Video Article

Modified Roller Tube Method for Precisely Localized and Repetitive Intermittent Imaging During Long-term Culture of Brain Slices in an Enclosed System

Benjamin B. Fixman*[1], Isaac W. Babcock*[1], Laurie S. Minamide*[1], Alisa E. Shaw[1], Marina I. Oliveira da Silva[1,2], Avery M. Runyan[1], Michael T. Maloney[1,3], Jeffrey J. Field[1], James R. Bamburg[1]

[1]Department of Biochemistry and Molecular Biology and Molecular, Cellular and Integrated Neuroscience Program, Colorado State University

[2]IBMC-Instituto de Biologia Molecular e Celular, i3S-Instituto de Investigaçãoe Inovação em Saúde, ICBAS, Universidade do Porto

[3]Denali Therapeutics

*These authors contributed equally

Correspondence to: James R. Bamburg at James.Bamburg@colostate.edu

URL: https://www.jove.com/video/56436
DOI: doi:10.3791/56436

Keywords: Neuroscience, Rodent hippocampus, viral-mediated gene expression, confocal microscopy, photoetched coverslips, neurodegenerative diseases, cofilin pathology, Alzheimer's disease

Date Published: 12/9/2017

Citation: Fixman, B.B., Babcock, I.W., Minamide, L.S., Shaw, A.E., Oliveira da Silva, M.I., Runyan, A.M., Maloney, M.T., Field, J.J., Bamburg, J.R. Modified Roller Tube Method for Precisely Localized and Repetitive Intermittent Imaging During Long-term Culture of Brain Slices in an Enclosed System. *J. Vis. Exp.* (), e56436, doi:10.3791/56436 (2017).

Abstract

Cultured rodent brain slices are useful for studying the cellular and molecular behavior of neurons and glia in an environment that maintains many of their normal *in vivo* interactions. Slices obtained from a variety of transgenic mouse lines or use of viral vectors for expression of fluorescently tagged proteins or reporters in wild type brain slices allow for high-resolution imaging by fluorescence microscopy. Although several methods have been developed for imaging brain slices, combining slice culture with the ability to perform repetitive high-resolution imaging of specific cells in live slices over long time periods has posed problems. This is especially true when viral vectors are used for expression of exogenous proteins since this is best done in a closed system to protect users and prevent cross contamination. Simple modifications made to the roller tube brain slice culture method that allow for repetitive high-resolution imaging of slices over many weeks in an enclosed system are reported. Culturing slices on photoetched coverslips permits the use of fiducial marks to rapidly and precisely reposition the stage to image the identical field over time before and after different treatments. Examples are shown for the use of this method combined with specific neuronal staining and expression to observe changes in hippocampal slice architecture, viral-mediated neuronal expression of fluorescent proteins, and the development of cofilin pathology, which was previously observed in the hippocampus of Alzheimer's disease (AD) in response to slice treatment with oligomers of amyloid-β (Aβ) peptide.

Video Link

The video component of this article can be found at https://www.jove.com/video/56436/

Introduction

Primary culture of dissociated neurons from regions of rodent brain is an important tool used by researchers to observe responses to pathologically implicated stimuli. However, such studies have the disadvantage of looking at neurons in only 2D and without their glial support system. Furthermore, unless grown under conditions of very high density (640 neurons/mm^2 or about 16% of surface area) in which it becomes impossible to follow the random outgrowth of a dendrite or axon for more than a short distance from its cell body, hippocampal neuronal viability over 4 weeks declines significantly[1], limiting the use of dissociated cultures for extended studies of age-related pathologies. The culturing of slices prepared from rodent brain is an attractive option that overcomes these limitations by maintaining an organized cell architecture and viability for weeks or months. Conditions for maintaining many different regions of rodent brain in slice culture have been described[2].

Two major methods are widely used for long-term culture of brain slices: culturing on membranes at the air-liquid interface[3] or culturing on coverslips in sealed tubes allowed to rotate in a roller incubator to provide aeration[4]. Slices cultured on membranes can be directly imaged with high-resolution fluorescence microscopy using an upright microscope and water immersion objectives[5]. Alternatively, slices cultured on membranes have been transferred to glass bottom dishes to achieve good resolution of dendritic spines using an inverted microscope[6]. However, both methods of imaging slices grown on membranes are open systems that require medium changes and often use antifungal and/or antibiotics to prevent or reduce contamination[5,6]. Slices on a membrane at the air-medium interface maintain excellent morphology and survival, but returning to precise locations during repetitive imaging at high magnification is extremely difficult unless the experiment is following only small groups of cells expressing a fluorescent marker. Although slices grown on membranes have been used with viral-mediated expression of transgenes[5,6], biosafety protocols may require an enclosed culture system be employed for certain viral vectors that are used for expressing fluorescently tagged proteins and reporters of cell physiology. Furthermore, immersion objectives require decontamination between samples that

Copyright © 2017 Journal of Visualized Experiments

will be followed in culture[5]. One major application of membrane interface cultures is combining high-resolution imaging with electrophysiology at single time points[7].

The roller tube method with coverslips inside the plastic tube does not permit any electrophysiology or high-resolution imaging without removing the coverslip. Thus, this method has been most often applied to long-term studies in which post-fixation observations have been made[8]. Described here is a method that utilizes the roller tube culture technique but on drilled-out tubes with slices on coverslips that can be imaged repetitively for as long as the cultures are maintained. The enclosed system requires no medium change for imaging and utilizes photoetched coverslips to provide fiducial marks that allow imaging at high magnification, after days or weeks, the precise fields previously imaged.

We apply this method to examine changes in the rodent hippocampus, a major brain region involved in memory and learning. The rodent hippocampus is often studied as a model for pathological or age-related changes observed during development of cognitive impairment[9], such as those that occur in AD. Our method is particularly well suited to study pathological changes that develop within a single slice over time in response to environmental changes, such as increases in Aβ peptides, which is characteristic of AD[8]. One pathology associated with human and rodent AD brain is the presence of cofilin-actin aggregates and rods, the latter containing bundles of filaments in which cofilin and actin are in a 1:1 molar ratio[10-12]. Rods have been observed in fixed slices of rat hippocampus following Aβ treatment, as well as within a live rodent brain slice expressing cofilin-GFP subjected to hypoxia[8], and they may contribute to the synaptic dysfunction seen in AD and stroke. Here we use this new culturing method to observe the time course and distribution within slices of expressed exogenous chimeric fluorescent proteins introduced by different viruses. We then utilize the neuronal specific expression of a cofilin reporter construct to follow the development of cofilin rod and aggregate pathology in hippocampal slices in response to treatment with soluble Aβ oligomers ($A\beta_o$).

Protocol

Animal use follows approved breeding and animal use protocols that conform to the Animal Care and Use Guidelines of Colorado State University.

NOTE: The protocol below describes the preparation and culture method for the long-term incubation and intermittent imaging of hippocampal slices. A single hippocampal slice is attached to a specially prepared photoetched coverslip using a plasma clot, and then the coverslips are sealed onto the flat side of a drilled-out roller tube, which is maintained in a roller incubator. Plasma clots are dissolved with plasmin before viral infection for fluorescent protein expression and high-resolution imaging. A fluorescent neuronal vital dye is used to image neurons within the slices.

