OPTIMIZATION OF WHOLE BRAIN CLEARING TECHNIQUES AND MOLECULAR INVESTIGATION OF IEGS INVOLVED IN SPATIAL MEMORY PROCESSES

By

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AUTHOR'S DISCLOSURE

The author participated in the development and refinement of methods, sacrifice and brain extractions of animals, and collection and analysis of data. The author was mentored by Dr. Carol A. Barnes and Dr. Monica K. Chawla, both of whom provided immense guidance throughout the project. All funding for the project was provided by Dr. Carol A. Barnes.

Abstract

Fluorescence *in situ* hybridization (FISH) has been used to track the localization of *Arc* transcripts as a molecular method for quantifying behavior-driven activity in the hippocampus. But with the development of several tissue clearing techniques like CUBIC, CLARITY, and passive-CLARITY, the opportunity to use *Arc* FISH on transparent brains to map out intact neural networks is now feasible. Convention has been to use the full length *Arc* riboprobe (~3.1 kb) for FISH in 20 micron thick sections. However, in whole cleared brains, the full length *Arc* cRNA probe cannot easily penetrate the brain due to its thickness. We sought a solution to the issue of penetration with hybridization chain reaction (HCR). HCR is a technique that utilizes DNA probes instead of RNA riboprobes. Since DNA probes are inherently more stable, and HCR probes are less than 150 bases in length, this should drastically improve penetration. In addition, HCR fluorescence hairpins self-polymerize off of the DNA probe and thus, amplify the fluorescence signal. Fischer 344 (F344) rats were given maximal electroconvulsive shock (MECS) to induce transcription, were anesthetized, and then decapitated with a rodent guillotine. The brains were extracted, fixed in paraformaldehyde, and cleared using the CUBIC reagent (containing aminoalcohols) for 6-7 weeks. Using HCR amplified fluorescence *in situ* hybridization, we expect to visualize *Arc*-positive cells in rat hippocampus.

Background and Introduction

*Activity-regulated cytoskeleton-associated protein (*Arc*) is an immediate-early gene (IEG) that has been identified to be important for synaptic plasticity and therefore has a pertinent role in learning and memory[1,2]. 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*[1,3,4]. In the case of *Arc*, it 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[1,2,5,6]. By using antisense oligodeoxynucleotides (ODNs), which would hybridize to sense *Arc* mRNA and inhibit translation, it was shown that the impairment of Arc protein expression disrupted LTP maintenance and consolidation of long term memory (LTM) on hippocampal-dependent spatial Morris water maze task without affecting LTP induction or short term memory[5]. *Arc* knockout (KO) mice also showed disrupted LTP, specifically an enhanced early LTP, while also drastically reducing long term depression (LTD)[6]. This suggests a decrease in AMPA receptor endocytosis. 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. It was confirmed in Chowdhury et al., 2006, that Arc protein interacts with endocytosis proteins to promote AMPA receptor reinternalization[7]. Thus, *Arc* has an integral function in the stabilization of activity-dependent hippocampal plasticity[5,7].

Arc as an IEG has a unique expression profile that was elucidated by fluorescence *in situ* hybridization for *Arc* mRNA transcripts. Maximal electroconvulsive shock (MECS) induces global transcription, especially of IEGs, when applied to rats[3]. Between 5 minutes and 15 minutes after an application of MECS, *Arc* was rapidly transcribed and localized to foci within

the nucleus[4]. By 15 minutes, intranuclear fluorescent labeling started to decrease while cytoplasmic staining started to appear, and by 30 minutes, neurons with intranuclear labeling had decreased to caged control levels, with *Arc* transcripts mainly localized to the cytoplasm[4]. By 60 minutes, *Arc* mRNA labeling disappeared due to rapid transport to dendrites to be locally translated near the synapse[4]. This time course was also seen in rats who were allowed to explore given environments, in which *Arc* was transcribed in hippocampal neurons activated by the exploration[4]. This unique expression of *Arc* transcripts paired with its important role in modulating synaptic plasticity, allowed for a new method of tracking neuronal activation with a temporal specificity at a subcellular resolution by using fluorescence *in situ* hybridization. This lead 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 localization of *Arc* mRNA[4]. If *Arc* was found in the nucleus, then that neuron was active within the last 15 minutes; if *Arc* was found in the cytoplasm, then that neuron was active 30 minutes or later. And if *Arc* was found in both the nucleus and the cytoplasm, then that neuron was active at both time points.

The method of catFISH involves RNA riboprobes complimentary to the *Arc* transcript binding and hybridizing to the transcript. Digoxigenin is linked to the riboprobe, which allows for the anti-Dig antibody to bind to the digoxigenin-riboprobe-*Arc* complex[4]. The antibody is conjugated to a horseradish peroxidase enzyme, which catalyzes the localization of tyramide fluorescent signals around the complex[4]. This allows neurons activated by spatial memory tasks containing the now fluorescently tagged *Arc*-riboprobe complex to be visualized and quantified under confocal microscopy. The catFISH method is typically done in brain sections no thicker

than 20 μm, which would each have to be labeled and then imaged. This presents a meticulous and time-intensive challenge if an entire brain were to be digitally reconstructed.

