

OPTIMIZATION OF WHOLE BRAIN CLEARING TECHNIQUES FOR THE MOLECULAR  
INVESTIGATION OF ARC

by

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8/16/2017  
Date

## AUTHOR'S DISCLOSURE

A portion of this thesis has been previously submitted to The Honors College and the Department of Chemistry and Biochemistry at the University of Arizona as an undergraduate senior capstone. The author was mentored by Carol A. Barnes and Monica K. Chawla, both of whom provided immense guidance and support throughout the project. All funding for the project was provided by Carol A. Barnes.

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## Abstract

The immediate early gene *Arc* has been implicated in synaptic plasticity and has also been shown to be unique in its behavior-driven expression and localization. Thus, it has been used as a molecular marker for behavior-driven neuronal activity, especially in hippocampal and cortical neurons. Fluorescence *in situ* hybridization (FISH) for *Arc* mRNA reveal distinct compartmentalization of the transcript: *Arc* is found in the nucleus of neurons activated within the last few minutes, moves to the cytoplasm staining in neurons activated over ~25 minutes ago, and both intranuclear foci and cytoplasmic *Arc* mRNA is observed in neurons activated at both time points if the behavior at the two time points is identical. With the development of several tissue clearing techniques like CUBIC, CLARITY, and EDC-CLARITY, it is now possible to use *Arc* FISH to label whole brains in order to map out intact neural networks in response to behavior. Conventional *Arc* FISH utilized full length antisense *Arc* riboprobes (~3.1 kb) in 20 micron thin sections, however in cleared whole brains, the full length riboprobes may not fully penetrate the tissue. We sought to resolve the issue of tissue penetration with hybridization chain reaction (HCR), which uses DNA probes less than 150 bases in length. The DNA probes can also be amplified by using HCR fluorescence hairpins thus providing better tissue penetration and signal amplification. Male Fischer 344 rats were given maximal electroconvulsive shock (MECS) to induce *Arc* transcription in a high percentage of hippocampal and cortical neurons. The animals were sacrificed and the brains were extracted to be cleared by CUBIC or EDC-CLARITY. Utilizing HCR amplified FISH, *Arc*-positive cells were found in the dentate gyrus of the hippocampus and in the cerebral cortex.

## Introduction

Activity-regulated cytoskeleton-associated protein (*Arc*) is an immediate-early gene (IEG) that has been identified to be important for synaptic plasticity and has been shown to have an important role in learning and memory<sup>1,2</sup>. IEGs refer to a group of genes that are expressed transiently and rapidly in response to cellular stimuli; some other IEGs include *c-fos*, *zif268*, and *Homer 1A*<sup>1,3,4</sup>. *Arc* is expressed in response to excitatory, LTP-inducing stimuli including high-frequency stimulation and neuronal spiking, linking *Arc* to long term potentiation (LTP) and plasticity<sup>1,2,5,6</sup>.

By using *Arc* antisense oligodeoxynucleotides, that can sequester sense *Arc* mRNA to inhibit translation, it was shown that the reduction of *Arc* protein expression disrupted LTP maintenance and consolidation of long term memory (LTM) without affecting LTP induction nor short term memory<sup>5</sup>. *Arc* knockout (KO) mice also showed disrupted LTP. Specifically, early LTP was enhanced, while long term depression (LTD) was significantly reduced<sup>6</sup>. The mechanism for this phenomenon was said to be a reduction in activity-dependent AMPA receptor endocytosis thus allowing a larger pool of synaptic AMPA receptors to facilitate early LTP while at the same time dampening LTD<sup>6</sup>. Canonically, synaptic plasticity is the balance between AMPA receptors being placed on the synaptic membrane (exocytosis/LTP), and AMPA receptors being reinternalized (endocytosis/LTD), in which a synapse can be strengthened or weakened, respectively<sup>7</sup>. It was shown in Chowdhury et al. (2006), that *Arc* protein interacts with endocytosis proteins dynamin and endophilin in order to promote AMPA receptor reinternalization<sup>8</sup>. Thus, *Arc* plays an integral role in the stabilization of activity-dependent hippocampal plasticity<sup>5,8</sup>.

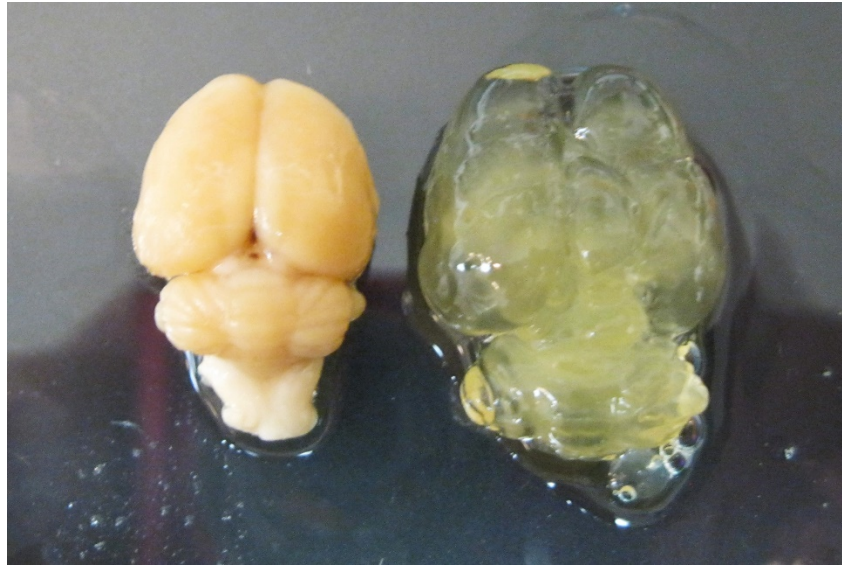
As an IEG, *Arc* has a unique profile of expression as shown by fluorescence *in situ* hybridization (FISH) of *Arc* transcripts in Guzowski et al. (1999)<sup>4</sup>. They found four distinct *Arc* staining profiles following exploratory behavior: cells with no staining, cells with intranuclear foci staining, cells with cytoplasmic-only staining, and cells with both intranuclear foci and cytoplasmic staining<sup>4</sup>. In behavioral studies, rats were allowed to explore an environment for 5 minutes, after which, they were either immediately sacrificed or returned to their cage for 25 minutes before being sacrificed<sup>4</sup>. Rats that were immediately sacrificed after exploration showed 45% of hippocampal CA1 region cells with nuclear foci staining as compared to nuclear foci staining in 8% of CA1 neurons of rats who remained in their cages without exploration<sup>4</sup>. Rats that were sacrificed after the 25 minute delay, showed predominantly cytoplasmic staining with nuclear foci staining falling to caged control levels<sup>4</sup>. In these delay rats, 44% of CA1 neurons showed cytoplasmic staining. In another behavioral study, rats were allowed to explore environment A for 5 minutes before returning to their cages for 20 minutes<sup>4</sup>. The rats were then either returned to environment A (A/A group) or to different environment B (A/B group). A/A group rats showed 90% of the CA1 neurons activated by the first exploration being activated again in the second exploration as visualized by cells with both foci and cytoplasmic labeling<sup>4</sup>. The rats from the A/B group showed 22% cytoplasmic-only, 23% nuclear foci-only, and 16% foci and cytoplasmic staining of CA1 neurons<sup>4</sup>. This suggested that *Arc* was expressed in specific cellular compartments under a specific time course of localization in neurons in a task-specific manner.

In order to investigate the intranuclear foci as sites of RNA transcription, Guzowski et al. (1999), used maximal electroconvulsive shock (MECS) to induce global transcription, especially of IEGS, in rats<sup>4</sup>. *Arc* was rapidly transcribed and localized to nuclear foci in 95% of CA1

neurons within a few minutes after having received a MECS<sup>4</sup>. By 15 minutes, intranuclear staining had begun to decrease while cytoplasmic staining started to appear<sup>4</sup>. By 30 minutes, *Arc* labeling had been mainly localized to the cytoplasm with intranuclear labeling returning to cage control levels<sup>4</sup>. Due to rapid transport to dendrites for local translation near the synapses, by 60 minutes, *Arc* mRNA staining had disappeared from the cytoplasm<sup>4</sup>. These MECS studies lined up with the expression profile of *Arc* that was found in the behavior studies. With its important role in modulation of synaptic plasticity and its unique pattern of expression, a novel method of tracking neuronal activation at a subcellular resolution with a temporal specificity was developed by utilizing *Arc* FISH. This led to the development of cellular compartment analysis of temporal activity by fluorescence *in situ* hybridization (catFISH), where the neuronal activation history of a particular spatial task could be tracked by the cellular localization of *Arc* mRNA<sup>4</sup>. If *Arc* was found in the nucleus, then that neuron was activated within the last 5 minutes. If *Arc* was found in the cytoplasm, then that neuron was activated ~25 minutes ago. And if *Arc* was found in both the nucleus and the cytoplasm after two identical behavioral experiences, then that same neuron was activated at both time points.