1. Preparation of Roller Tube Rack

1. Use the template shown in **Figure 1A** printed to the size shown on the scale bar. With a nail, punch small holes (large enough for a fine point marker) in the template centered on the holes.
2. Set the template on the bottom of a 15 cm tissue culture dish (nominal diameter of 14 cm) and mark the position of the holes. Repeat this on a second dish.
3. With drill bits designed for use on plastic, drill six 1.5 cm diameter holes on each dish in a hexagonal array (4.8 cm center-to-center) with hole centers 2.5 cm from the edge of the dish. Drill three holes (3 mm in diameter) 12 mm from the edge that are placed equidistantly between two of the larger holes as shown in **Figure 1A**.
4. With the bottoms of each dish facing each other, place a 2.5 inch long machine screw (3/16 inch diameter) with a flat washer through one of the small holes followed by a second flat washer, a piece of polyethylene tubing (spacer, 4.7 cm), another flat washer, the second tissue culture dish, another flat washer, a locking washer, and a nut.
5. Repeat step 1.4 on the other two machine screws, and tightening only loosely until all machine screws are in place. Then tighten the nuts securely.
6. Work the grommets (5/16 inch thick, 5/8 inch hole diameter) into the holes of the bottom dish to obtain the final roller tube rack (**Figure 1B**; shown with two tubes in place). Place a sticker on each rack with a unique number.

2. Preparation of Roller Tubes and Coverslips

1. **Making the jig for drilling the hole in roller tubes**
    1. Drill a 1.5 cm hole 8 cm deep in the center side of a 2 x 4 x 5.5 inch wooden block at an angle such that the flat side of the roller tube will be nearly parallel with the block when inserted (**Figure 2A**).
    2. Enlarge the hole using a round wood file to both widen and taper the hole to allow tube insertion (roller tubes are slightly larger in diameter near the cap end) (**Figure 2B**).
    3. Drill a 1.5 cm diameter vertical hole, 5.5 cm from the side of the block, and centered over the side hole (**Figure 2C**).
    4. When the side hole is tapered enough, insert a roller tube that is marked at the desired spot to center the hole for the slice and position the tube so the marked spot is centered in the 1.5 cm vertical hole.
    5. Remove the tube and measure the distance from the spot to the end of the tube. Mark this distance from the center of the hole in the jig and insert a nail to provide a stop to correctly position the tube for drilling (arrow in **Figure 2C**).
    6. Use a hacksaw to cut the nail off flush with the surface of the wood block to prevent injury.
    7. Add spring clips on the bottom of the jig if there is a drill press with slots that allow it to be anchored (**Figure 2D** black arrow). Otherwise, use C-clamps to hold the jig securely onto the drill press.

2. Using the jig described above to hold and position a flat sided 11 cm plastic culture tube with the flat side up (**Figure 2A**), drill a 6 mm diameter hole with the center 1.0 cm from the bottom and centered between the sides of the tube.
NOTE: A drill bit designed for plastic should be used.

3. With a swiveling deburring tool, smooth the edges of the hole (**Figure 2E**) and make 4 grooves on the inside edge of the hole (**Figure 2E**, inset) to facilitate draining of the hole during rotation.
4. With a 12 mm hole punch, cut 12 mm diameter disks from non-toxic double sided adhesive silicon rubber sheets. Using a standard one hole paper punch (6 mm diameter), make a hole in the center of each disk.
5. Rinse the drilled tubes with 70% ethanol, air dry them in a biological safety cabinet, and sterilize the tubes and the punched adhesive discs for 40 min under the UV lamp (30 W at 70 cm average distance) in the biological safety cabinet.
6. Reposition the tubes and discs after 20 min so that all exposed surfaces are sterilized. Under sterile conditions, peel off the white backing from an adhesive disc and affix the silicone rubber to the outside of a tube, aligning the holes (**Figure 2F**).
   Caution: To avoid UV exposure, wear eye protection and close the cabinet before turning on the UV lamp.
7. Clean 12 mm diameter photoetched (100 center numbered 1 mm squares) German glass coverslips. Hold the coverslips gently with forceps and dip in absolute ethanol, followed by water, followed by absolute ethanol again, and finally dip the coverslips in a flame to burn off the ethanol. Allow coverslips to cool.
8. Holding the coverslips with forceps, dip into 2% 3-aminopropyltriethoxysilane in acetone for 10 s. Rinse the coverslips with ultrapure water and allow to air dry.
9. Set the coverslips on sterile filter paper inside of a biological safety cabinet and turn on the UV light. Expose each side of the coverslips for 20 min.
   Caution: To avoid UV exposure, wear eye protection and close the cabinet before turning on the UV lamp.

3. Hippocampal Slice Preparation

1. Before starting the dissection, prepare halves of double-edged razor blades for the tissue chopper. Fold the blades lengthwise carefully with fingers and snap in half.
2. Rinse the blade halves with acetone using a cotton swab to clean them, followed by rinsing in absolute ethanol and air drying. Before mounting a half blade on the tissue chopper, sterilize it by rinsing with 70% ethanol.
3. Follow protocols approved by the institutional Animal Care and Use Committee; after isoflurane anesthesia, euthanize a 4-7 day old mouse or rat pup by decapitation and remove the head with scissors or guillotine.
   NOTE: The brain slice culture protocol is independent of mouse or rat strain or genotype. Many transgenic mouse lines with different genetic backgrounds have been used.
4. Rinse the head with 70% ethanol, and place it in a 60 mm Petri dish. Until the final mounting of the brain slice onto the roller tube, all of the following steps are performed in a laminar flow hood to maintain sterility.
5. Using a #21 surgical blade, make a sagittal cut through the skin and skull. With a #5 Dumont forceps, peel back the skin and skull to expose the brain.
6. With closed forceps, gently tease out the whole brain, releasing it by pinching with the forceps through the brain stem behind the cerebellum. Place the brain in a sterile 60 mm dish containing 4 °C Gey's Balanced Salt Solution/0.5% glucose (GBSS/glucose).
7. **Using a dissection microscope to visualize the brain (Figure 3A), place the brain dorsal side up and cut off the front third of the brain and the cerebellum with a surgical blade (Figure 3B).**
   1. With forceps, hold the trimmed brain posterior side up and ventral side against the side of the Petri dish for stability. Gently tease away meninges around the sagittal midline and remove the midbrain tissue using a fine tipped Dumont #5 forceps (**Figure 3C**, dashed circle).
   2. Make two cuts along the side of the brain to spread it open (**Figure 3C**, dashed lines). Once the brain is placed dorsal side down and spread open, the hippocampal fissure should be visible (**Figure 3D**, arrow).
   3. Transfer the spread open brain to a piece of polychlorotrifluoroethylene plastic film and position it for slicing on the stage of a tissue chopper. Wet the blade with GBSS/glucose and chop the hippocampus into ~300 µm thick slices.
   4. With a transfer pipette, flush the sliced brain off the plastic film into a fresh 60 mm dish containing GBSS/glucose (**Figure 3E**). Gently pinch off and tease away, with fine tipped forceps, the remaining meninges and other non-hippocampal tissue (**Figure 3F**) from the slices (**Figure 3G, H**).