However, over the past few years, several tissue clearing techniques have been developed: CLARITY[8], passive-CLARITY[9], iDISCO[10], and CUBIC[11]. All of these methods clear tissue in a variety of ways, but in general, they remove lipids from the tissue while keeping nucleic acids and proteins intact and they also help match the refractive indices of the clear tissue and imaging solution to reduce light-scattering. Lipids are prone to light-scattering, so by removing lipids from the tissue, optical penetration can increase dramatically[11]. This could potentially bypass the sectioning and digital reconstruction of the separate images, since clear and transparent brains could possibly be imaged intact due to the minimal light-scattering of the clear tissue. This project utilized the CUBIC (clear, unobstructed brain imaging cocktails) technique, in which whole rat brains fixed in 4% paraformaldehyde were incubated in Sca/eCUBIC-1 (CUBIC reagent-1) for 6-7 weeks at 37°C until the brains were cleared[11] (**Figure 1**).

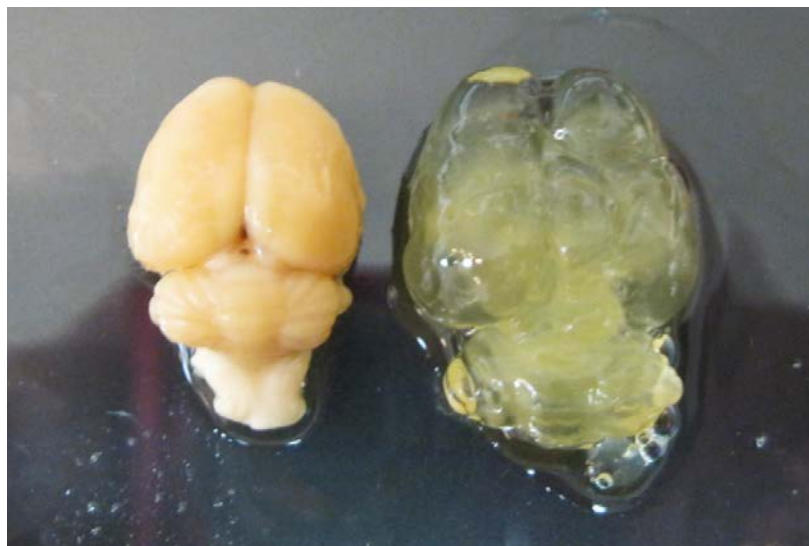

Figure 1: **Left)** A fixed whole rat brain prior to the CUBIC protocol.
Right) A fixed whole rat brain following 6-7 weeks of CUBIC reagent-1 incubation at 37°C with fresh reagent-1 being replaced every 2 weeks. The cleared brain shows preserved structure and morphology with only some swelling.

The issue with combining catFISH with CUBIC cleared brains is that traditional catFISH methods were developed on brain sections no thicker than 20 µm, whereas whole brains are a few centimeters thick. The riboprobes used are full-length *Arc* cRNA that are 3.1 kilobases (kb) long[1,4]. This presents a fundamental challenge for probe penetration – as the probes have to be able to penetrate the tissue completely to label *Arc* throughout the whole brain. These catFISH riboprobes used are made of RNA, which are not only expensive reagents, but notoriously unstable and prone to degradation[11]. 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.

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 uses DNA initiator probes (**Figure 2**), 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 4a**), have DNA HCR hairpin amplifiers containing fluorophores that self-polymerize orthogonally off of the DNA initiator probe[12] (**Figure 3 and 4b**). As more and more hairpins polymerize onto the growing chain, fluorescence signal is amplified to increase the ratio of signal compared to background noise (**Figure 3 and 4b**). These hairpins are only 72 nt long and should also have improved tissue penetration[12]. HCR was

designed so that the two initiator sequences flanking the 10 sets of variable *Arc* recognition sequences (**Figure 2**) would initiate the hairpins to unfold and hybridize to the flanking initiators[12]. This is the case since half of one hairpin is complementary to the initiator and the other hairpin is half complementary to the first hairpin allowing for the two types of hairpins to self-assemble off of each other (**Figure 3**).

