold was broken just prior to DPT17 which corresponds to DIV24 (**Fig 7I**).

Optimization of Optical Methods and Image Acquisition

In order to obtain the high-resolution images necessary for visualizing cell-specific morphologies and migrations, several parameters of the imaging platform were optimized. Maintaining the disk like structure of the three-dimensional microtissues across several weeks of culture requires the neural cells to be seeded into the stationary agarose mold placed in the bottom of a standard 24 well cell culture plate (see methods). We found that the thickness of the agarose and cell culture plate ultimately placed the microtissue outside the working distance of the 30x objective, thus limiting us to use of the 10x objective with an optical zoom which severely decreased the image resolution (**Fig 8A, i-iii**). Bringing the microtissues within working distance of the 30x objective required the fabrication of a specialized injection mold designed to place the microfeature agarose mold within 200um of the bottom of the cell culture plate (**Fig 8B**). With the microtissue within the working distance of the 30x objective we were able to obtain images where high -resolution cell-specific morphologies and features could be easily distinguished (**Fig 8C, i-iii**). Where previous unique morphological features such as the bushy extensions in astrocytes were not clearly visible in the 10x objective (**Fig 8A, iii**), the injection mold allowed for use of the 30x objective. The increased objective power allowed for a drastic increase in image resolution where the morphological features were not clear and distinguishable (**Fig 8C, iii**).

In addition to the adjustment made on the physical constraints of the imaging platform, a unique spectral deconvolution protocol was developed (**Fig 9**). With multiple spectrally close fluorescent probes it was necessary to develop a method to visually differentiate the labeled cell types. Microtissues were co-transduced on DIV1 with the neuron-targeting AAV1.CaMKII(1.3).eYFP (3.8e10vg/mL) and the astrocyte-targeting (5.2e10 vg/mL). In post-fixing analysis of co-transfected microtissues we are able to determine individual neuron-related viral expression and astrocyte-related viral expression by looking at the cell-specific