4. Plating Slices

1. Once slices have been obtained, place 2 µL of chicken plasma on the center of the photoetched side of a prepared coverslip. Spread the plasma slightly to achieve a 3-4 mm diameter spot.
   NOTE: The photoetched side is the top side of the coverslip when viewed through a dissection microscope such that the numbers are oriented correctly.
2. Transfer 1 brain slice with a sterile narrow-tip spatula (**Figure 4A**) to the plasma spot (**Figure 4B**). Use closed forceps to keep the slice on the spatula tip while lifting the slice from the GBSS/glucose.
3. Touch the spatula to the plasma spot on the coverslip, and with closed forceps, push the slice onto the coverslip.
4. Mix 2.5 µL of plasma with 2.5 µL of thrombin in a separate tube. Quickly place 2.5 µL of this mixture over and around the slice and pipet up and down gently to mix it (**Figure 4B**).
   NOTE: The plasma will clot within 10-15 s, so this must be done quickly. If slice adhesion is a problem, mix 5 µL plasma with 5 µL thrombin and use 4-5 µL on the slice, removing some after mixing so that the slice lies flat on the coverslip.
5. Remove the clear plastic covering from the exposed side of the silicone rubber adhesive previously affixed to a roller tube and place the coverslip with the brain slice onto the adhesive aligning the slice within the hole (**Figure 4C**).
6. To ensure adhesion, apply soft, even pressure to the coverslip with the thumb by pressing the coverslip down evenly and holding it for about 1 min while transferring it to the biological safety cabinet.
7. In a biological safety cabinet, add 0.8 mL of complete Neurobasal A culture medium (**Table of Materials**) to each tube (**Figure 4D**).
8. Flow a 5% CO_2/95% air mixture through a sterile cotton-plugged Pasteur pipette held securely by a clamp. Flush the roller tube with the gas mixture and rapidly cap the tube as it is withdrawn from around the pipette.

9. Label the tubes with the slice number and rack number. Insert tubes into a roller rack, ensuring they are geometrically balanced. If there is an odd number of tubes, add tubes to balance.
10. Place the racks in a 35 °C roller incubator with rollers turning the roller rack at about 10-13 RPH (**Figure 4E**). To keep the medium in the bottom of the tubes, tilt the incubator back approximately 5° by raising its front on a board.
11. Enter the slice and tube number onto a spreadsheet, which is used to record all information of slice treatments and observation dates.
12. On approximately day 6 in culture, add 1 µL (0.002 U) of active plasmin to each tube.
13. After the clots dissolve completely (usually within a few hours), remove the medium and replace it with fresh medium without plasmin. If necessary, slices can be incubated with plasmin overnight and the medium changed the next day.
14. Slices are usually incubated for at least 7-10 days before use in experiments. Aspirate medium and replace it on day 3 or 4, again on day 7, and every 7 days thereafter.

5. Preparation of Viral Vectors for Transgene Expression

NOTE: Expression of transgenes in neurons of slice cultures is achieved either by using brains from genetically engineered rodents or by introducing the transgene by infection with recombinant replication deficient viruses. Adenoviruses (AV), adeno-associated viruses (AAV), and recombinant lentivirus vectors have all been used in our hippocampal slice cultures for expression of different fluorescent protein chimeras in brain slices.

1. Prepare replication deficient AV for expressing the RNA of interest according to methods described elsewhere[13,14]. Titer the viruses for infectious U/mL by the serial dilution method using an antibody to a virally expressed protein as described[14]. To observe cofilin aggregates and cofilin-actin rod formation, utilize the cofilin-R21Q-mRFP cDNA (plasmid #51279)[16].
   NOTE: The synapsin 1 promoter is an excellent choice for neuronal specific expression[15], whereas the cytomegalovirus promoter is useful for driving high expression levels in many cell types[13].
2. Prepare AAV by co-transfection of transfer plasmid containing the gene of interest and a rep/cap plasmid, with or without a helper plasmid, into HEK293 packaging cells, which supply the viral E1 gene, as previously described[17,18].
   NOTE: Recombinant AAV can also be made for targeted insertion into the host cell genome[19]. For the transfer plasmid, we use human cofilin 1 with a C-terminal mRFP1 fluorescence protein tag (plasmid #50856) cloned into a synapsin promoter-containing AAV plasmid downstream from the calcium sensor GCaMP5G[20]. A piece of DNA encoding the P2A self-cleaving peptide sequence is inserted by PCR between GCaMP5G and cofilin-RFP during the preparation of the transfer plasmid to provide expression of both proteins from a single AAV transcript[21].
3. Prepare recombinant lentivirus vectors by co-transfection of transfer plasmid containing the gene or cDNA of interest and integration signals, along with a third-generation lentivirus packaging mixture that divides the viral gag, pol, rev, and vsv-g genes onto three separate plasmids[22,23].
4. For the transfer plasmid, use a single step cloning system[24] to assemble the synapsin promoter and cofilin-R21Q-mRFP cDNA (from plasmid #51279) into pLKO.1-GFP (plasmid #30323), with the synapsin promoter and cofilin-R21Q-mRFP replacing the hPGK promoter and GFP cDNA, respectively.
5. Transfect the final plasmid into HEK293T cells by calcium phosphate as previously described[23].
6. Collect medium from four 10 cm dishes, concentrate to 500 µL using 150K-cutoff centrifugal concentrators, and store the final lentivirus at -80 °C in small aliquots after quick freezing in liquid nitrogen. Thaw an aliquot only once for infecting cells.
7. Determine empirically the volume of each virus type prepared to achieve the degree of expression desired by setting up a number of different slice cultures to follow the expression of the transgenes after infection with various volumes of virus.
   NOTE: Typically, 1-10 µL of virus is used per slice.

6. Slice Treatments

1. **Infecting slices with virus**
   1. Working in a biological safety cabinet approved for virus work at the biological safety level appropriate for the vector, mix an aliquot of the virus (usually 1-10 µL) with 0.8 mL of complete medium.
   2. Aspirate the medium from the slice using a sterile Pasteur pipette into a collection trap containing bleach. A secondary trap is always used between the first trap and the vacuum source.
   3. Replace this medium with the virus-containing aliquot prepared above, return the culture tubes to a rack, and place in the incubator.
   4. After 2-5 days of incubating the slices with virus in the biological safety cabinet, remove the virus-containing medium with a sterile transfer pipette and place it into a bottle containing an approved antiviral agent to kill virus.

2. **Staining of neurons with vital dye**
   1. Prepare and store aliquots of fluorescent neuronal vital dye[25] by quick freezing 4 µL of aliquots in liquid nitrogen at a concentration of 100 µM and store these at -20 °C. Do not freeze/thaw the dye more than once.
   2. To label neurons for visualization by fluorescence microscopy, thaw one aliquot of the neuronal vital dye and dilute to 4 mL in complete Neurobasal medium (final dye concentration is 100 nM).
   3. Remove the medium from slices by aspiration (or with a transfer pipette if the medium contains virus) and replace it with 0.8 mL of the medium containing 100 nM neuronal vital dye. Return the slices to the roller apparatus in the incubator.
   4. After incubating the slices for 2 h, aspirate the dye-containing medium and replace it with 0.8 mL of fresh complete medium in a biological safety cabinet.
      NOTE: Labeling of neurons in slices with vital dye requires several hours of incubation. The first images are usually taken 24 h after dye treatment. Although neurons are specifically labeled, there is background fluorescence that declines over 2-3 days to give better neuronal imaging. Intensity of the vital dye declines after 72 h.
   5. To follow changes in slice morphology over time, relabel the slices every 7 days.