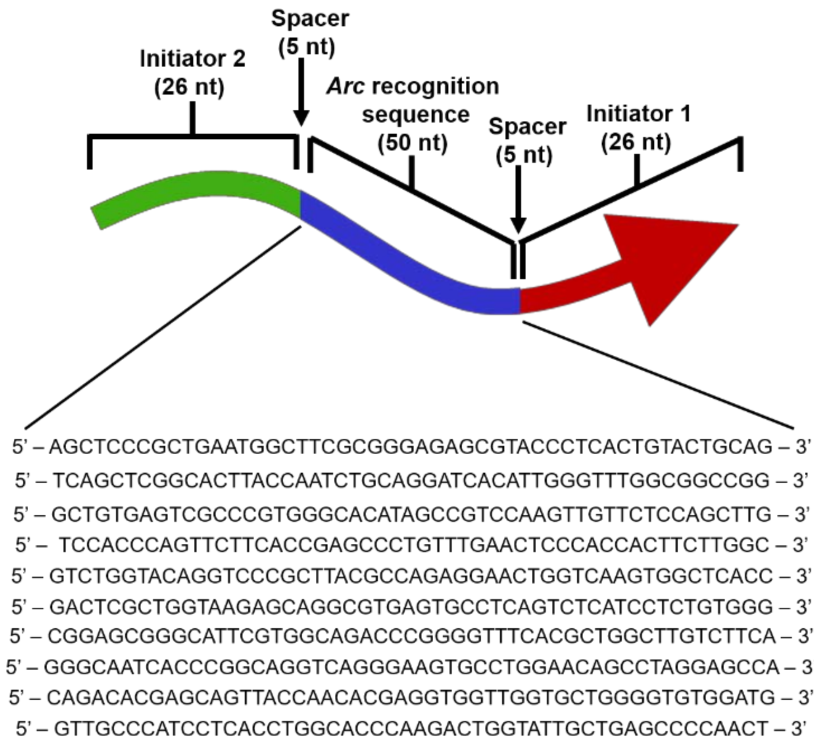

Figure 2: Composition of a single DNA initiator probe. Total length of each probe is 132 deoxy-nucleotides (nt) long with two 26 nt long initiator segments, two 5 nt spacers, and a 50 nt long *Arc* mRNA recognition sequence containing any of the 10 sequences pictured, each sequence corresponding to the 10 different probe sets. All strands are oriented 5' to 3'.

Beyond pairing the neuronal activity tracking system of catFISH utilizing HCR with the brain clearing technique of CUBIC, 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 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 CUBIC 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.

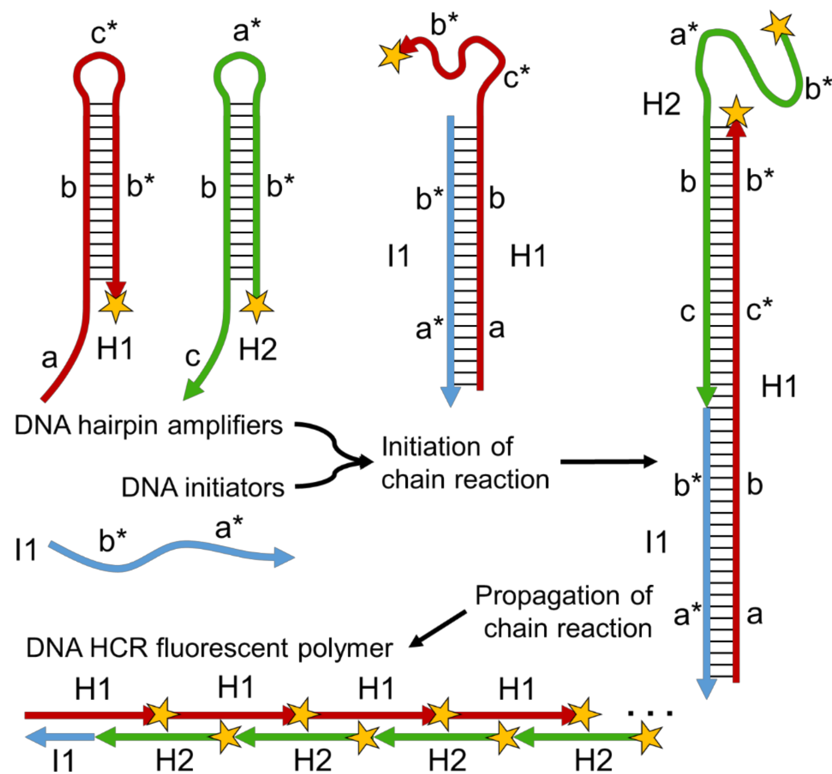

Figure 3: Schematic of the hybridization of DNA hairpin amplifiers to the initiator segments of the DNA initiator probes. The design of the two hairpins and the initiator segments promote the initiation of the chain reaction off of the initiator and then the elongation of HCR fluorescent polymers. Asterisks (*) 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.

Methods

Brain Preparation

Two seven month old male Fischer 344 (F344) rats were given maximal electroconvulsive shock (MECS) to induce global transcription. Saline-soaked ear clips were attached to the rats' ears and then an electroshock of 1 s, 100 Hz, 85 mA stimulus of 0.5 ms square-wave pulses was given using an UGO Basile ECT unit[3]. The rats were allowed to rest for 25 min in a warm incubator before they were given another MECS. They rested for another 5 min in the incubator before they were placed in an isoflurane chamber until rendered unconscious. Quickly, they were decapitated using a rodent guillotine and then brains were rapidly extracted into isopentane cooled by an ethanol/dry ice slurry to be quick frozen. The frozen brains were then fixed in 4% paraformaldehyde over two days.

CUBIC Tissue Clearing

Sca/eCUBIC-1 (reagent-1) was prepared by combining 25% w/v urea, 25 % w/v N,N,N',N'-tetrakis(2-hydroxypropyl)ethylenediamine, and 15% w/v polyethylene mono-p-isooctylphenyl ether/Triton X-100. The fixed brains were submerged in reagent-1 and allowed to incubate at 37°C in a shaker incubation oven for 6-7 weeks. Fresh reagent-1 was replaced every 2 weeks. The CUBIC method diffuses lipids out of the brain while maintaining subcellular structures, proteins, and nucleic acids in place[11]. The removal of light-scattering lipids makes the rat brains transparent, while only causing some swelling of the tissue (**Figure 1**). Following CUBIC clearing, both brains had their cerebellum cleaved off followed by hemisection using a razor blade. The removal of the cerebellum and the hemisection was done in order to increase surface area access for the probes to penetrate the brain tissue as well as to reduce the volume that would have to be imaged later. Right hemispheres were kept for future studies, while the left

hemispheres were taken through to fluorescent *Arc* labeling by fluorescence *in situ* hybridization with hybridization chain reaction (HCR) amplification.