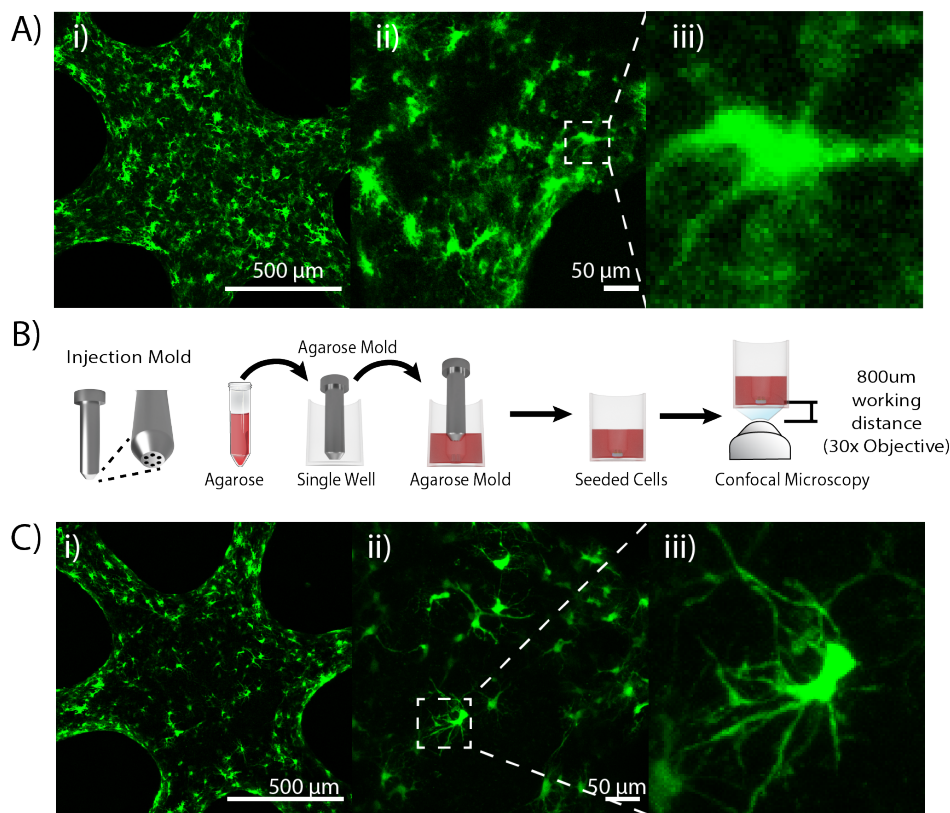


Figure 8: High Resolution Imaging

Limited spatial resolution of images taken with 10x objective (Fig 8A, i) and 10x objective with 2.5 optical zoom (Fig 8A, ii) prior to the development of specialized injection mold (Fig 8B) designed to bring microtissues within working distance of the 30x objective while remaining in agarose molded cell culture well. 10x (Fig 8C, i) images compared to 30x (Fig 8C, ii) demonstrate the increased resolution with implementation of the injection

immunohistochemical markers NeuN and GFAP (**Fig 9A**). However, in a live-imaging format an alternative method using peak deconvolution was implemented to achieve similar cell-specific