7. Slice Imaging

1. View slices on an inverted microscope. For brightest fluorescence imaging, at 24 h before imaging, exchange culture medium with complete Neurobasal A medium without the Phenol Red pH indicator.
   NOTE: For experiments reported here, slices are viewed on an inverted spinning disc confocal fluorescence microscope equipped with a linear encoded x, y stage with piezo z control and a sensitive high-resolution digital camera.
2. Transfer the tube with the slice culture to be imaged from the roller tube apparatus to the custom-made tube holder (**Figure 5A**), which is placed in the stage adapter (**Figure 5B**), to keep the coverslip perpendicular to the objective and maintain the slice in the same orientation during repetitive imaging sessions over long intervals.
3. Push the slider on the stage adapter tight against the tube to hold the tube in position (**Figure 5B**). Maintain slice temperature at 35 °C during imaging by use of heating strips and a thermoregulatory controller built into the custom-made stage adapter (**Figure 5C**).
   NOTE: Details of the tube holder, stage adapter, and heater can be accessed at: https://vpr.colostate.edu/min/custom-machining/roller-tube-holder/.
4. Using a low power (*e.g.*, 4X) objective and bright field transillumination, focus on the photoetched grid pattern (**Figure 6A**) under the slice. For the initial imaging session, quickly scan around the slice to locate and record the number for the various regions where higher magnification imaging is desired (*e.g.*, cornu ammonis (CA)1, CA3, dentate gyrus (DG), *etc.*).
5. Move the stage to the first grid square containing a region of interest. Switch to the 20X air objective and locate a fiducial mark (*e.g.*, tip of an etched number) (**Figure 6B**).
6. Switch to a higher power objective (40X or 60X), localize the fiducial mark, and then record the x and y position of the stage. Move the stage to find the field(s) of interest nearby and record their x and y offsets from the fiducial mark (**Figure 6B**, arrow). Repeat in other grid areas if desired.
   NOTE: These off-set positions from the fiducial mark allow consistent pinpointing of the same coverslip location when the slice is imaged, even though the original x, y setting of the fiducial mark changes when the tube or stage adapter are removed and replaced.
7. Capture an image Z-stack within each selected field using either the microscope objective control or a piezo stage control, if available.
   NOTE: Image planes are usually obtained at intervals of 0.5 µm to 2 µm, depending on the size and desired resolution of the image features. Building a quality 3D image requires that image features extend over multiple planes of acquisition, and so to visualize smaller features, smaller intervals are required between planes.
8. Keep the total imaging time for each slice as short as possible.
   NOTE: Most imaging sessions reported here were under 18 min/slice. However, we have imaged some slices ten or more times, and even as long as 40 min in a single session, without apparent harm in the long-term survival of the slice.

Representative Results

To determine how accurately fiducial marks can be utilized to reimage the same cells within the same fields over time, we examined slices grown on photoetched coverslips (**Figure 6A**). Neurons were visualized by staining with a vital dye (100 nM for 2 h; does not stain non-neuronal cells), which disappears from neurons over time without harming the cells[25]. We identified a fiducial mark in a single grid square (**Figure 6A, B**), found a region of vital dye-labeled neurons 24 h after labeling, recorded the x and y offset positions from the fiducial mark (**Figure 6B**), and collected, using a 60X objective, confocal image stacks of this region, repeating the imaging on 4 consecutive days. The maximum projection images of a 30 µm image stack taken in the same location are shown (**Figure 6C-F**). Although some morphological changes occur within the region over 4 days, the identical cells (several of which are marked) can be followed over time. The fluorescence intensity of the vital dye declined over time but neurons were still clearly identifiable 4 days after labeling. Although most of the neuronal vital dye fluorescence was diffuse within the cytoplasm, some punctate staining was always observed, which became more noticeable as background fluorescence declined. In unhealthy slices, a punctate staining of non-neuronal cells was also observed as slices deteriorated. These results demonstrate that identified cells in slices can be repetitively imaged by using fiducial marks to find them.

To examine time-dependent changes in neuronal organization and viability within slices during long-term culture, we followed the same slices over 5 weeks, labeling with fluorescent neuronal vital dye once per week 24 h before imaging. Multiple rounds of staining with this vital dye over several weeks increased accumulation of aggregates. Neurons within freshly plated slices that were still in plasma clots were loaded with dye and imaged at one day *in vitro* (1 DIV). The same slices were imaged again weekly for 5 weeks. Images obtained from a single slice with a 4x objective are shown (**Figure 7A-E**). The pyramidal cell layers of the CA and DG regions are brightly labeled when excited at 488 nm and fluorescence emission measured at > 620 nm. Over a 5-week period of observing 19 slices, three slices came off the coverslip and two others lost their typical morphology and became opaque, an indication of their death. Thus, a survival rate of about 70% for experiments should be considered, and extra slices prepared to assure an adequate number for analysis. Slices were prepared at a nominal thickness setting on the tissue chopper of 300 µm. After 5 weeks in culture, we measured the slice thickness by imaging neuronal vital dye-stained slices from the coverslip up through the slice with a 40X oil objective on a spinning disc confocal microscope. Loss of focus of neurons occurred at an average of 257 nm (n = 5 slices with multiple locations used per slice), demonstrating that very little thinning of the slice had occurred from the time of plating. We could not accurately measure the slice thickness by fluorescence microscopy at the time of plating because the vital dye entrapped in the plasma clot gave diffuse fluorescence making it difficult to accurately measure the position at which loss of focus occurred. However, 3D images of neurons within slices are easily obtained in slices after the plasma clot is removed. The 21 DIV slice, shown at low magnification in **Figure 7F**, was imaged with a 60X objective on a confocal microscope (1 µm steps) 3 days after loading with the neuronal vital dye. A 60 µm 3D image was built from the focal planes (**Figure 7G**). Neurons and their neurite processes that are labeled with the vital dye can be followed in 3D. Morphology and 3D structure of slices were well-maintained over at least 3 months, and the longest times were used in the current study.

Major changes in the morphology of a previously imaged position also occurred in some slices, suggesting that movement of the slice on the coverslip may take place. Certainly, over longer intervals between imaging it became more difficult to know with certainty that the cells in the field being imaged were identical to the ones observed in previous imaging sessions. Thus, maximum projection images of confocal stacks of vital dye-labeled slices acquired at the identical location with the 60x objective at weekly intervals spanning 4 weeks (**Figure 8**) show that neuronal viability is well maintained but that it is difficult to identify a specific neuron over time when images are obtained with long time intervals between sessions. Presumably the pattern of cells labeled with multiple fluorophores would be more easily recognized, as are cells in localized groups when observed by scrolling through an image stack.