Hybridization Chain Reaction Coupled Fluorescence in situ Hybridization

The HCR protocol was adapted from Slywestrak et al., 2016[13]. Prior to the HCR *in situ* hybridization, both half-brains were washed three times in enough 0.1 M phosphate buffered saline (PBS) to adequately cover the half-brains for 10 min on a shaker at room temperature to remove excess reagent-1. Both half-brains were then pre-incubated in 2 mL of probe hybridization buffer (Molecular Instruments, California Institute of Technology) in a 15 mL centrifuge tube at 37°C for 1 hr. Meanwhile, probe solutions were prepared for each brain. One half-brain was treated with a solution containing five randomly selected probe sets (**Figure 2**), each at a final concentration of 1 nM diluted in probe hybridization buffer to a final volume of 1 mL. The second half-brain was treated with seven randomly selected probe sets (**Figure 2**), each at a final concentration of 1 nM diluted in probe hybridization buffer to a final volume of 1 mL. Five probe sets and seven probe sets were chosen as the two conditions based on the recommendation to start with five probe sets and to increase as needed[13]. Both probe solutions were vortexed using a miniature vortexer and heated to 37°C while the half-brains pre-incubated. All ten DNA initiator probe sets (**Figure 2**) were designed by Molecular Instruments (Caltech) to hybridize to various 50 nt sequences of *Rattus norvegicus Arc* (**Supplemental Figure 1**).

After pre-incubation, the half-brains were placed into a new 15 mL centrifuge tube with their respective 1 mL probe solutions of either 5 probe sets or 7 probe sets. They were then incubated overnight (14 hr) at 37°C in the shaker incubation oven. After probe incubation/hybridization (**Figure 4a**), the half-brains were subjected to 5 × 1 hr washes in enough probe wash buffer (Molecular Instruments, Caltech) to adequately submerge the half-

brains at 37°C in the shaker oven. This was done to remove excess and unhybridized probes from the tissue.

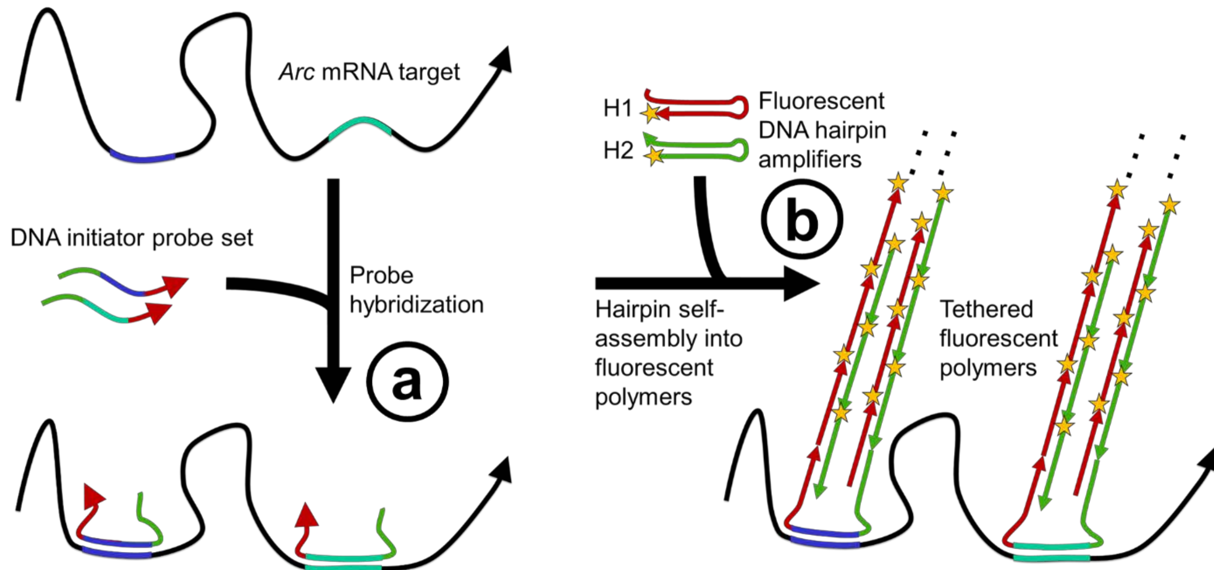

Figure 4: Fluorescence *in situ* hybridization of *Arc* mRNA with HCR amplification. **(a)** Sets of DNA probes hybridize to target sequences (colored purple and teal to differentiate different sets) on *Arc* mRNA. (**b**) Fluorescent DNA hairpins H1 and H2 self-polymerize off hybridized probes forming tethered fluorescent polymers. Yellow stars represent the fluorophore Alexa Fluor 647.

Following probe washings, the half-brains were subjected to hairpin amplification (**Figure 4b**). Half-brains were pre-incubated in 2 mL of amplification buffer (Molecular Instruments, Caltech) in 15 mL centrifuge tubes for 1 hr at room temperature. Meanwhile, hairpins (Molecular Instruments, Caltech) were prepared by pipetting 40 µL of hairpin 1 into a 0.5 mL microcentrifuge tube and 40 µL of hairpin 2 into a separate 0.5 mL microcentrifuge tube for each half-brain. All four microcentrifuge tubes were snap cooled by heating to 95°C for 90 s and cooling to room temperature for 30 min in the dark. For each half-brain, hairpin solutions were prepared by adding 40 µL of hairpin 1, 40 µL of hairpin 2, and 920 µL of amplification buffer for a final concentration of 120 nM for each hairpin in a total volume of 1 mL. Both half-brains were transferred to new 15 mL centrifuge tubes containing the prepared hairpin solutions. Both centrifuge tubes were then wrapped in tin foil to prevent photobleaching of the Alexa Fluor

647 fluorophore conjugated to the hairpins. The half-brains were then allowed to incubate in hairpin solution overnight (10 hr) at room temperature. To wash out excess hairpins, both half-brains were subjected to 5 × 1 hr washes in 10 mL of 5×SSC (saline sodium citrate buffer) with 0.1% Tween20 at room temperature.