The method of catFISH involves RNA riboprobes designed to be complimentary to the *Arc* mRNA. The riboprobes bind and hybridize to the transcript. Digoxigenin is conjugated to the riboprobe, which allows for the anti-Dig antibody to bind to the digoxigenin-ribo-*Arc* complex<sup>4</sup>. The antibody is conjugated to a horseradish peroxidase enzyme, which catalyzes the localization of tyramide fluorescence signal to the complex<sup>4</sup>. Neurons activated by spatial memory tasks now containing the fluorescently tagged *Arc*-ribo-*Arc* complex can be visualized using confocal microscopy. The catFISH method is typically done in brain sections no thicker

than 20  $\mu\text{m}$ , where each section is labeled and then imaged. This presents a meticulous and time-intensive challenge if an entire brain were to be digitally reconstructed.



**Figure 1:** **Left)** A fixed whole rat brain prior to the CUBIC protocol. **Right)** A fixed whole rat brain following 6-7 weeks of CUBIC reagent-1 incubation at 37°C with fresh reagent-1 being replaced every 2 weeks. The cleared brain shows preserved structure and morphology with only some swelling.

However, with the recent development of several tissue clearing techniques such as: CLARITY<sup>9</sup>, passive-CLARITY<sup>10</sup>, iDISCO<sup>11</sup>, and CUBIC<sup>12</sup>, ClearT<sup>13</sup>, SeeDB<sup>14</sup> it is now possible to do whole brain imaging. All of these methods clear tissue in a variety of ways, but in general, they remove lipids from the tissue using a detergent while maintaining the macromolecular structures intact. Lipids are prone to light-scattering, so by removing lipids from the tissue, optical penetration can increase dramatically<sup>12</sup>. This could potentially alleviate the tissue sectioning and digital reconstruction of the catFISH sections, since clear and transparent brains could possibly be imaged intact.

## Background

This project initially utilized the CUBIC (clear, unobstructed brain imaging cocktails) technique, in which whole rat brains fixed in 4% paraformaldehyde were incubated in ScaleCUBIC-1 (reagent-1) for 6-7 weeks at 37°C until the brains were cleared<sup>12</sup> (**Figure 1**). CUBIC was favored among the other tissue clearing techniques due to its much simpler protocol (compared to CLARITY) which utilized non-toxic water-soluble reagents. Previous comparisons between CUBIC and CLARITY also demonstrated that CUBIC was faster at clearing the tissue, while also causing less tissue swelling and damage. It became clear, however, that CUBIC was optimized for protein labeling and not nucleic acid labeling. With the development of EDC-CLARITY<sup>15</sup> (will now be referred to as CLARITY), which is passive-CLARITY<sup>10</sup> with an additional RNA fixation step, the project shifted to this tissue clearing method for its added RNA preservation. Brains cleared by CLARITY underwent fixation via a transcardial perfusion of acrylamide hydrogel monomer solution. The brains had to be degassed to allow for the polymerization of the hydrogel to occur (oxygen gas impedes the polymerization), which crosslinked proteins and nucleic acids to the hydrogel. Whole rat brains took at least 4 months to clear in the CLARITY clearing solution (**Figure 2**).

The issue with combining catFISH with brain clearing techniques is that traditional catFISH methods were developed on brain sections no thicker than 20  $\mu\text{m}$ , whereas whole brains are a few centimeters thick. The riboprobes used are full-length *Arc* cRNA that are 3.1 kilobases (kb) long<sup>1,4</sup>. This presents a fundamental challenge for probe penetration – as the probes have to penetrate the tissue completely to label *Arc* throughout the whole brain. These catFISH riboprobes are made of RNA, which are not only expensive reagents, but notoriously unstable and prone to degradation<sup>16</sup>. As part of developing the protocol to carry out catFISH on cleared

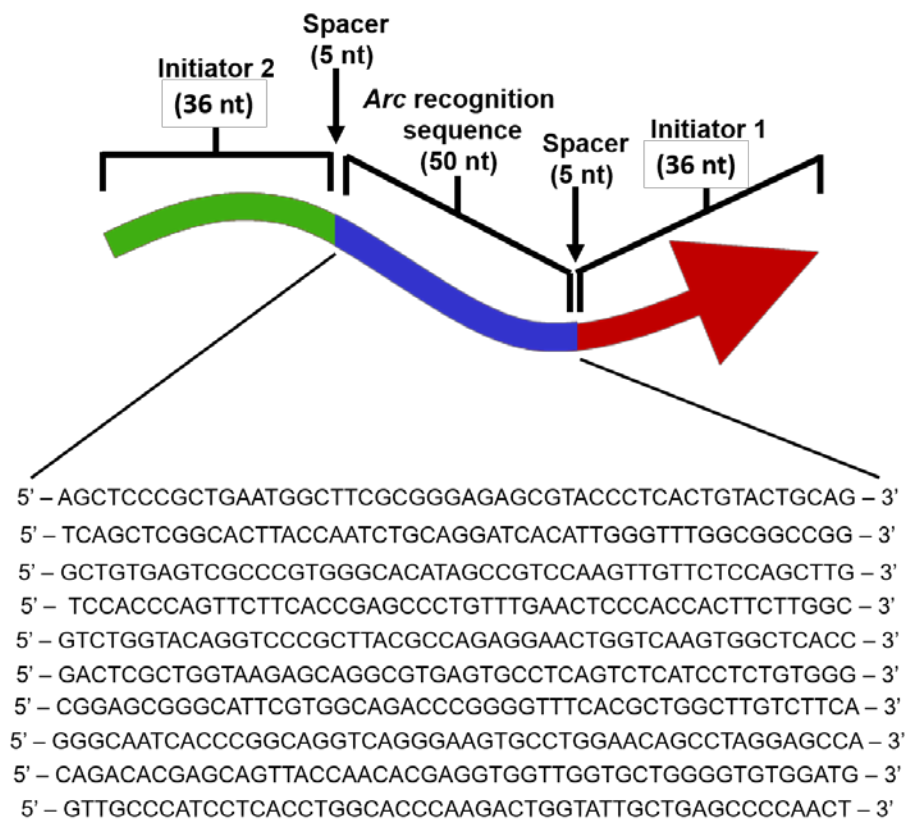
brains, probe design is paramount to enable the combination of both technologies. Many considerations have to be taken into account when designing probes: probe length and methods to boost signal-to-background. Probes have to be long enough to specifically hybridize to the *Arc* mRNA sequence, but not too long that it cannot penetrate the tissue or cause unfavorable hybridization energetics and kinetics. In order to boost signal-to-background, hybridization conditions, washing protocols, and signal amplification all need to be considered.



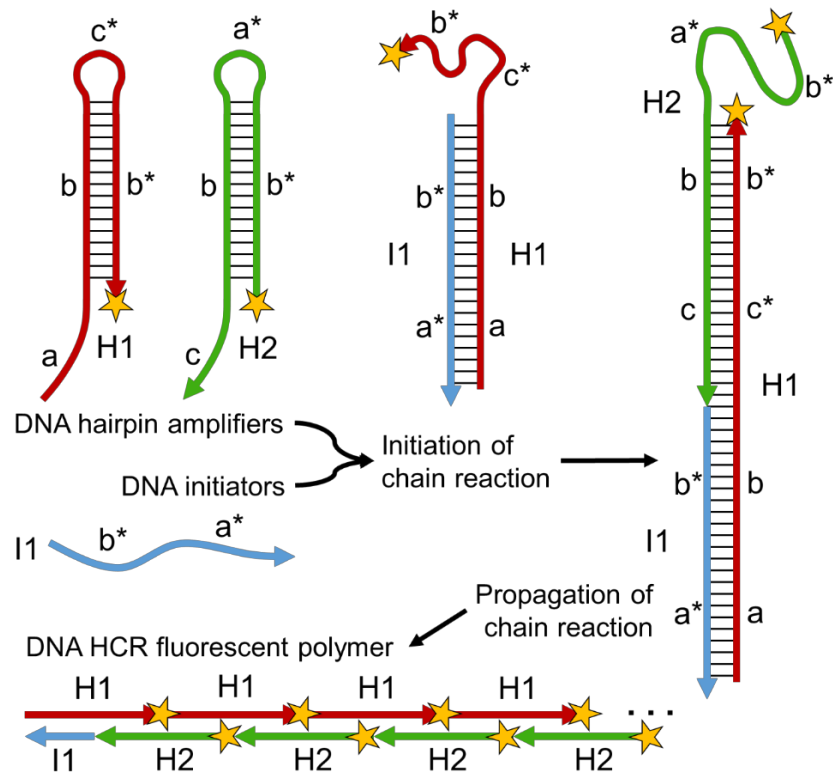
**Figure 2:** Whole rat brain after transcardial perfusion with a cold hydrogel monomer solution. **Left)** After passive CLARITY clearing for ~4 months. Note the transparency of this brain with minimum swelling. **Right)** A whole rat brain before the passive CLARITY protocol.