To assess the usefulness of different viral vectors for introduction of exogenous genes in neurons of hippocampal slices, we compared slice infectivity using AV, AAV, and recombinant lentiviral vectors, each expressing different fluorescent tags or using different promoters to drive expression. AV (2 x 10^7 infectious U/slice) expressing cofilin-mRFP behind a strong, non-cell specific CMV promoter was used to infect a mouse hippocampal slice that had been cultured 9 weeks on coverslips. Expression of cofilin-mRFP was found throughout the slice at 5 days post-infection, with expression most intense around the slice periphery as observed with a 4x objective (**Figure 9A**). Cells within the slice expressing cofilin-mRFP were also observed with a 20X objective (**Figure 9B**) with some bright punctate staining and also diffuse expression in both neurons and non-neuronal cells. After 17 weeks in culture (8 weeks post-infection), spontaneous cofilin-rods had formed in some cells, presumably driven by overexpression of wild type cofilin-mRFP (**Figure 9C**)[26,27].

We also demonstrated that AAV (10^{10} particles) could be used for expression in slices. Images of slices infected at 9 weeks in culture with AAV, in which a neuronal specific synapsin promoter drove expression of GCaMP5-cofilin-mRFP with a self-cleaving P2A peptide sequence in the linker of the translated polyprotein[21], were captured 8 weeks post-infection (17 weeks in culture). In neurons expressing the GCaMP5 and cofilin-mRFP, some cofilin rods/aggregates formed (**Figure 9D-F**). The fluorescence intensity of the rods/aggregates was so strong that very little fluorescence of a diffuse cofilin-mRFP could be observed without complete saturation and blossoming of the cofilin fluorescence image of rods. Spontaneous cofilin rods appear in neurons in which wild-type cofilin-fluorescent protein chimeras have been over-expressed[26,27], as well as in stressed neurons[10]. Based on the titers of adenovirus, which are determined on the basis of infectivity[14] and the particle counts used for determining AAV titer, about 100 to 500 fold higher particle numbers of AAV are needed to obtain approximately the same infectivity/expression in slices compared to AV.

To follow recombinant lentivirus-mediated expression of fluorescent proteins in slices, slices were infected at 6 DIV with 1, 3, 10, and 30 µL aliquots of a recombinant lentivirus for neuronal specific expression (synapsin promoter) of cofilin-R21Q-mRFP, developed as a live cell imaging probe for cofilin-actin rod formation[16]. Slices were labeled with neuronal vital dye at 11 DIV and imaged in specific regions for the dye and cofilin-R21Q-mRFP expression on 12 and 14 DIV. The slice infected with the 30 µL aliquot of virus did not survive for imaging but triplicate slices treated with the other volumes of virus showed a dose-dependent expression of mRFP. **Figure 10** shows images of slices infected with 1 µL and 10 µL of lentivirus at 6 and 8 days post-infection. Multiple regions of two different slices were quantified for co-staining of neurons with the vital dye and mRFP expression. For slices infected with 1 µL of lentivirus, about 28% of neurons expressed mRFP at 6 days post-infection, increasing to 85% by 8 days post-infection. For slices infected with 10 µL of lentivirus, about 58% of neurons expressed mRFP at 6 days post-infection, increasing to 86% by 8 days post-infection. Thus, 1 µL of the lentivirus was sufficient to provide widespread slice infectivity and neuronal expression by 8 days post-infection.

To demonstrate that this culture system is useful in following development of cofilin pathology, slices infected with lentivirus for expressing cofilin-R21Q-mRFP in neurons were left untreated or treated with various concentrations (1 µM, 333 nM, and 100 nM) of synthetic human Aβ protein that had undergone incubation to form oligomers[28]. Results from previous studies demonstrated that synthetic $A\beta_o$ induce cofilin-actin rods in up to 25% of dissociated hippocampal neurons[29-31]. All three slices treated with the 1 µM concentration of Aβ came loose from the coverslip in the first 24 h, whereas all vehicle treated slices (control) and those treated with the 333 nM and 100 nM concentrations of Aβ survived for the two weeks that they were followed. The same cellular regions (CA1, CA3, and DG) in a slice treated with 100 nM $A\beta_o$ were imaged (60x objective) over several days. Control slices that were infected on 6 DIV with lentivirus for expressing synapsin driven cofilinR21Q-mRFP had diffuse cellular mRFP expression by 15 DIV (**Figure 11A**). Slices exposed to 100 nM $A\beta_o$ at 14 DIV and imaged at 15 DIV showed that the distribution of cofilin-R21Q-mRFP became punctate, appearing in both rod shaped structures and aggregates (**Figure 11B**). These structures became even more prominent 6 days after Aβ-treatment (**Figure 11C**, which is the same field of cells as **Figure 11B**). In many places rich in neurites where neuronal cell somas are absent (**Figure 11D**), punctate and rod-like arrays of cofilinR21Q-mRFP developed (arrows in **Figure 11C**), similar to the distribution of cofilin-actin rods previously reported within neurites of Aβ-treated neurons in culture[29-31]. Thus, this new method for culturing and observing hippocampal slices will allow users to determine the long-term viability of cells in which cofilin aggregates and rods form and the reversibility of the pathology at various stages of development, and to easily perform dose-response measurements on reagents that could block or reverse the formation of cofilin pathology in a more *in vivo*-like cellular organization.

Copyright © 2017 Journal of Visualized Experiments

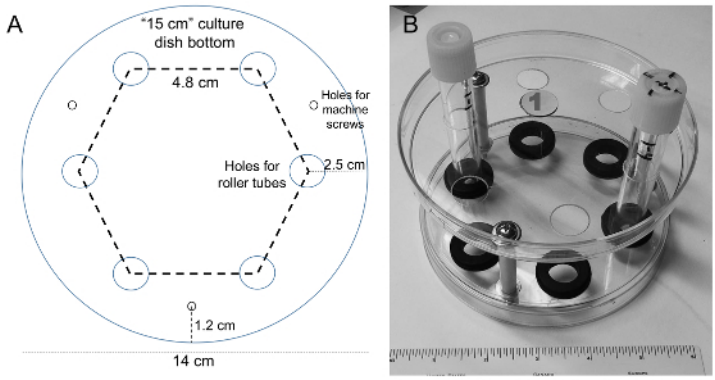

Figure 1: Preparation of roller tube rack. (**A**) Template for marking hole positions for drilling out the 15 cm tissue culture dish bottoms. If the figure is printed to the size of the scale bar shown, it can be cut out and used for marking the positions on a 15 cm culture dish for drilling the holes shown. (**B**) Completed roller tube rack with two tubes inserted. Each rack is numbered on a sticker easily visible on the top of the rack. Please click here to view a larger version of this figure.