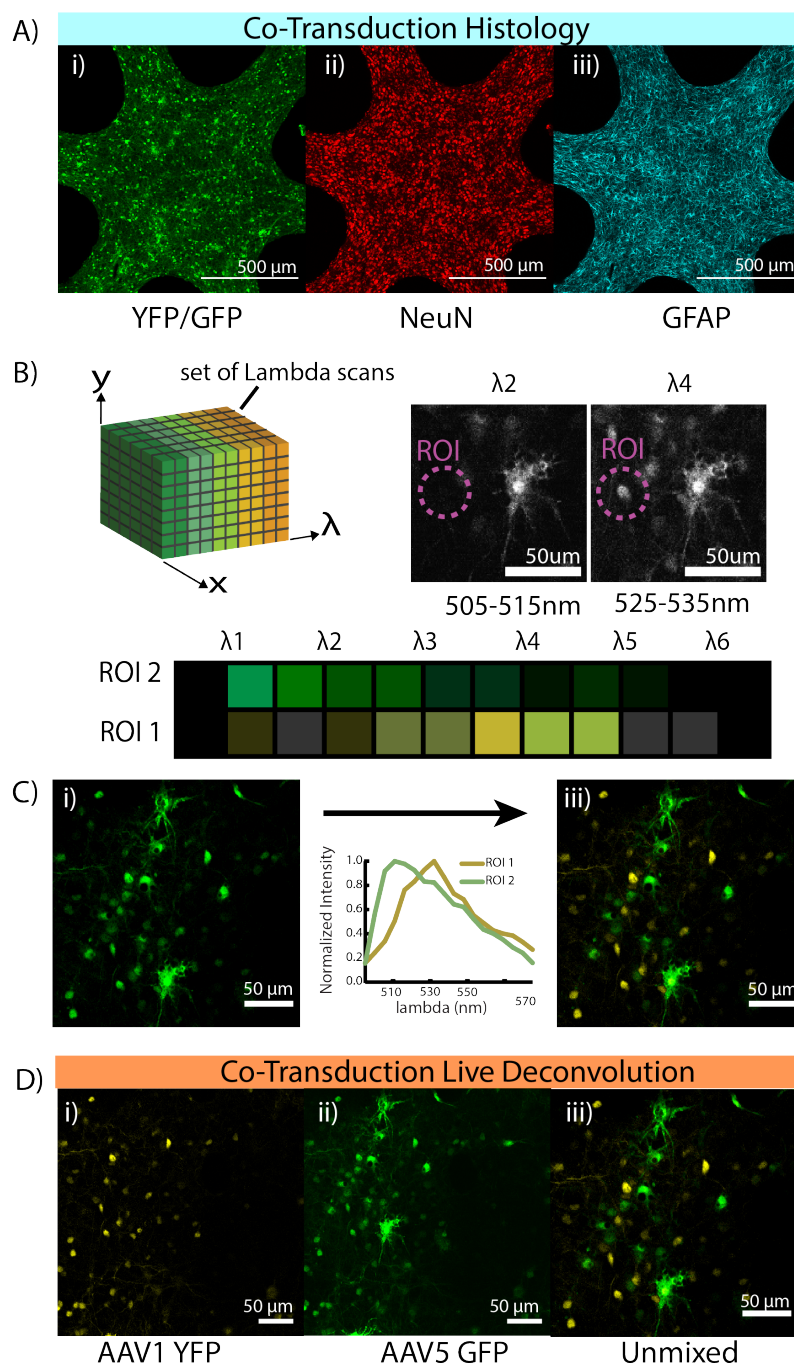


Figure 9: Optimized Optical Methods

Comparison of immunolabeled neurons (NeuN) and astrocytes (GFAP) in co-transfected sample (Fig 9A, i-iii). Spectral deconvolution (Fig 9B) applied to live imaging of a co-transfected sample where YFP-labeled neurons were visibly indistinguishable from GFP-labeled neurons due to limited emission peaks between the two fluorophores. Successful peak deconvolution reveals visible differentiable cell types in co-transfected sample.

fluorescent labeling with the existing YFP and GFP viral tags in neurons and astrocytes (**Fig 9B**). The co-transfected microtissues was imaged on the confocal microscope at DPT7. The YFP (530nm peak emission) tag in excitatory neurons is spectrally very close to the GFP (510nm peak emission) tag in astrocytes and is not distinguishable by eye. However, the capabilities of the Olympus VF3000 confocal microscope allowed us to spectrally deconvolve these two fluorophores. This was done by acquiring a set of lambda scans across a linear range of wavelengths (500-560nm) in 10 nm increments. Using the distinct morphologies of astrocytes and neurons, two ROIs were selected, each associated to a particular cell type. The lambda stack of each cell-specific ROI was then assessed to determine which 10nm scan contained the peak fluorescence associated with the cell-specific fluorescent labels (astrocytes-GFP=510nm, neurons-YFP=530). The spectral information in the whole image was binned into three broad spectral ranges. The onboard software offers linear unmixing which enables the precise determination of spectral profiles at each pixel in the image to overcome overlap and binning artifacts. The spectral profile is calculated and plotted across the lambda scans of the whole image resulting in a spectrally deconvolved image (**Fig 9C**). Upon assigning a false color to one of the ROI groups, the image was linearly unmixed and the two unique fluorophores were then visibly distinguishable. This live imaging capability allows us to distinguish, in real-time, cell-specific fluorescent labeling of GFP-tagged Astrocytes and YFP-tagged neurons in a single sample.

Additionally, a detailed set of confocal microscope acquisition parameters were developed to provide a high-resolution dataset that could be analyzed longitudinally in an experimental pipeline. The confocal microscope and imaging stage does not provide the capability of placing the cell culture plate in the exact same position across every imaging session. This means that in order to monitor cell-specific behaviors and migrations over time it is necessary to spatially align the high-resolution 30x images taken across several days. The agarose pegs were used as reference points from which tissue contraction and shifts in the (x,y) plane could be determined. However, it is not possible to capture the agarose pegs and the whole tissue center in a single

30x field of view. In order to obtain such images, an acquisition map was created, establishing a 4x4 grid of individual 30x images spanning the entire microtissue. Once each 30x image with a set z-depth was captured for each portion of the grid, the 16 images were 'stitched' together to create a larger image. The robust confocal imaging techniques and strategies described above were created for the purpose of longitudinally monitoring interactive and dynamic cellular behavior of three key neural cell types of interest.