In the search for increased probe penetration and signal amplification techniques, a variation of *in situ* hybridization utilizing DNA probes instead of RNA probes called hybridization chain reaction (HCR) was found. This technique used DNA initiator probes (**Figure 3**), which are only 132 nucleotides (nt) long; being significantly shorter than the 3.1 kb length of the traditional catFISH riboprobes, tissue penetration should be greatly increased. After hybridizing to the target mRNA (**Figure 5a**), the initiator probes provide a scaffold for DNA HCR hairpin amplifiers containing fluorophores to self-polymerize from (**Figure 4 and**

**5b)**<sup>16</sup>. As more and more hairpins polymerize onto the growing chain, fluorescence signal is amplified and increases the signal to background ratio (**Figure 4 and 5b**). These hairpins are only 72 nt long and should also have improved tissue penetration<sup>16</sup>. HCR was designed so that the two initiator sequences flanking the 10 sets of variable *Arc* recognition sequences (**Figure 3**) would initiate the hairpins to unfold and hybridize to the flanking initiators<sup>16</sup>. This is the case since one hairpin is half complementary to the initiator and half complementary the other hairpin allowing for the two types of hairpins to self-assemble off of each other (**Figure 4**). MECS was also used in order to induce expression of *Arc* mRNA in a high percentage (90% in CA1<sup>4</sup>) of neurons to enable more possible targets for the HCR probes to hybridize to.



**Figure 3:** Composition of a single DNA initiator probe. Total length of each probe is 132 deoxynucleotides (nt) long with two 36 nt long initiator segments, two 5 nt spacers, and a 50 nt long *Arc* mRNA recognition sequence containing any of the 10 sequences pictured, each sequence corresponding to the 10 different probe sets. All strands are oriented 5' to 3'.



**Figure 4:** Schematic of the hybridization of DNA hairpin amplifiers to the DNA initiator probes. The design of the two hairpins and the initiator segments promote the initiation and chain reaction polymerization of the HCR fluorescent polymers. Asterisks (\*) denoted the complementary strand. Red strands represent hairpin 1 (H1), green strands represent hairpin 2 (H2), blue strands represent initiator 1 (I1), and yellow stars represent the conjugated fluorophore Alexa Fluor 647.

Beyond pairing the neuronal activity tracking system of catFISH utilizing HCR with the brain clearing techniques of CUBIC or CLARITY, the ability to image the labeled whole brain still presents an issue due to the working distance limitations of a conventional confocal microscope. The Deisseroth lab (CLARITY) and the Ueda lab (CUBIC) have successfully imaged their cleared brains using selective-plane illumination microscopy (SPIM) and light-sheet fluorescence microscopy (LSFM), respectively. We do not have access to such microscopy equipment, but in collaboration with the University of Arizona Optical Science's Dr. Rongguang Liang, a High speed, High resolution, Long working distance, Large field of view Confocal

Fluorescence Microscope ( $H^2L^2$ -CFM) is being developed. With the long working distance and large field of view of this new microscope, we will be able to potentially utilize clearing techniques and catFISH in order to map out behaviorally-induced neural networks in whole brains. In the meantime, a combination of LagoX luminescence and fluorescence camera imaging and advanced intravital multi-photon microscopy will be used to optimize the HCR protocol for cleared rat brains. Once this tri-pronged toolkit can be established, many questions involving whole brain networks can start to be answered in a more feasible time frame.

## Methods

### *Brain Preparation*

Male Fischer 344 rats between 7 and 24 months were used in these experiments. To induce global transcription in the brain, these rats were given maximal electroconvulsive shock (MECS). Saline-soaked ear clips were attached to both ears of the animal and then, an electroshock of 85 mA stimulus of 0.5 millisecond square-wave pulses at 100 Hz for 1 second was given using an UGO Basile ECT unit<sup>3</sup>. Under the CUBIC protocol, the animals were allowed to rest in a warm incubator for 15 minutes before receiving a second MECS. The rats then rested for another 5 minutes in the incubator. They were then placed in an isoflurane chamber until rendered unconscious, which was followed very quickly by decapitation by rodent guillotine and quick brain extraction and freezing in isopentane cooled in dry ice/ethanol slurry<sup>4</sup>. The frozen brains were then fixed in 4% paraformaldehyde for 2 days. Under the CLARITY protocol, the animals were allowed to rest in a warm incubator for 15 minutes after the first MECS. They would then receive a second MECS, followed by an intraperitoneal injection of 0.35 mL of Euthasol euthanasia solution. Once the animal was deemed fully anesthetized via the toe pinch-response method, it was transcardially perfused with cold 0.01 M phosphate buffered saline (PBS; pH 7.4) at 40 mL/min until the PBS ran clear and then perfused with 20 mL of cold 1% acrylamide hydrogel monomer (HM) solution at 10 mL/min<sup>10,17</sup>. These brains were then submerged in additional HM solution at 4°C for 3 to 5 days.

### *CUBIC Tissue Clearing*

As adapted from Susaki et al. (2014), the ScaleCUBIC-1 (reagent-1) solution was prepared by combining N,N,N',N'-tetrakis(2-hydroxypropyl)ethylenediamine (25% w/v), urea (25% w/v), and Triton X-100 (15% w/v) in nanopure water<sup>12</sup>. The paraformaldehyde-fixed

brains incubated in reagent-1 in a shaker oven at 37°C for 6-7 weeks. Every 2 weeks, fresh reagent-1 was replaced to help facilitate faster tissue clearing. Following tissue clearing, the brains were hemisected and liberated from the cerebellum to improve probe penetration while also reducing the volume to be imaged by a microscope. The right hemispheres were kept for future comparative studies, while the left hemispheres were labeled for *Arc* with fluorescence *in situ* hybridization with hybridization chain reaction (HCR) amplification.

### ***EDC-CLARITY Tissue Clearing***

As adapted from Tomer et al. (2014), the 1% acrylamide HM solution was prepared on ice by combining 40% acrylamide, 2% bis-acrylamide, VA-004 thermally triggered initiators, 16% paraformaldehyde and 0.1 M PBS in nanopure water to reach final concentrations of 1% acrylamide, 0.0125% bis-acrylamide, 0.25% w/v VA-004 initiator, 4% paraformaldehyde, and 0.01 M PBS<sup>10</sup>. After sitting in the HM solution for 3-5 days at 4°C, the brains were degassed under a small vacuum pressure to remove oxygen gas, which inhibits the polymerization of the acrylamide hydrogel. The degassed brains in HM solution were incubated at 37°C for 5 hours to allow the thermally triggered initiator to initiate the polymerization of the acrylamide monomers to form a hydrogel mesh which crosslinks and incorporates proteins and nucleic acids into the mesh. Then the brains were transferred to 50 mL of CLARITY clearing solution overnight.

The hydrogel-embedded brain underwent an RNA fixation step with 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC), which links the 5' phosphate group of RNA to the amine group of a protein. This allowed for RNA species to be better retained by linking the RNA molecules to the proteins already crosslinked to the other biomolecules and the hydrogel mesh<sup>15,18</sup>. The EDC fixation solution was prepared by combining 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (0.1 M final concentration), 5-ethylthio-1H-tetrazole (0.1 M final

concentration), and methylimidazole buffer (0.8% final concentration) in nanopure water, and then the solution was buffered to a pH of 8.5 with sodium hydroxide. The brains were first washed in methylimidazole buffer (0.8% in nanopure water) alone for 1 hour at 37°C in a shaker oven. Then they were allowed to incubate in the EDC solution overnight at 37°C.

The brains were then sectioned into 1 mm coronal sections using a brain matrix, which allowed for faster tissue clearing times and offered a better view of the hippocampus for targeted imaging. CLARITY clearing solution was prepared by combining sodium dodecyl sulfate (SDS) and boric acid in nanopure water to reach final concentrations of 4% w/v and 0.2 M, respectively<sup>9,10</sup>. The clearing solution was adjusted to a pH of 8.5 with sodium hydroxide. These brain sections were incubated at 37°C in the CLARITY clearing solution for 2-3 weeks to allow for passive diffusion of lipids out of the brain facilitated by SDS detergent. Every 2 days, the clearing solution was replaced to increase the speed of delipidation. Once cleared, the brain sections were washed in 50 mL of PBST (0.1% Triton X-100 in 0.01 M PBS) overnight at 37°C on a shaker. The PBST was replaced and the brain sections underwent another overnight wash. The PBST washes were intended to wash the SDS micelles out of the tissue to prevent precipitation later in mounting solution.

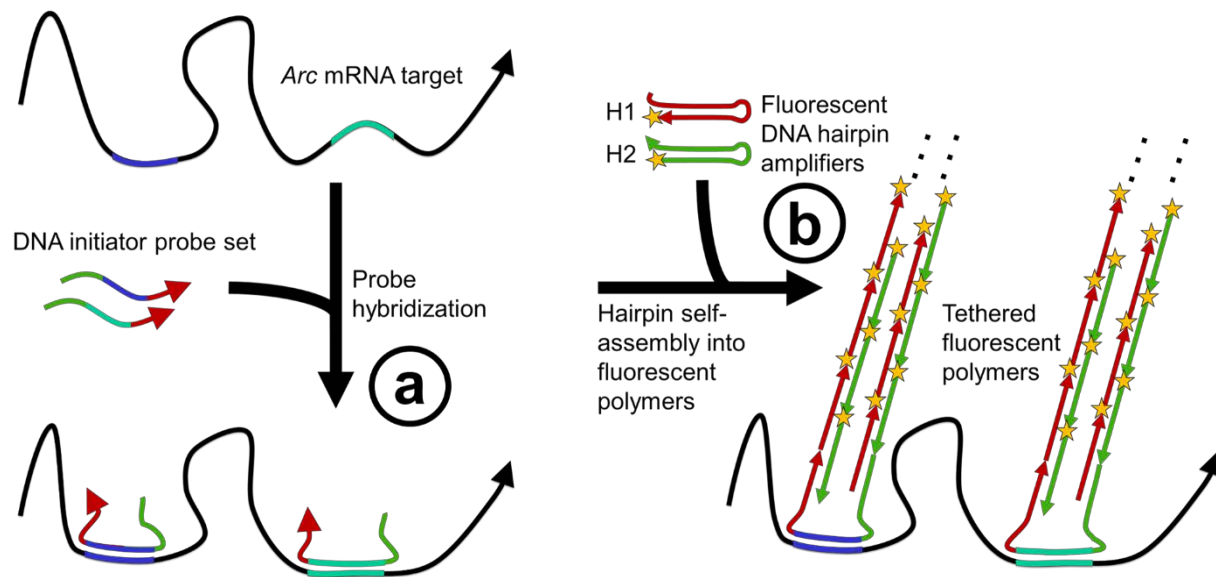
### ***Hybridization Chain Reaction Coupled Fluorescence in situ Hybridization***