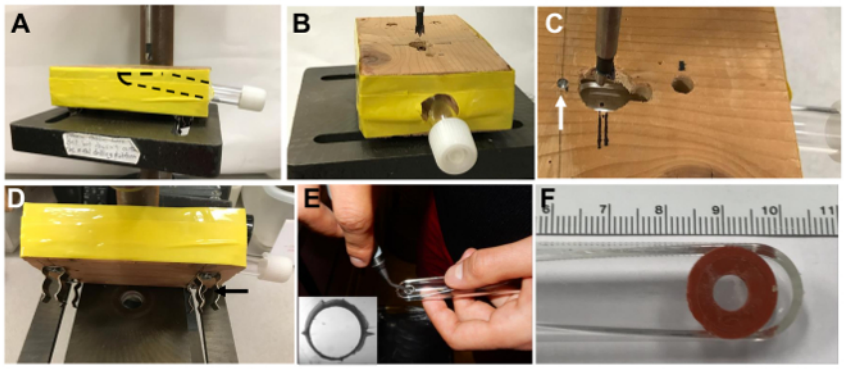

Figure 2: Preparation of the roller culture tubes. (**A**) Front view of the roller tube inserted in a jig on a drill press for drilling out the 6 mm hole in the tube. Dashed line shows the position of the flat sided roller tube in the jig. (**B**) End view of the jig with the tube inserted and drill bit aligned over hole. (**C**) Top view of the hole in the jig for drilling out roller tubes with a 6 mm drill bit. White arrow shows the position of the cut-off nail inserted as a stop for positioning the tubes, and black double lines are for bit alignment. (**D**) Spring clips (black arrow) installed on the jig bottom to securely hold it in position when drilling tubes. (**E**) After drilling out the hole, the edges are smoothed with a deburring tool and grooves are cut on the inner side of the hole (inset shows the hole viewed through a dissection microscope) to enhance medium draining from the hole during tube rotation. (**F**) Culture tube with the hole aligned to a hole in the silicone rubber adhesive, to which the coverslip will be attached. Please click here to view a larger version of this figure.

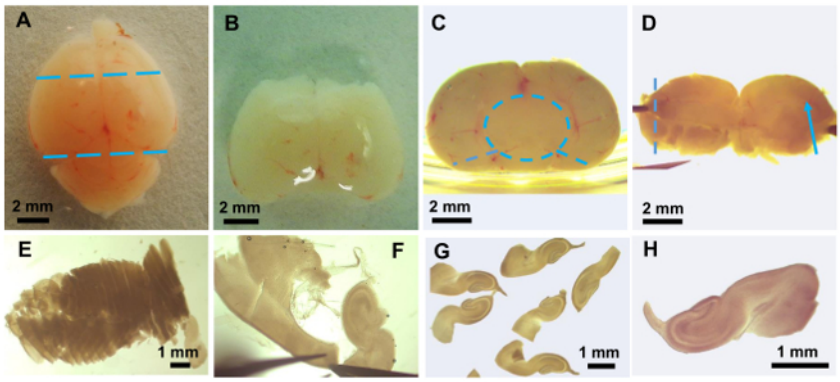

Figure 3: Preparation of hippocampal brain slices. Photos taken with a dissection microscope showing: (**A**) intact mouse brain. Position of cuts to remove forebrain and cerebellum are shown as blue dashed lines. (**B**) after removal of forebrain and cerebellum. (**C**) piece of brain from **B** is flipped 90° with posterior region (toward the cerebellum) facing up. Positioning the piece next to the side of the dish helps with the removal of the midbrain (blue dashed circle) which can be teased away from the remaining hippocampus, thalamus, and hypothalamus. Two cuts with the forceps (blue dashed lines) allow the remaining piece containing the hippocampus from both hemispheres to be spread flat. (**D**) The flattened brain piece showing the blood vessel running along the hippocampal fissure (blue arrow). This tissue is placed on plastic film and transferred to the tissue chopper for slicing in the direction of the dashed line. (**E**) Sliced tissue showing slightly more than half the hippocampus after being returned to GBSS/glucose. (**F**) Final dissection of the hippocampus and cleaning of the slices to remove non-hippocampal material. (**G**) Several floating slices after final clean-up. (**H**) Enlarged photo of a single slice for transfer to coverslip. Please click here to view a larger version of this figure.

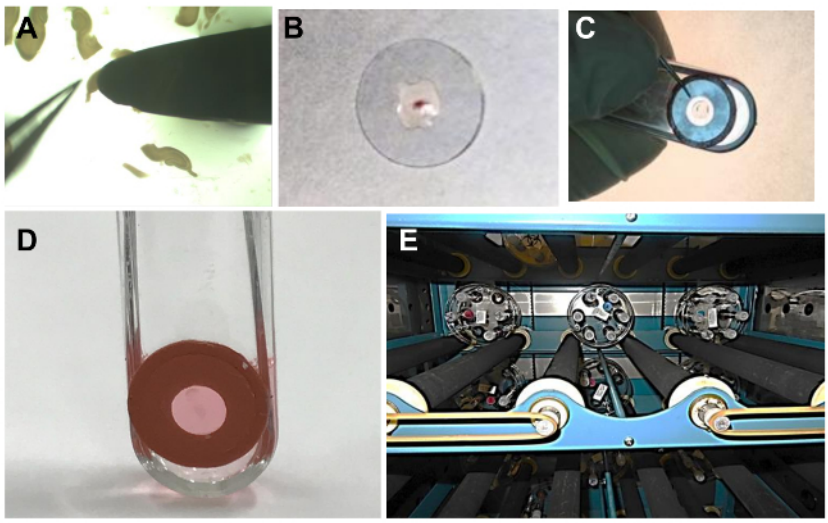

Figure 4: Plating and incubating slices. (**A**) A mouse hippocampal slice is removed from the culture dish on the tip of a spatula using the tip of forceps to help lift it free from the solution. (**B**) The slice is placed flat in the center of a photoetched and treated 12 mm coverslip on 2 µL of chicken plasma and another 2.5 µL of a 1:1 plasma/thrombin mixture is added to generate a clot. (**C**) After the clot is set (about 1-2 min), the covering is removed from the silicone rubber adhesive circle on a roller tube and the coverslip is positioned with the clot centered into the hole; then the coverslip is pressed in place with a thumb and held in position for about 1 min. (**D**) Add 0.8 mL of complete culture medium. (**E**) Roller tube holders inside of a large roller incubator with the front raised to tilt 5° to keep the medium at the bottom of the tubes. Please click here to view a larger version of this figure.

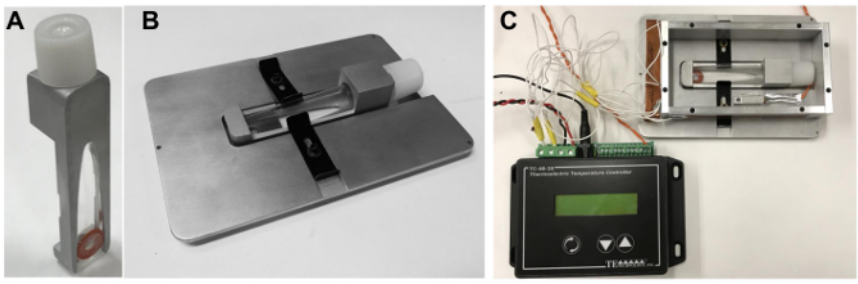

Figure 5: Roller tube holder and stage plate incubator. (**A**) Tube holder that positions the tube such that the coverslip is maintained in position for imaging. (**B**) Tube holder mounted in a microscope stage adapter plate. Sliders on the side hold the tube securely for imaging. (**C**) Stage adapter with the tube holder and side panels added containing heating strips connected to a thermoelectric temperature controller. Once tubes are mounted and positioned, a solid top to the box can be added to help maintain the temperature during imaging. Orange wire is the thermocouple lead. Plans for the design and building of the stage adapter and heater are available at: https://vpr.colostate.edu/min/custom-machining/roller-tube-holder/. Please click here to view a larger version of this figure.