Imaging of Fluorescent Labeled Arc in Cleared Half-Brains

Following the probe and hairpin washes, the transparency of the tissue was lost due to the mismatched refractive indices between the cleared tissue and wash solutions. Prior to imaging, transparency was reestablished by submerging the half-brains in fresh CUBIC reagent-1 overnight (16 hr) at 37°C in the shaker oven. Half-brains were mounted to petri dishes for imaging; agarose gel was added to the petri dishes until a thin layer was established. Right before the agarose solidified, the half-brains were placed on the layer of agarose and drops of agarose were added around the base to secure the half-brains in the dishes. After the agarose solidified, the rest of the dish was filled with 85% glycerol for refractive index matching. Both half-brains were taken to be imaged on the LagoX luminescence and fluorescence camera, which can image fluorescence in animals *in vivo* (**Figure 5**). The half-brain treated with 5 probe sets was unmounted and a 2 mm coronal section slab was cut out of the middle of the half-brain using a razor blade and a rat brain matrix to guide the blade. This 2 mm slab was remounted to a petri dish and taken to be 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 6 and 7**).

Results

Due to the current inability to image all the way through the half-brains without SPIM or LSFM microscopes, the 5 probe set and 7 probe set 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). The image of the 5 probe set half-brain showed Alexa Fluor 647 fluorescence radiance intensities between 8.0×10^7 and 1.0×10^8 Radiance [$W \cdot sr^{-1} \cdot m^{-2}$] 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 5A and 5B**). The 5 probe set half-brain also showed more Alexa Fluor 647 fluorescence between 1.0×10^8 and 1.8×10^8 Radiance [$W \cdot sr^{-1} \cdot m^{-2}$] (**Figure 5A and 5B**).

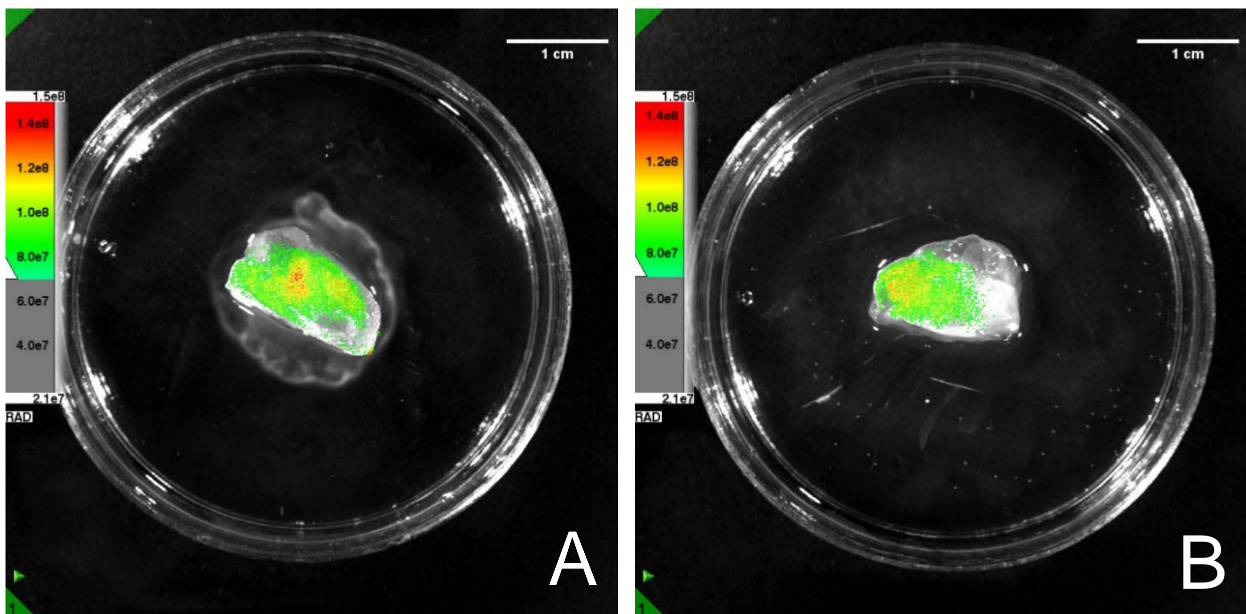

Figure 5: Images taken on the LagoX luminescence and fluorescence camera, where regions of red and yellow have higher radiance [$W \cdot sr^{-1} \cdot m^{-2}$] intensity corresponding to more Alexa Fluor 647 fluorescence and therefore more *Arc* labelling. **(A)** Left hemisphere treated with 5 randomly selected sets of DNA probes. Dorsal side up, rostral to the SE corner. **(B)** Left hemisphere treated with 7 randomly selected sets of DNA probes. Ventral side up, rostral to the west.