The hybridization chain reaction (HCR) protocol was adapted from Choi et al. (2014)<sup>16</sup> and Sylwestrak et al. (2016)<sup>15</sup>. Brains cleared by either CUBIC or CLARITY were submitted to an overnight wash in PBST at room temperature to remove excess clearing reagents. The two hemisected CUBIC brains pre-incubated for 1 hour in 2 mL of pre-made hybridization buffer (Molecular Instruments, California Institute of Technology) at 37°C, while the 1 mm CLARITY brain sections pre-incubated for 1 hour in 1 mL of freshly prepared hybridization buffer at 37°C.

The pre-made hybridization buffer from Molecular Instruments contained 50% formamide, 5× saline sodium citrate (SSC), 9 mM citric acid, 0.1% Tween 20, 50 µg/mL heparin, 1x Denhardt's solution, and 10% dextran sulfate. Freshly made hybridization buffer was prepared according to the optimized concentrations from Sylwestrak et al. (2016), which was comprised of 40% formamide, 2×SSC, 0.5 mg/mL yeast tRNA, and 10% dextran sulfate.

While the brains were pre-incubating for 1 hour in hybridization buffer, the probe solution was prepared. There were 10 possible DNA initiator probe sets designed by Molecular Instruments (**Figure 3**) to target various 50 nt sequences of *Rattus norvegicus Arc* (**Supplemental Figure 1**). The two hemisected CUBIC brains were either probed with 5 random, non-duplicative probe sets or were probed with 7 random, non-duplicative probe sets. In both of these conditions, each probe set was diluted in hybridization buffer to a final concentration of 1 nM in a total volume of 1 mL of probe solution. Both hemisected CUBIC brains incubated in their respective 1 mL of probe solution in a shaker oven at 37°C overnight (~14 hours) in order to hybridize to *Arc* mRNA targets. The CLARITY brain sections were probed with different combinations of 5 randomly selected probe sets diluted with hybridization buffer to a final concentration of 1 nM in a total volume of 0.5 mL of probe solution. These CLARITY sections incubated in 0.5 mL of their respective probe solution at 37°C in a shaker oven overnight (~20 hours) in order to hybridize to *Arc* mRNA targets.

After the hybridization phase of HCR (**Figure 5a**), the CUBIC brains were subjected to 5×1 hour washes in probe wash buffer (Molecular Instruments) to wash out excess and unhybridized DNA probes. CLARITY brains were subjected to 3×1 hour washes in 40% formamide and 2×SSC, followed by 2×1 hour washes in 5×SSC and 0.1% Tween 20 (5×SSCT).



**Figure 5:** Fluorescence *in situ* hybridization of *Arc* mRNA with HCR amplification. (a) Sets of DNA probes hybridize to target sequences (colored purple and teal to differentiate different sets) on *Arc* mRNA. (b) Fluorescent DNA hairpins H1 and H2 self-polymerize off hybridized probes forming tethered fluorescent polymers. Yellow stars represent the fluorophore Alexa Fluor 647.

Following the washes, all brains were taken through the hairpin amplification phase of HCR (**Figure 5b**). The two CUBIC brains pre-incubated for 1 hour at room temperature in 2 mL of amplification buffer (5×SSC, 0.1% Tween 20, and 10% dextran sulfate), while the CLARITY sections pre-incubated in 1 mL of amplification buffer for 1 hour at room temperature. While the brains were incubating, the hairpin solutions were prepared in the dark to prevent photobleaching of the fluorescent hairpins conjugated with Alexa Fluor 647. For each CUBIC brain, 40  $\mu$ L of hairpin 1 (H1) and 40  $\mu$ L of hairpin 2 (H2) were pipetted into separate 0.5 mL microcentrifuge tubes. For each CLARITY brain, 20  $\mu$ L of H1 and 20  $\mu$ L H2 were pipetted into separate 0.5 mL microcentrifuge tubes. All of the tubes of either H1 or H2 were snapcooled by being heated to 95°C for 90 seconds followed by being cooled to room temperature for 30 minutes, which causes the hairpins to unfold. For each CUBIC brain, 40  $\mu$ L of H1, 40  $\mu$ L of H2,

and 920  $\mu\text{L}$  of amplification buffer were combined resulting in a final concentration of 120 nM for each hairpin in a total volume of 1 mL. For each CLARITY brain, 20  $\mu\text{L}$  of H1, 20  $\mu\text{L}$  of H2, and 460  $\mu\text{L}$  of amplification buffer were combined resulting in a final concentration of 120 nM for each hairpin in a total volume of 0.5 mL. All brains were allowed to incubate in their respective hairpin solution overnight (10 hours for CUBIC and 20 hours for CLARITY) at room temperature to allow for hairpin self-polymerization off of the *Arc* mRNA-hybridized DNA initiator probes (**Figure 5b**). All brain-containing tubes were covered in foil in order to prevent photobleaching. To wash out excess hairpins, all brains were subjected to 5 $\times$ 1 hour washes in 5 $\times$ SSCT at room temperature.

### ***Imaging of Fluorescent Labeled Arc mRNA***

Tissue transparency was lost during the HCR process, so prior to imaging, transparency was achieved again by incubating the brains in their respective clearing solutions overnight at 37°C. CLARITY cleared brains had an additional overnight wash in 0.2 M boric acid buffer adjusted to pH 8.5 at 37°C to wash out the SDS from the CLARITY clearing solution. All brains were mounted to petri dishes with 1% agarose gel by allowing the agarose to cool and solidify. Right before the point of solidification, the brains were placed on the agarose with additional drops of warm agarose added to secure the brains to the dish. Once the gel was fully solid, the rest of the dish was filled with 85% glycerol to match the refractive index of the cleared tissue to decrease optical scattering.

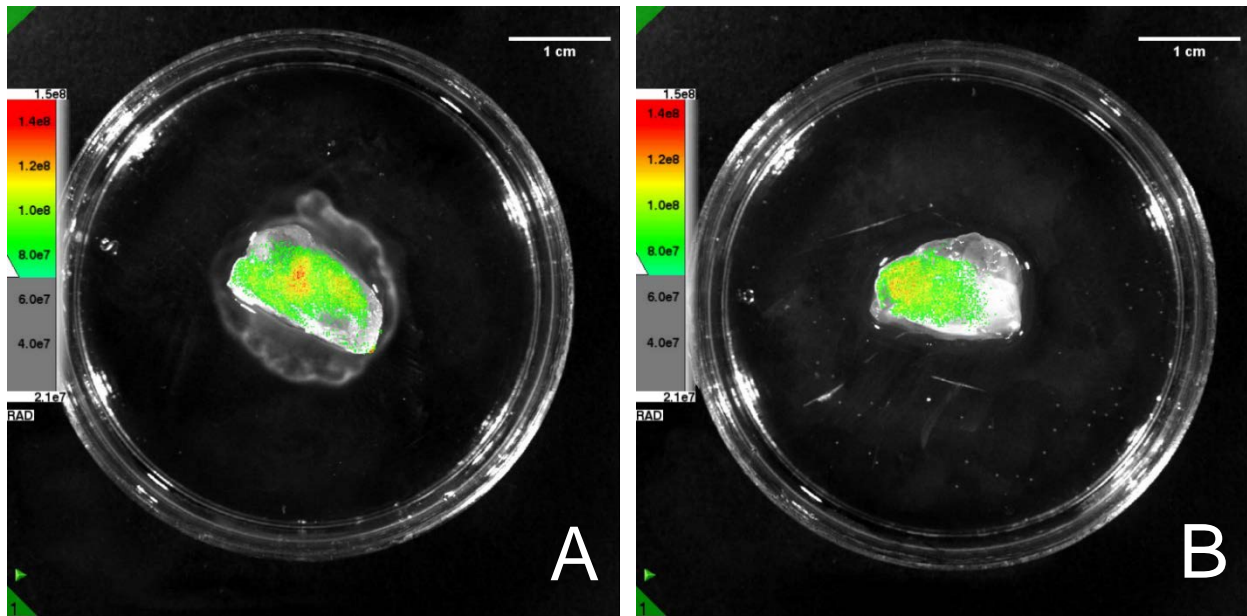
Both of the CUBIC hemisected brains were taken to be imaged on the LagoX luminescence and fluorescence camera, which can image fluorescence in animals *in vivo*. This camera allowed for imaging of the hemisected brains, which is a volume too large to be imaged on a typical confocal microscope (**Figure 6**). However, this LagoX camera did not provide

cellular resolution of the *Arc* labeling. The CUBIC brain was unmounted from the dish, and a 2 mm coronal section slab was cut out of the middle of the brain using a brain matrix to access the deepest tissue that the HCR probes had to penetrate to. This 2 mm slab was remounted to a petri dish and imaged on an advanced intravital multi-photon microscope (2-photon microscope). Images from the 2-photon microscope were processed and rendered in three-dimensions using ImageJ software (**Figures 7 and 8**).