Discussion

Immense efforts have been made to further our understanding of the complex neural environment and the effects of neurological diseases. The design of effective tools and neurotechnologies for use in these investigations has become of critical importance. Implantable electrode devices and advanced optical methods have developed as innovative modalities for interfacing with the brain. And although these impactful research tools provide valuable information regarding physiology and function, they lack a means of visualizing the cellular interactions between the technology and the biological neural environment in real time. Using virally mediated expression of fluorescent reporter proteins in three key cell types (neurons, astrocytes and microglia), we present an in vitro model for real time capture of dynamic cellular behavior in cultured three-dimensional primary cortical microtissues.

Three unique viral vectors targeting neurons, astrocytes, and microglia were investigated in this study. Successful AAV transduction methods were optimized for each cell type. The neuron-targeting AAV1.CamKII.eYFP vector was added to microtissues at DIV1 and a fluorescent expression curve was calculated which indicated stable fluorescence onset at 7 days post transduction (DPT7). Similarly, for the astrocyte-targeting AAV5.GFAP.eGFP viral vector, a DIV1 transduction resulted in stable fluorescence onset by DPT7. The microglia-targeting AAV1.CD69.h4MD(Gi).mCherry viral vector only displayed stable fluorescence at DPT17 following a DIV7 transduction. Based on this data, we demonstrate that AAV induced

fluorescence could be used for visualization of cell-specific dynamics in a three-dimensional neural microtissue.

With the objective of simultaneously visualizing the three cell types in real time, the fluorescence expression profiles for each virus were used to create a triple transduction protocol. The neuron-targeting AAV1.CamKII.eYFP vector was added to microtissues at DIV12 and a fluorescent expression curve was calculated and compared to the previous TDIV1 profile. As predicted the expression profile repeated at the shifted timeline. The TDIV12 method produced stable fluorescence onset at DPT7. Expression profiles, for the astrocyte-targeting AAV5.GFAP.eGFP viral vector were also assessed for the shifted timeline. The TDIV17 method produced stable fluorescence onset at DPT7. These results indicate that projected triple transduction times for each virus would allow for stable fluorescence onset by DIV24 in all three cell types.

The optical methods and imaging acquisition parameters optimized for longitudinal high-resolution imaging. The design, fabrication, and implementation of a specialized injection mold allowed for capture of high-resolution live images of the microtissue by minimizing the distance between the 30x confocal objective and the microfeature seated in the cell culture plate. The ability to acquire the 30x higher-resolution images dramatically increases the spatial resolution of the experimental pipeline.

In addition to the adjustments made on the physical constraints of the imaging platform, a unique spectral deconvolution protocol was developed to increase. As a part of the triple transduction protocol, deconvolution was necessary in order to visibly distinguish the spectrally close YFP and GFP tags in neurons and astrocytes respectively. Successful deconvolution of the YFP and GFP fluorophores was achieved in a sample co-transduced with neuron-targeting AAV1 and astrocyte-targeting AAV5. The ability to spectrally pull-apart and visibly differentiate the YFP and GFP fluorophores is an additional indication that simultaneous visualization of

multiple cell types in real-time is possible using the AAV-mediated fluorescence in the three-dimensional microtissue.

The final optical method optimization was the development and implementation of confocal acquisition parameters that ensure image alignment and tracking of cells over multiple imaging sessions across several days. Image 'stitching' of 16 separate 30x z-stack images was completed to create a single high-resolution image that included stationary features that could be used as (x,y) reference position. This ability to spatially orient the image across multiple imaging days will be essential in determining microtissue contractions and single cell migrations over time.

This model provides a methodology for potential longitudinal data collection of the neural microenvironment by tracking single cell dynamics. The application of fluorescent tagging via viral transduction in the three-dimensional neural microtissues offers new characterization of cellular interactions in real time. Although this work provides continuous visualization of an in vitro neural microenvironment under normal conditions, we predict that the platform could be implemented as a research tool for in vitro assessment against a variety of perturbations. Specifically, a next step goal of this research would be to monitor the dynamic inflammatory response to implantable electrodes by longitudinally tracking changes in morphology in real-time.

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