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 3D rendering of a stack of 41 optical sections through the slab taken in 5 µm steps (**Figure 6A**), 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 6B**). Another 3D rendering of a stack of 21 optical sections through a different region of the slab taken in 5 µm steps (**Figure 7A**), a video of the 21 section stack rotating about the y-axis (**Supplemental Video 2**), and a single optical section pulled out of the stack (**Figure 7B**). All of these images show Alexa Fluor 647 labelling of *Arc* transcripts.

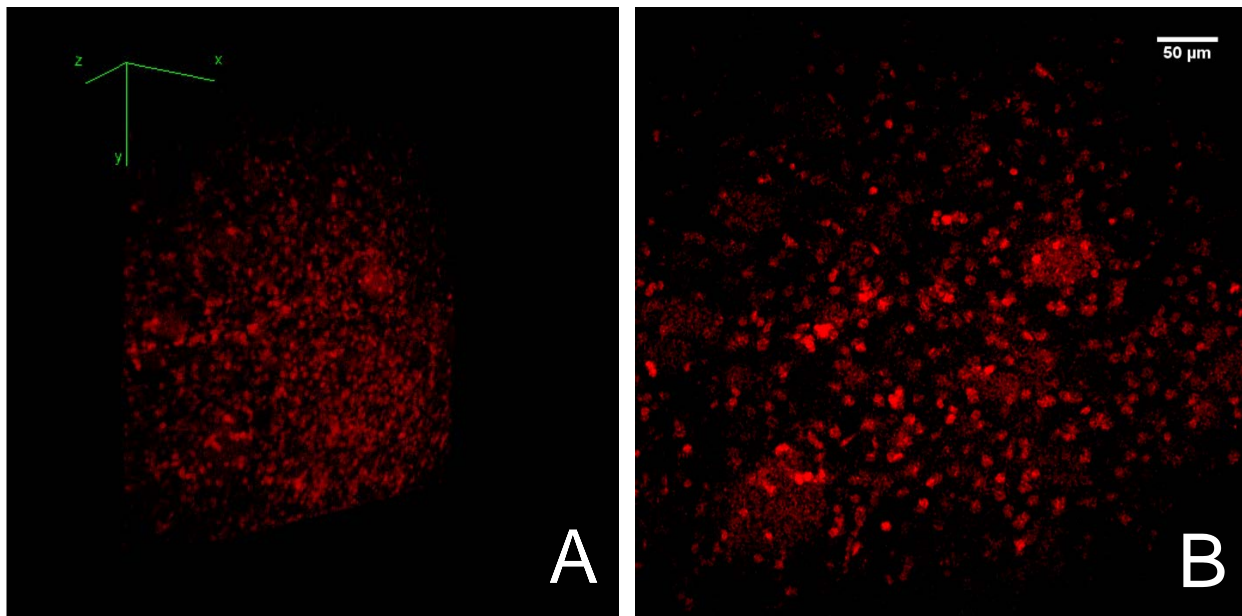

Figure 6: Images taken on an advanced intravital multi-photon microscope with a 20X objective of a 2 mm slab cut out from the half-brain treated with 5 DNA probe sets. **(A)** The three dimensional rendering of 41 optical sections taken in 5 µm steps. **(B)** A single section taken from the stack of 41 optical sections. *Arc* transcripts were fluorescently labeled with HCR probes and hairpins conjugated with Alexa Fluor 647. *Arc* labeling is seen in red.

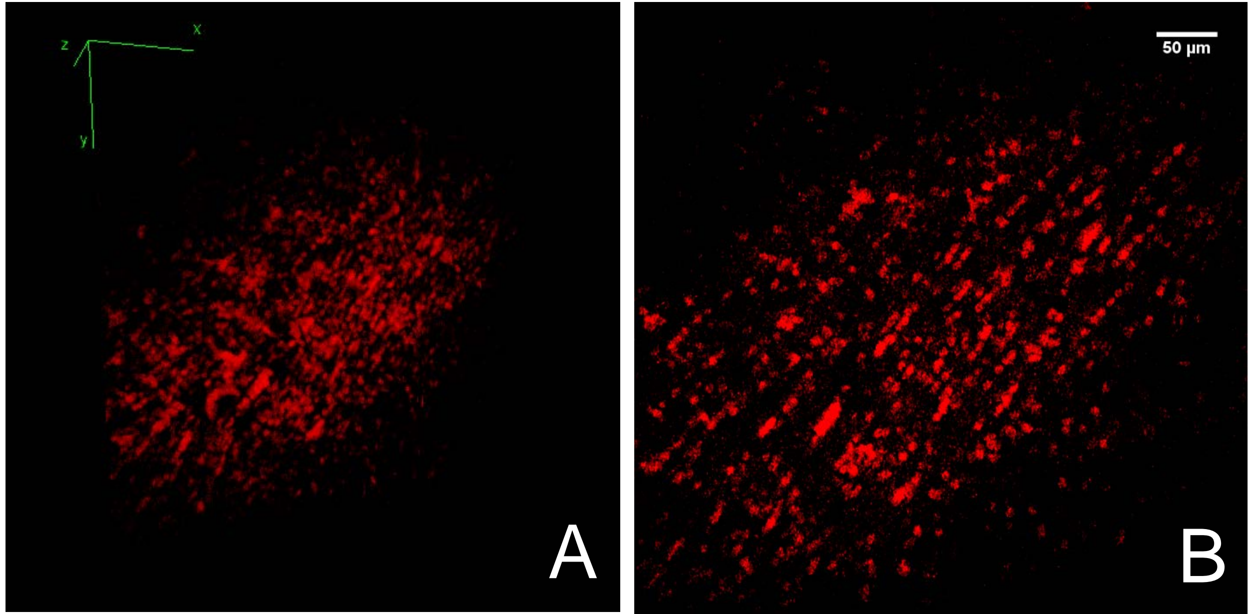

Figure 7: Images taken on an advanced intravital multi-photon microscope with a 20X objective of a 2 mm slab cut out from the half-brain treated with 5 DNA probe sets. **(A)** The three dimensional rendering of 21 optical sections taken in 5 µm steps. **(B)** A single section taken from the stack of 21 optical sections. *Arc* transcripts were fluorescently labeled with HCR probes and hairpins conjugated with Alexa Fluor 647. *Arc* labeling is seen in red.