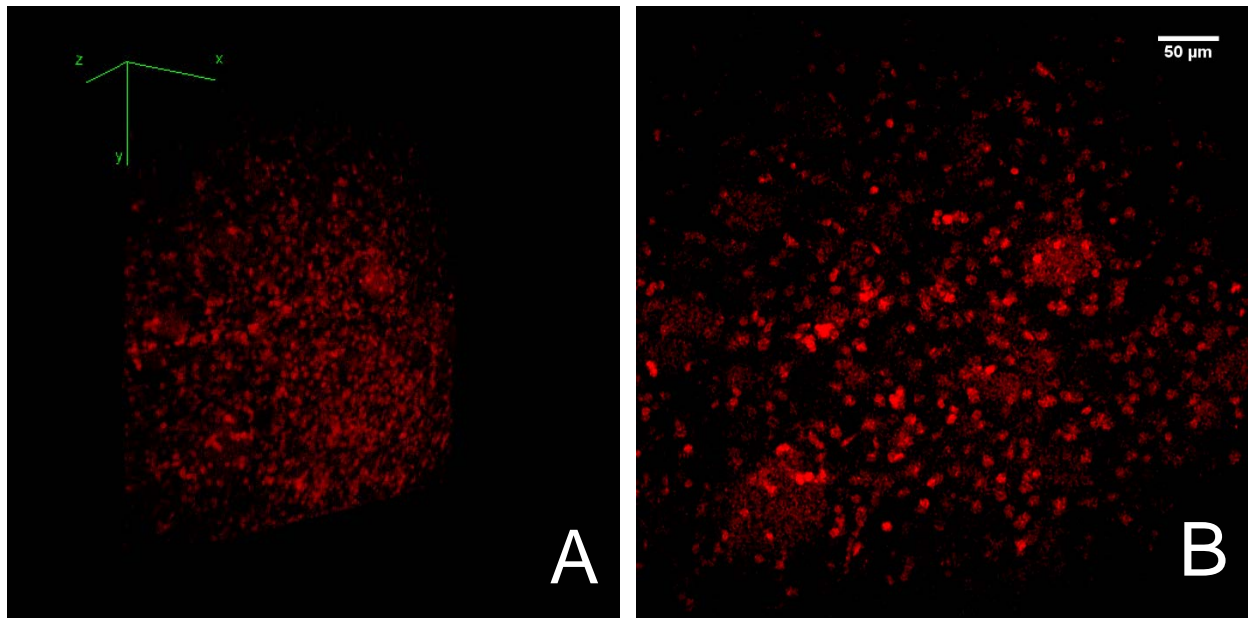
Once mounted in agarose and submerged in glycerol, the 1 mm CLARITY sections were also taken to be imaged on the 2-photon microscope. The images collected from this microscope were processed and rendered in three-dimensions using both ImageJ and FluoRender softwares (**Figures 9 and 10**).

## Results

Due to the current inability to image all the way through brains without SPIM or LSFM microscopes, the 5 probe set and 7 probe set CUBIC cleared half-brains were taken to be imaged on the LagoX luminescence and fluorescence camera. This camera has the capability to image fluorescence from within animals *in vivo* (rats and mice) and thus, be able to also image the hemisected brains. The image of the 5 probe set half-brain showed Alexa Fluor 647 fluorescence radiance intensities between  $8.0 \times 10^7$  and  $1.0 \times 10^8$  Radiance [ $\text{W}\cdot\text{sr}^{-1}\cdot\text{m}^{-2}$ ] throughout most of the half-brain, while the 7 probe set half-brain only showed a similar radiance range for about two-thirds of the half-brain (**Figure 6A and 6B**). The 5 probe set half-brain also showed more Alexa Fluor 647 fluorescence between  $1.0 \times 10^8$  and  $1.8 \times 10^8$  Radiance [ $\text{W}\cdot\text{sr}^{-1}\cdot\text{m}^{-2}$ ] (**Figure 6A and 6B**).



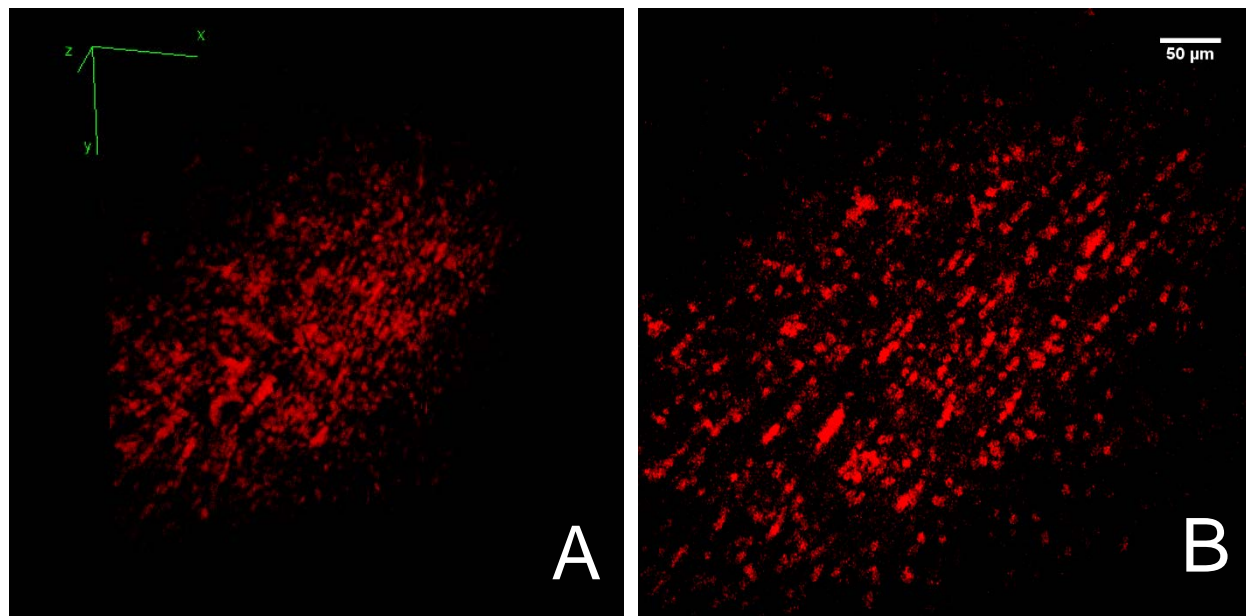
**Figure 6:** Images taken on the LagoX luminescence and fluorescence camera. The heatmap shows regions of red having the highest radiance [ $\text{W}\cdot\text{sr}^{-1}\cdot\text{m}^{-2}$ ] intensity corresponding to more Alexa Fluor 647 fluorescence and therefore more *Arc* labeling. **(A)** Left hemisphere of a CUBIC cleared rat brain treated with 5 randomly selected sets of DNA probes. Dorsal side up, rostral to the SE corner. **(B)** Left hemisphere of a CUBIC cleared rat brain treated with 7 randomly selected sets of DNA probes. Ventral side up, rostral to the west.



**Figure 7:** Images taken on an advanced intravital multi-photon microscope with a 20X objective of a 2 mm slab cut out from the CUBIC cleared hemisected rat brain treated with 5 DNA probe sets. **(A)** The three-dimensional rendering of 41 optical sections taken at 5  $\mu\text{m}$  steps totaling 200  $\mu\text{m}$  deep into the tissue. Video of a rotation of the rendering can be found in **Supplemental Video 1**. **(B)** A single section taken from the stack of 41 optical sections. *Arc* transcripts were fluorescently labeled with HCR probes and hairpins conjugated with Alexa Fluor 647. *Arc* labeling is seen in red.