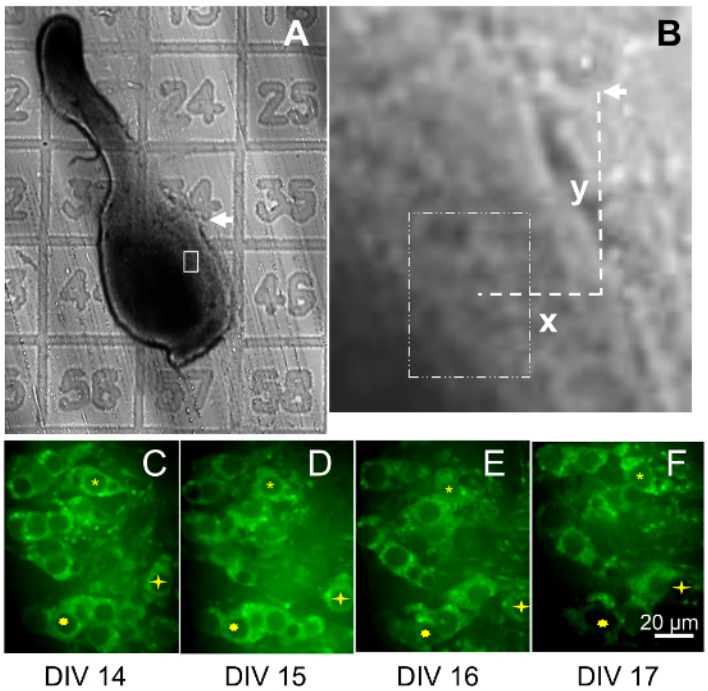

Figure 6: Using fiducial marks for repetitive imaging of same cells. (**A**) Hippocampal slice on photoetched coverslip (1 mm squares) with subiculum unfolded (tail) viewed with 4x objective and bright field illumination. Curvature of plastic roller tube helps create an oblique illumination that enhances the visualization of the grid. The box shows the position and size of a 60X field. (**B**) A view of the same slice with a 20x objective for finding the fiducial mark as the tip of the bottom of the 4 from square 34. The y and x offsets are shown to reproducibly locate the center of the desired box for higher magnification confocal imaging. (**C-F**) A slice labeled with neuronal vital dye 13 DIV was imaged using a 60x objective and making a 30 µm projection image on 4 consecutive days (14-17 DIV). Identical neurons were imaged each day. The position of the nucleus in each of three neurons is marked with a different symbol. They are more easily identified by scrolling through image stacks as their 3D position changes slightly. Please click here to view a larger version of this figure.

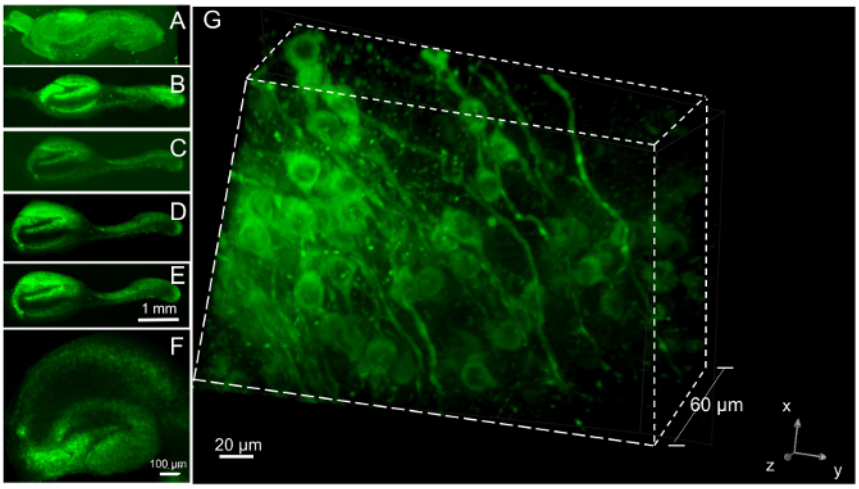

Figure 7: Imaging neurons in slices with a neuronal vital dye. (**A**) Neuronal vital dye stained slice in plasma clot taken 24 h after plating with 4X objective. Dye (100 nM) was added when the slice was first placed in the roller tube holder and washed out 2 h later. Subiculum is curled around the hippocampus in the clot. (**B-E**) Following clot dissolution by plasmin added at 6 DIV, the slice was reloaded 5 times with the vital dye 24 h in advance of imaging at weekly intervals. Images shown were collected at 8, 21, 28, and 35 DIV (**B-E**, respectively). (**F**) Hippocampal slice cultured for 3 weeks and stained with neuronal vital dye 24 h in advance of imaging with 4X objective. (**G**) Confocal stack of images on the same slice as in **F** showing a 3D view of 61 planes taken at 1 µm intervals. Neuronal vital dye clearly labels both neurites and cell body but it is excluded from the nucleus. Please click here to view a larger version of this figure.

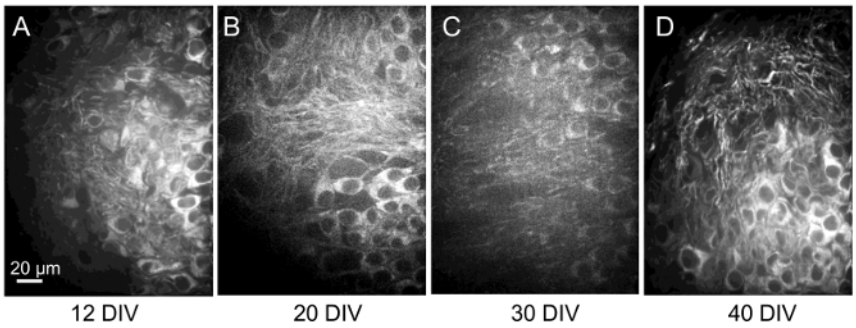

Figure 8: Repetitive imaging of neurons in a single location of the slice over 4 weeks. Maximum projection images of 30 µm confocal image stacks of the same field of cells (by positioning) from vital dye-labeled slices taken at 12, 20, 30, and 40 DIV (**A-D**, respectively). It is difficult to repetitively identify individual cells over the longer time frames in projection images. However, even over these long periods, identification of the same cells is often possible by scrolling through the image stacks or building 3D images that can be rotated, such as shown in **Figure 7G**. Please click here to view a larger version of this figure.