Discussion and Future Directions

With its role in synaptic plasticity and learning and memory, along with its unique transcript compartmental localization, *Arc* is a powerful tool capable of being utilized to track neurons activated in response to a specific task to map out behavior-driven neural connections with a temporal sensitivity at a subcellular resolution. When coupled with a tissue clearing technique like CUBIC, a fluorescence *in situ* hybridization method with the full tissue penetrance of HCR, and a suitable microscope to unlock the full potential of the combination of these techniques, *Arc* can be used to fluorescently label intact, whole-brain neural networks. This will cultivate and enable further research on how normal aging, Alzheimer's, hypertension, or any other medical phenomena with a neural component affect these connections and networks on a molecular scale throughout the whole brain. But in order to develop this toolkit to a practical usage standpoint, more work needs to be done to optimize the pairing of all these methods.

The usage of *Arc* fluorescence *in situ* hybridization with HCR amplification seems to be promising with the 2-photon micrographs (**Figure 6 and 7**) that show Alexa Fluor 647 labelling of *Arc* from a 2 mm coronal slab taken from the center of the 5 probe set half-brain. This indicates increased tissue penetrance of the HCR DNA probes over traditional catFISH riboprobes. In the future, we hope to be able to simultaneously image the Alexa Fluor 647 with the nuclear counterstain DAPI (4',6-diamidino-2-phenylindole) in order to determine *Arc* compartmentalization (localization to nuclei, cytoplasm, both, or neither). With the current set of filters for the 2-photon microscope, in order to image Alexa Fluor 647, DAPI signal had to be sacrificed due to the filters in place. We would also like to expand HCR fluorescence *in situ* hybridization to the whole rat brain instead of just half-brains. The LagoX luminescence and fluorescence camera images (**Figure 5A and 5B**) support that the usage of 5 randomly selected

probe sets has higher Alexa Fluor 647 fluorescence radiation intensity over the usage of 7 randomly selected probe sets. Moving forward, we would like to use the 2-photon microscope to image *Arc* labeling in 5 probe set brains and 7 probe set brains from comparable coronal sections from both brains in order to quantify the difference in fluorescence signal at the cellular level. Once we have determined the optimal protocol for HCR in cleared whole rat brains, we would then move back to spatial exploration tasks to elicit *Arc* expression in task-specific neurons instead of utilizing MECS to induce global transcription. The complete development of the toolkit to be able to image fluorescently labeled *Arc* in whole cleared brains with an account of neuron-activation history at a subcellular resolution would be a significant gain to the field of systems neuroscience.

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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.