The 5 probe set half-brain was then taken to be imaged on an advanced intravital multi-photon microscope, but the sample was too thick to image on the microscope, which has a maximal working distance of only a few millimeters. Resulting images only captured crude surface morphology, so the half-brain was resectioned. Using a rat brain matrix, a 2 mm coronal section was cut out of the half-brain approximately midway between the most rostral and most caudal portion of the half-brain. This 2 mm slab was then imaged on the 2-photon microscope using a 20X objective, which resulted in a three-dimensional rendering of a stack of 41 optical sections through the slab taken in 5  $\mu\text{m}$  steps totaling a total tissue depth of 200  $\mu\text{m}$  (**Figure 7A**), a video of the 41 section stack rotating about the y-axis (**Supplemental Video 1**), and a single optical section pulled out of the stack (**Figure 7B**). Another 3D rendering of a stack of 21

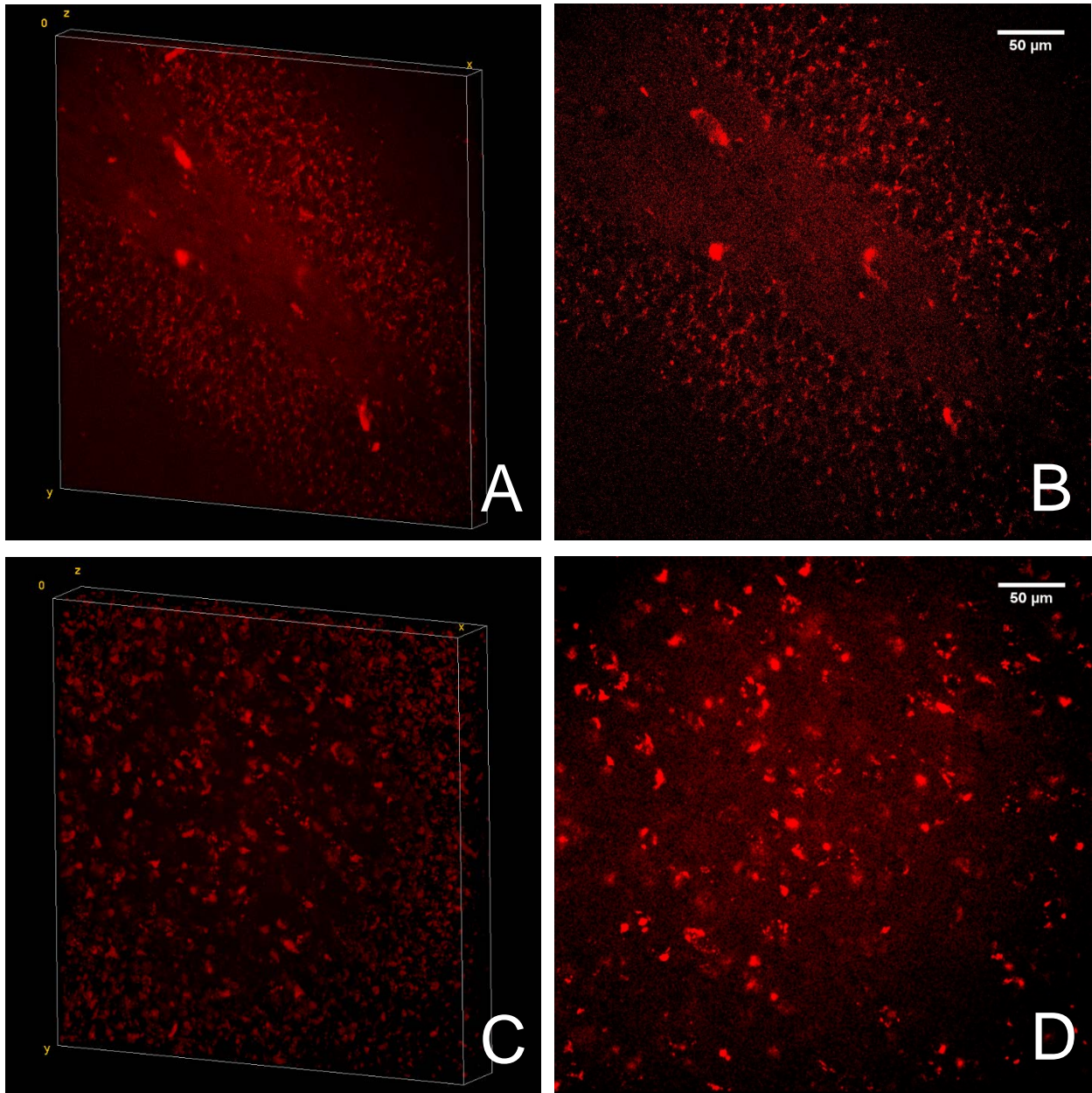
optical sections through a different region of the slab was taken in 5  $\mu\text{m}$  steps to a tissue depth of 100  $\mu\text{m}$  (**Figure 8A**). A video of the 21 section stack rotating about the y-axis (**Supplemental Video 2**) and also a single optical section pulled out of the stack (**Figure 8B**) resulted from this second stack. All of these images showed Alexa Fluor 647 labelling of *Arc* transcripts.



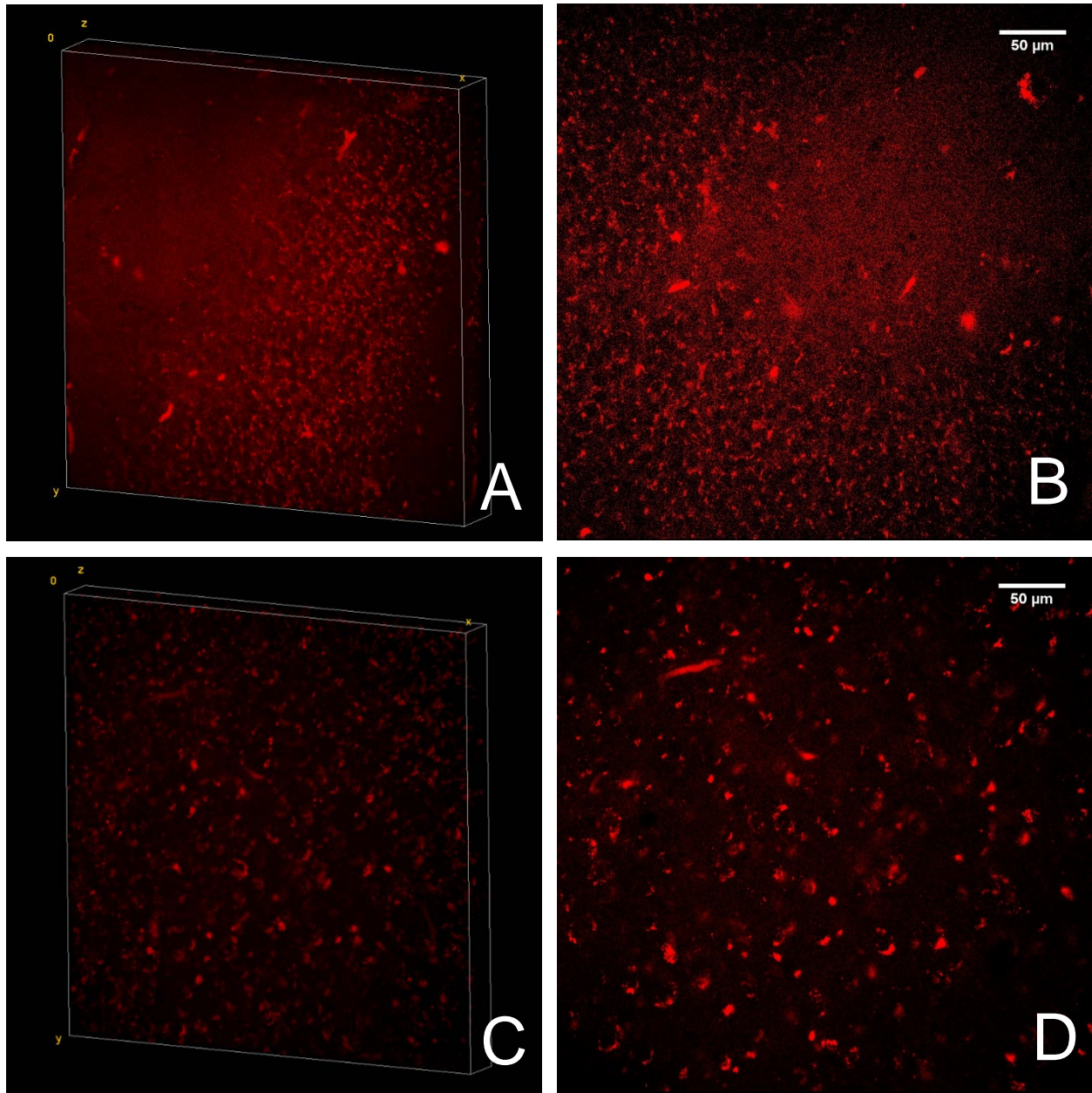
**Figure 8:** Images taken on an advanced intravital multi-photon microscope with a 20X objective of a 2 mm slab cut out from the CUBIC cleared hemisected rat brain treated with 5 DNA probe sets. **(A)** The three-dimensional rendering of 21 optical sections taken in 5  $\mu\text{m}$  steps totaling 100  $\mu\text{m}$  deep into the tissue. Video of a rotation of the rendering can be found in **Supplemental Video 2**. **(B)** A single section taken from the stack of 21 optical sections. *Arc* transcripts were fluorescently labeled with HCR probes and hairpins conjugated with Alexa Fluor 647. *Arc* labeling is seen in red.

With the development of EDC-CLARITY and its added retention of nucleic acids like RNA, brains were cleared by CLARITY. It was also evident from the CUBIC experiments, that once a brain had been cleared, it was difficult to find orientation within the tissue, due to its transparency. Brains that underwent CLARITY were sectioned to 1 mm before the tissue clearing procedures in the attempt to have better tissue orientation. A CLARITY brain section was then labeled using HCR and 5 randomly selected probe sets (**Figure 9**). With the ability to

be oriented in the tissue during 2-photon microscopy, images were taken from the tip of the dentate gyrus of the hippocampal region (**Figure 9A and 9B**). A three-dimensional rendering of 75 optical sections taken every 3  $\mu\text{m}$  to a tissue depth of 222  $\mu\text{m}$  (**Figure 9A and Supplemental Video 3**) of this dentate tip was taken. One optical slice was pulled aside to demonstrate the *Arc* labeling in the upper and lower blade as well as the hilus of the dentate gyrus (**Figure 9B**). Images were also taken from the cortex, which produced a three-dimensional rendering of 141 optical sections taken at 5  $\mu\text{m}$  steps to a tissue depth of 700  $\mu\text{m}$  (**Figure 9C and Supplemental Video 4**). An optical slice pulled out of this stack demonstrated what appeared to be cytoplasmic staining (labeling that surrounds the nucleus) of the neuron (**Figure 9D**). Another CLARITY brain section was labeled with the other 5 remaining probes not used in Figure 9 (**Figure 10**). Images taken at the tip of the dentate gyrus produced a rendering of 134 optical sections taken at 3  $\mu\text{m}$  increments for a total tissue depth of 399  $\mu\text{m}$  (**Figure 10A and Supplemental Video 5**). One optical section pulled out of the stack showed staining in upper and lower blades and hilus of the dentate (**Figure 10B**). The parietal cortex of this brain section also showed *Arc* staining, producing a rendering of 101 optical sections taken at 5  $\mu\text{m}$  steps for a depth of 500  $\mu\text{m}$  (**Figure 10C and Supplemental Video 6**). The optical section pulled out of this stack also displayed some cytoplasmic staining (**Figure 10D**).



**Figure 9:** Images taken on an advanced intravital multi-photon microscope with a 20X objective of a 1 mm coronal rat brain section cleared by CLARITY and treated with 5 random DNA probe sets. **(A-B)** These images were taken of the tip of the dentate gyrus. **(A)** The three-dimensional rendering of 75 optical sections taken in 3  $\mu\text{m}$  steps totaling 222  $\mu\text{m}$  deep into the tissue. Video of a rotation of the rendering can be found in **Supplemental Video 3**. **(B)** A single section taken from the stack of 75 optical sections. **(C-D)** These images were taken within the region of the cortex. **(C)** The three-dimensional rendering of 141 optical sections taken in 5  $\mu\text{m}$  steps totaling 700  $\mu\text{m}$  deep into the tissue. Video of a rotation of the three-dimensional rendering can be found in **Supplemental Video 4**. **(D)** A single section taken from the stack of 141 optical sections. *Arc* transcripts were fluorescently labeled with HCR probes and hairpins conjugated with Alexa Fluor 647. *Arc* labeling is seen in red.



**Figure 10:** Images taken on an advanced intravital multi-photon microscope with a 20X objective of a 1 mm coronal rat brain section cleared by CLARITY and treated with the other 5 DNA probe sets from the 10 possible sets. **(A-B)** These images were taken of the tip of the dentate gyrus. **(A)** The three-dimensional rendering of 134 optical sections taken in 3  $\mu\text{m}$  steps totaling 399  $\mu\text{m}$  deep into the tissue. Video of a rotation of the rendering can be found in **Supplemental Video 5**. **(B)** A single section taken from the stack of 134 optical sections. **(C-D)** These images were taken within the region of the parietal cortex. **(C)** The three-dimensional rendering of 101 optical sections taken in 5  $\mu\text{m}$  steps totaling 500  $\mu\text{m}$  deep into the tissue. Video of a rotation of the rendering can be found in **Supplemental Video 6**. **(D)** A single section taken from the stack of 101 optical sections. *Arc* transcripts were fluorescently labeled with HCR probes and hairpins conjugated with Alexa Fluor 647. *Arc* labeling is seen in red.

## Discussion and Future Directions

Until recently, tissue clearing techniques have been primarily used to enhance the molecular characterization and structural interrogation of different tissues and organs by offering the capability to label proteins over large, intact volumes<sup>9-13,19,20</sup>. These approaches have left the more transient biomolecules such as coding RNAs like mRNA and non-coding RNAs like microRNA untapped. These molecules have been shown to provide an additional layer of information such as the expression of untranslated RNA species and their possible effects in diseases that is often not captured by protein expression alone, and more relevant to our work, the ability for *Arc* mRNA to investigate activity-regulated transcriptional events.

Due to its role in synaptic plasticity and learning and memory, in addition to its unique compartmental expression and localization, *Arc* has been shown to be a powerful tool in tracking the history of neuronal activation in response to behavior. catFISH can be used for the mapping of behavior-driven neural networks involved in spatial tasks with temporal sensitivity and subcellular resolution. When coupled with a tissue clearing technique like EDC-CLARITY or CUBIC, and fluorescence *in situ* hybridization method with the tissue penetrance of HCR, and a microscope capable of unlocking the full potential of these combined techniques, *Arc* can be used to label intact, whole-brain neural networks.

Here, we show that with 2-photon microscopy and the usage of HCR amplified FISH increased tissue penetration was achieved in a 2 mm coronal slab taken from the center of the CUBIC cleared half-brain (**Figure 7 and 8**). Although it was difficult to orient the tissue and locate key regions of *Arc* expression like the hippocampus and cortex<sup>1</sup> due to transparency of the brain, the HCR probes were able to access and label *Arc* in the centermost region of the tissue, indicating that the smaller DNA probes were able to penetrate the tissue effectively.

With the transition from CUBIC to EDC-CLARITY for its enhanced RNA preservation, 1 mm coronal sections were used in order to shorten the amount of time to clear the tissue from ~4 months for whole brains to 2-3 weeks for the 1 mm sections. This made it easier to locate the hippocampus and cortex during microscopy. We showed *Arc* labeling in both the dentate gyrus of the hippocampus and the cortex in a CLARITY cleared brain section that was probed with 5 randomly selected probe sets (**Figure 9**). *Arc* labeling was also present in the dentate gyrus and cortex of another brain section probed with the remaining 5 probe sets not used in Figure 9 (**Figure 10**). In both figures, cytoplasmic staining can be seen as staining that surrounds a nucleus, which appears to be dark circles in the images. But without a nuclear counterstain like DAPI (4',6-diamidino-2-phenylindole), to clearly mark those dark circles as cell nuclei, it cannot definitively be called cytoplasmic staining. The available microscope filter set could not fully separate the DAPI signal from the Alexa Fluor 647 signal without signal bleed-through, in which signal in one channel may not be true signal, but rather, bleed-through signal from the other channel. For this reason, we imaged the brains without a nuclear counterstain.

Our results are in agreement with Sylwestrak et al. (2016), in which they performed *Arc* HCR FISH in 0.5 mm sections of EDC-CLARITY cleared brains and also observed *Arc* staining in the hippocampus<sup>15</sup>. However, instead of inducing expression of *Arc* with MECS, they used kainic acid induced seizures. They have also been able to label *somatostatin* with HCR FISH in a 2 mm thick section through the cortex, in which they observed staining throughout the tissue depth<sup>15</sup>. In addition to *Arc* and *somatostatin*, they were also successful in labeling other genes like *parvalbumin*, *neuropeptide Y*, *tyrosine hydroxylase*, and *tachykinin1*<sup>15</sup>. In the development of HCR, Choi et al. (2014) demonstrated the ability of HCR to allow for concurrent multiple gene target labeling in the same tissue<sup>16</sup>. With successive HCR FISH labeling of multiple genes,

this may open up the possibility to simultaneously label other IEGs involved in synaptic plasticity.

With our current lack of access to microscopes designed to image large volumes, we are limited to imaging brain sections 1-2 mm thick. We have been able to image up to a maximum tissue depth of 700  $\mu\text{m}$  (**Figure 9C**). To be able to image the entire volume of a cleared rat brain, we have been in collaboration with the University of Arizona Optical Science's Dr. Rongguang Liang, who is developing a High speed, High resolution, Long working distance, Large field of view Confocal Fluorescence Microscope ( $\text{H}^2\text{L}^2\text{-CFM}$ ). This novel microscope design will hopefully enable us to image whole and intact brains using the new tissue clearing techniques<sup>21</sup>.

In order to develop this toolkit to a practical usage standpoint, much more work needs to be done to optimize the combination of these methods. Moving forward, additional work also needs to be done in order to identify the probe sets (from the 10 possible sets) that provide good signal and also identify the sets that contribute to noise. In addition, a better choice of fluorophore needs to be investigated so that there is less signal bleed-through of nuclear counterstain and actual *Arc* stain. Even with the technological limitations stemming from the microscope we used and the tissue volume we attempted to image, we have shown here that the coupling of *Arc* fluorescence *in situ* hybridization with hybridization chain reaction amplification and tissue clearing methods like EDC-CLARITY and CUBIC shows promise in the pursuit to map out three-dimensional neural networks driven by behavior.