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   1 AGTGCTCTGGCGAGTAGTCCTCCCTCAGCCGCAGTCTCTGGGCCTCTTCAGCTTGAGCGGCGGCGAGCCTGCCACACTCGCTAAGCTCCT
  91 CCGGCACCGCGCACTTGCCACTGCCACTGCCGCTTCGCGCCCGCTGCAGCCGCCGGCTCTGAATCCTTCTGGCTTCCGCCTCAGAGGAGT
 181 TCTTAGCCTGTCCCGAACCGTAACCCCGGCGAGCAGATGGAGCTGGACCATATGACGACCGGCGGCCTCCACGCCTACCCTGCCCCGCGG
 271 GGTGGGCCGGCCGCCAAACCCAATGTGATCCTGCAGATTGGTAAGTGCCGAGCTGAGATGCTGGAGCACGTACGGAGGACCCACCGGCAT
 361 CTGTTGACCGAAGTGTCCAAGCAGGTGGAGCGAGAGCTGAAAGGGTTGCACAGGTCGGTGGGCAAGCTGGAGAACAACTTGGACGGCTAT
 451 GTGCCCACGGGCGACTCACAGCGCTGGAAGAAGTCCATCAAGGCCTGTCTCTGCCGCTGCCAGGAGACCATCGCCAACCTGGAGCGCTGG
 541 GTCAAGCGTGAGATGCACGTGTGGAGGGAGGTCTTCTACCGTCTGGAGAGGTGGGCCGACCGCCTGGAGTCCATGGGCGGCAAGTACCCA
 631 GTGGGCAGCGAGCCGGCCCGCCACACTGTCTCTGTAGGTGTGGGGGGTCCAGAGCCCTACTGCCAGGAAGCTGATGGCTACGACTACACT
 721 GTTAGCCCCTATGCCATCACCCCGCCACCTGCCGCAGGAGAGCTGCCTGAGCAGGAGTCAGTTGGGGCTCAGCAATACCAGTCTTGGGTG
 811 CCAGGTGAGGATGGGCAACCAAGCCCAGGTCTGGATACCCAGATCTTTGAGGACCCACGGGAGTTCCTGAGCCACCTGGAAGAGTACCTG
 901 CGGCAGGTGGGTGGCTCTGAAGAATATTGGCTGTCCCAGATCCAGAACCACATGAATGGGCCAGCCAAGAAGTGGTGGGAGTTCAAACAG
 991 GGCTCGGTGAAGAACTGGGTGGAGTTCAAGAAGGAGTTTCTGCAGTACAGTGAGGGTACGCTCTCCCGCGAAGCCATTCAGCGGGAGCTG
1081 GACCTGCCACAGAAGCAGGGTGAGCCACTTGACCAGTTCCTCTGGCGTAAGCGGGACCTGTACCAGACACTGTATGTGGACGCTGAGGAG
1171 GAGGAGATCATTCAGTATGTGGTGGGCACCCTGCAGCCCAAGTTCAAGCGCTTTCTGCGCCACCCACTTCCCAAGACCCTGGAGCAGCTC
1261 ATCCAGAGGGGCATGGAAGTTCAGGACGGCCTGGAGCAGGCAGCTGAGCCTTCTGTCACCCCTCTGCCCACAGAGGATGAGACTGAGGCA
1351 CTCACGCCTGCTCTTACCAGCGAGTCAGTAGCCAGTGACAGGACCCAGCCTGAATAGAGGGGCCAGCCCAGGGTCCCCAGCCTGCCTGCC
1441 ACACCCAGTCTGTGGCTTTTGTCAACTAGGACTTGATTGAGCTGGGGCTGACACCCAAGGGGATGCCCTGTCCAGCCAGACACCTTCTCA
1531 CCCACTGGCCTGACTCACAACTGCCACACAACCATGATTCATGGACATCAAGAAGCCCCTCTCCCATAGGGCTCCCACCTGCCACCTACC
1621 CCTCACCTGTCTGCCCTAGTCCTGGCCCTGTCTCCAGTGGCCTCACCCTCTACACTCTCAGACCATCACAGAACACCTTTGGCTTCCTCA
1711 TTCTGCATCAGTGTCCAGGGCCCTTTGGGTAGTCAAGAAATCAAGTGTCTGAAAGGCAATGAAAAGTAGGCACCAAACCCAAGGGGCATC
1801 CCAGGGCAGATGCTAAAGCAGAATCAGAGATGGCCGAAGGAACCTCTACTTCCGGGGATGCAGCCCGCTCCTACAGACACAGCAGATCCA
1891 GCTGGTGCCCTACCTGCCTCCCAGAGCAACTGGCCAGTCTTGGGCAGCATAGCTCCCCTCTCAGGGTGAGCTGAAGCAGCAGACCTGACG
1981 CGCTGGCGCCTCCTGGCCCCCAGCAGTGATTCATACCAGTGAAGAAAAGCAGACTTCGGCTCCATGACTCAGCCATGCCAGGCGGAGGGT
2071 CCCAGAGGGGCTGAGTCCTCAGCCCCAGCTGAGGCAGCAGCTGGAGTCTTCAGAGCCAGGTGAATGACACCAGGTCTCAAGCTGCTGAGA
2161 AGTCTTTCCGGCCATGTCTGGAAGGGGTACCACCCCAGCACCAGCACCGTCCCCTCCTCTCTTGAAGCTGCCTGCACAGAGGTTCCAAGA
2251 CACTTTCAAGGCAGAGAAAATAGGATTACAAAGAGGAGGTGCCTTGGCAGAGGGCAGCACCCAGCTCAGCCTCAGAGCTGAAGGTGAAGA
2341 CAAGCCAGCGTGAAACCCCGGGTCTGCCACGAATGCCCGCTCCGCTGGCCACTCACCAGCTGCCTGCCACAAGCCACTGCAGCTTGAGCA
2431 GGGTCTGTGCCCTCTCAGCACAGAGCCCAGTTCGCTGCGTGGCCTTTGGCCCCCGCCAGAACCTTGCAGGAGCCTTAAGGTTCGGGCCCT
2521 AGCCCAGCCTGACCTTACCTGCTGTGCCCTGCCTGCTGGTCAAGTCCAGTCCCAGGAGACCCCATGCCTTGGCTCCTAGGCTGTTCCAGG
2611 CACTTCCCTGACCTGCCGGGTGATTGCCCAGCTGGAACCTCATCCACACCCCAGCACCAACCACCTCGTGTTGGTAACTGCTCGTGTCTG
2701 TAGTCTGAGTAGGCCATGTTGAGGTTCCTCCATCTGCCTGGTCCATTGGTGTTCTGAGACCAGTTCCACTGCTGTTCTGACAGATCCCCC
2791 ACCCTGTGCCCCTGCCAGCCCCCACAGGTTTATTTTGCACATAAACCATGACCCATACTAATTTGGCTAGCTCTGGGGACTAGGGAGAC
2881 CCTGGAGATCTCAAGAGTGTGGCTATCCCCTATTTTCACCAAGCCTTCAATATCCAGCCAGGCCATCTGGCCCACACCATCTTACCTCAA
2971 AGACAGACATATATATATATATACATATATATGATTTTGTTAATAAAACTATGAAATTTAAA
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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 with an advanced intravital multiphoton microscope of a 2 mm slab cut out of the 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/162219955

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 with an advanced intravital multiphoton microscope of a 2 mm slab cut out of 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/162223867