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## Supplemental Materials

**Supplemental Figure 1:** Nucleotide sequence for *Rattus norvegicus Arc* with the total sequence length being 3032 bp. Regions where the 10 different initiator probe sets hybridize to *Arc* are highlighted in yellow. Translated region is highlighted red and untranslated region is highlighted green.

```
1 AGTGTCTTGGCGAGTAGTCCCTCCCTCAGCCGAGTCTCTGGGCTCTTTCAGCTTGAGCGGCGGCGAGCCTGCCACACTCGCTAAGCTCCT
91 CCGGCACCGCGCACTTGCCACTGCCACTGCCGCTTCGCGCCCGCTGCAGCCCGGCTCTGAATCCTTCTGGCTTCCGCCCTCAGAGGAGT
181 TCTTAGCCTGTCCCGAACCCTAACCCCGGCGAGCAGATGGAGCTGGACCATATGACGACCGGCGGCTCCACGCCTACCTTGCCCGCGG
271 GGTGGGCGCGGCCAAACCCAAATGTGATCCTGCAGATTGGTAAGTGCCGAGCTGAGATGCTGGAGCACGTACGGAGGACCCACCGGCAT
361 CTGTTGACCGAAGTGTCCAAGCAGGTGGAGCGAGAGCTGAAAGGTTGCACAGTTCGGTGGGCAAGCTGGGAGAACAACTTGGACGGCTAT
451 GTGCCACCGGGCGACTCACAGCGCTGGAAGAAGTCCATCAAGGCTGTCTCTGCCGCTGCCAGGAGACCATCGCAACCTGGAGCGCTGG
541 GTCAAGCGTGAGATGCACGTGTGGAGGGAGGTCTTACCGTCTGGAGAGGTGGGCCGACCGCTGGAGTCCATGGGCGGCAAGTACCCA
631 GTGGGACCGAGCCGCGCCACACTGTCTCTGTAGTGTGGGGGTCCAGAGCCCTACTGCCAGGAAGCTGATGGCTACGACTACACT
721 GTTAGCCCTATGCCATCACCCGCCACCTGCCGAGGAGAGCTGCCTGAGCAGGAGTCAAGTTGGGGCTCAGCAATACCAGTCTTGGGTG
811 CCAGGTGAGGATGGGCAACCAAGCCAGGTCTGGATACCCAGATCTTTGAGGACCCACGGGAGTTCCTGAGCCACCTGGAAGAGTACCTG
901 CGCAGGTGGGTGGCTCTGAAGAATATTGGCTGTCCAGATCCAGAACCACATGAATGGGCCAGCCAAGAAGTGGTGGGAGTTCAAACAG
991 GGTCTGGTGAAGAACTGGGTGGAATCAAGAAGGAGTTTCTGCAGTACAGTGGGGTACGCTCTCCCGCAAGCCATTGAGCGGGAGCTG
1081 GACCTGCCACAGAACGAGGTTGAGCCACTTGACCAGTTCCTCTGGCGTAAGCGGGACCTGTACCAGACACTGTATGTGGACGCTGAGGAG
1171 GAGGAGATCATTAGTATGTGGTGGGCACCTGCAGCCCAAGTTCAAGCGCTTCTGCGCCACCCTTCCCAAGACCTGGAGCAGCTC
1261 ATCCAGAGGGGCATGGAAGTTCCAGGACGGCCTGGAGCAGGAGCTGAGCCTTCTGTACCCCTCTGCCCACAGAGGATGAGACTGAGCCA
1351 CTCACGCCTGCTCTTACCAGCGAGTCAAGTAGCCAGTGACAGGACCCAGCCTGAATAGAGGGGCCAGCCAGGGTCCCCAGCCTGCCTGCC
1441 ACACCCAGTCTGTGGCTTTTGTCAACTAGGACTTGATTGAGCTGGGGCTGACACCCAAGGGGATGCCCTGTCCAGCCAGACACCTTCTCA
1531 CCCACTGGCCTGACTCACAACAGCCACACAACCATGATTTCATGGACATCAAGAAGCCCTCTCCCATAGGGCTCCACCTGCCACCTACC
1621 CCTCACCTGTCTGCCCTAGTCCCTGGCCCTGTCTCCAGTGGCCTCACCTCTACACTCTCAGACCATCACAGAACACCTTTGGCTTCTCTCA
1711 TCTGTCATCAGTGTCCAGGGCCCTTTGGGTAGTCAAGAAATCAAGTGTCTGAAAGGGAATGAAAAGTAGGCACCAAAACCAAGGGGCATC
1801 CCAGGGCAGATGCTAAAGCAGAAATCAGAGATGGCCGAAGAACCTTACTTCCGGGATGCAGCCCGCTCTACAGACACAGCAGATCCA
1891 GCTGGTGCCCTACCTGCCCTCCAGAGCAACTGGCCAGTCTTGGGCAGCATAGCTCCCTCTCAGGGTGAGCTGAAGCAGCAGACCTGACG
1981 CGTGGCGCCTCCTGGCCCCAGCAGTGATTCATACAGTGAAGAAAAGCAGACTTCGGCTCCATGACTCAGCCATGCCAGGCGGAGGGT
2071 CCCAGAGGGGCTGAGTCTCAGCCCGAGCTGAGGACGAGCTGGAGTCTTCCAGACCGGTAATGACACCAGGTCTCAAGCTGTGAGA
2161 AGTCTTTCGGCCATGTCTGGAAGGGGTACCACCCAGCACCAGCACCCTCCCTCTCTTGAAGCTGCCTGCACAGAGGTTCCAAGA
2251 CACTTTCAAGGCAGAGAAAATAGGATTACAAAGAGGAGGTGCCTTGGCAGAGGGCAGCACCAGCTCAGCCTCAGAGCTGAAGGTGAAGA
2341 CAAGCCAGCGTGAAACCCCGGGTCTGCCACGAATGCCCGCTCCGCTGGCCACTCACCAGCTGCCTGCCACAAGCCACTGCAGCTTGAGCA
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2611 CACTTCCCTGACCTGCCGGGTGATTGCCAGCTGGAACCTCATCCACACCCAGCACCACCAACCCTCGTGTGGTAACTGCTCGTGTCTG
2701 TAGTCTGAGTAGCCATGTTGAGGTTCCCTCCATCTGCCTGGTCCATTGGTGTCTGAGACCAGTCCACTGCTGTTCTGACAGATCCCCC
2791 ACCCTGTGCCCTGCCAGCCCCACAGTTTTATTTTGCACATAAACCATGACCATACTAATTTGGCTAGCTTGGGGACTAGGGAGAC
2881 CCTGGAGATCTCAAGAGTGTGGCTATCCCTATTTTCAACCAAGCCTTCAATATCCAGCCAGGCCATCTGGCCACACCATCTTACCTCAA
2971 AGACAGACATATATATATATATACATATATATGATTTTGTAAATAAACTATGAAATTTAAA
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**Supplemental Video 1:** Video shows a 360° rotation about the y-axis of a three-dimensional rendering of a stack of 41 optical sections taken every 5 μm totaling 200 μm deep into the sample. Images were taken with an advanced intravital multiphoton microscope of a 2 mm slab cut out of a CUBIC cleared half-brain treated with 5 probe sets. *Arc* transcripts were fluorescently labeled with HCR probes and hairpins conjugated with Alexa Fluor 647. *Arc* labeling is seen in red. The video can be accessed at: <https://vimeo.com/228262493>

**Supplemental Video 2:** Video shows a 360° rotation about the y-axis of a three-dimensional rendering of a stack of 21 optical sections taken every 5 μm totaling 100 μm deep into the sample. Images were taken with an advanced intravital multiphoton microscope of a 2 mm slab cut out of a CUBIC cleared half-brain treated with 5 probe sets. *Arc* transcripts were fluorescently labeled with HCR probes and hairpins conjugated with Alexa Fluor 647. *Arc* labeling is seen in red. The video can be accessed at: <https://vimeo.com/228262510>

**Supplemental Video 3:** Video shows a 360° rotation about the y-axis of a three-dimensional rendering of a stack of 75 optical sections taken every 3 μm totaling 222 μm deep into the sample. Images were taken with an advanced intravital multiphoton microscope of a 1 mm coronal section of a CLARITY cleared brain treated with 5 probe sets. *Arc* transcripts were fluorescently labeled with HCR probes and hairpins conjugated with Alexa Fluor 647. *Arc*

labeling is seen in red. The video can be accessed at: <https://vimeo.com/228262532>

**Supplemental Video 4:** Video shows a 360° rotation about the y-axis of a three-dimensional rendering of a stack of 141 optical sections taken every 5 μm totaling 700 μm deep into the sample. Images were taken with an advanced intravital multiphoton microscope of a 1 mm coronal section of a CLARITY cleared brain treated with 5 probe sets. *Arc* transcripts were fluorescently labeled with HCR probes and hairpins conjugated with Alexa Fluor 647. *Arc* labeling is seen in red. The video can be accessed at: <https://vimeo.com/228262421>

**Supplemental Video 5:** Video shows a 360° rotation about the y-axis of a three-dimensional rendering of a stack of 134 optical sections taken every 3 μm totaling 399 μm deep into the sample. Images were taken with an advanced intravital multiphoton microscope of a 1 mm coronal section of a CLARITY cleared brain treated with 5 probe sets. *Arc* transcripts were fluorescently labeled with HCR probes and hairpins conjugated with Alexa Fluor 647. *Arc* labeling is seen in red. The video can be accessed at: <https://vimeo.com/228262440>

**Supplemental Video 6:** Video shows a 360° rotation about the y-axis of a three-dimensional rendering of a stack of 101 optical sections taken every 5 μm totaling 500 μm deep into the sample. Images were taken with an advanced intravital multiphoton microscope of a 1 mm coronal section of a CLARITY cleared brain treated with 5 probe sets. *Arc* transcripts were fluorescently labeled with HCR probes and hairpins conjugated with Alexa Fluor 647. *Arc* labeling is seen in red. The video can be accessed at: <https://vimeo.com/228262480>