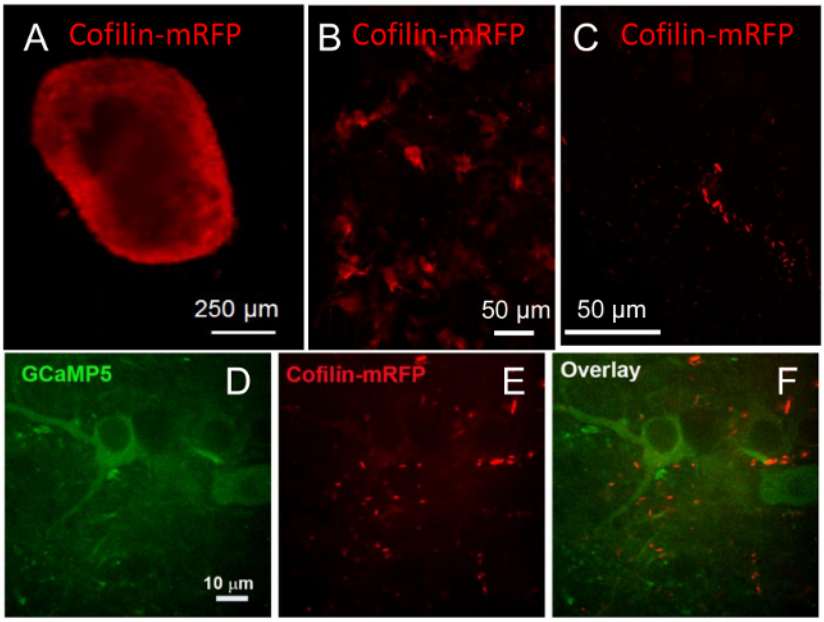

Figure 9: Viral-mediated expression and imaging of fluorescent proteins. (**A**) Hippocampal slice cultured for 9 weeks and infected with adenovirus for expressing cofilin-mRFP behind a CMV promoter. Expression was found throughout the slice at 5 days post-infection but fluorescence was brightest near the slice periphery. (**B**) Same slice showed expression in cells deeper within the slice when viewed with 20X objective. Image is a projection from a stack of 20 images spaced 2 µm apart. (**C**) The same slice was examined after 17 weeks in culture (8 weeks post-infection) and cofilin mRFP was observed in rod shaped aggregates as seen in this projection image from a 70 µm stack of 23 images, 3 µm apart, taken with a 40X objective. (**D-F**) Mouse hippocampal slice infected at 9 weeks in culture with an AAV expressing a GCaMP5-(P2A)-cofilin-mRFP behind a synapsin promoter. Fluorescence was visible in both red and green channels after 10 days. A single plane image of the slice showing the expression of (**D**) GCaMP5, a calcium sensitive reporter, (**E**) many cofilin-containing rods, and (**F**) an overlay image. Please click here to view a larger version of this figure.

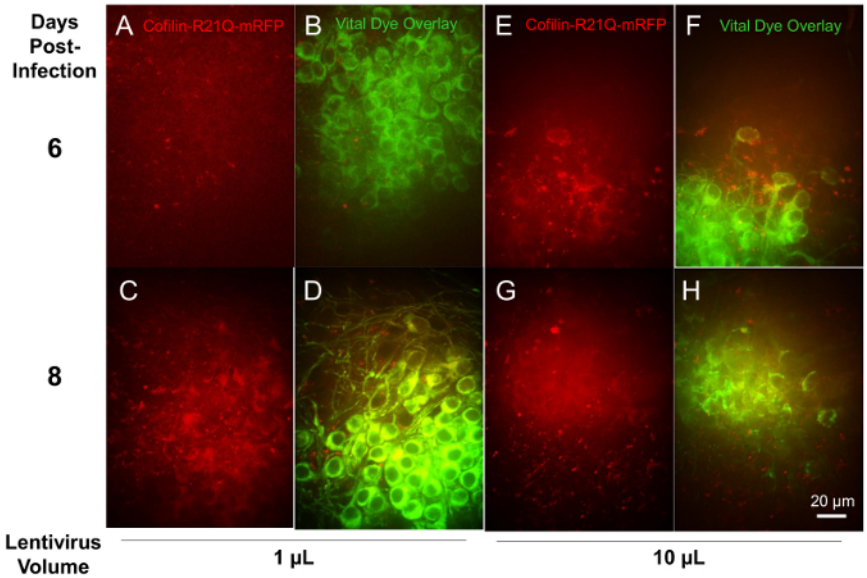

Figure 10: Expression of cofilin-R21Q-mRFP driven by a synapsin promoter in neurons infected with recombinant lentiviral vectors. Detection of a weak fluorescence signal is first observed by about 3-4 days post-infection using 10 µL of virus and becomes usable by 5-6 days, (**E**) as seen in these images acquired with a 60x objective. Although it takes longer to achieve the same levels of expression with 1 µL of virus, by 8 days post-infection a similar high percentage of neurons (vital dye labeled) were expressing the cofilin-R21Q-mRFP. Only 27% of neurons were positive for mRFP fluorescence at 6 days post-infection with 1 µL (**A**, **B**) but this increased to 85% (**C**, **D**) by 8 days. Please click here to view a larger version of this figure.

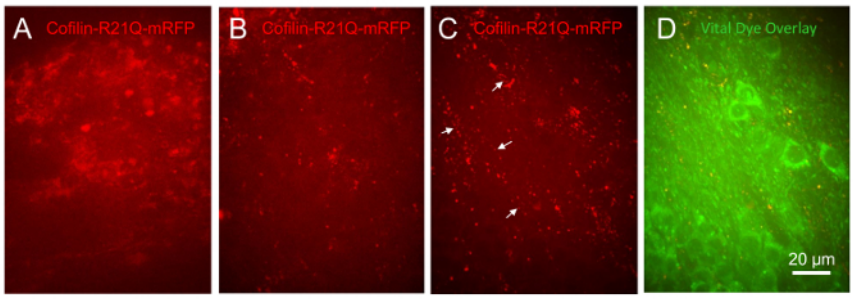

Figure 11: Aβ oligomer-induced cofilin pathology in mouse hippocampal slices. All images taken as 30 µm image stacks with a 60x objective and are shown as maximum projection images. Slices were infected with cofilin-R21Q-mRFP at 6 DIV. (**A**) 15 DIV slice treated on 14 DIV with vehicle (DMSO/HAMS F12 medium used to generate $A\beta_o$). (**B**) Slice 15 DIV treated with 100 nM $A\beta_o$. (**C**) Same field as in **B** taken at 20 DIV and shown in (**D**) as an overlay with neuronal vital dye label. Arrows show linear arrays of cofilin aggregates and rods in the region of the slice containing neurites but few cell bodies. Please click here to view a larger version of this figure.

Discussion

The roller tube method described here allows for long-term culturing and high-resolution live imaging of sliced brain tissue. One major issue with the slice technique as applied here is in the mounting and maintenance of slices. Coverslip coatings that support slice adhesion, promote slice thinning by enhancing the outgrowth of neurites and migration of cells out of the slice; thus, we avoided the use of these substrates. The insertion of amino groups onto the glass by treatment with 3-aminopropyltriethoxysilane improved the adherence of slices, but too little or too much chicken plasma on the coverslip can also cause adherence problems leading to slice loss. The volume of plasma needed for proper adhesion is dependent on the size of the cultured brain slice, and thus is greater for rat hippocampal slices, which are about 4 times larger in area than mouse brain slices. If too much plasma clots under the slice, cell adhesion to the coverslip is impaired and treatment with plasmin will loosen the slice so that it either changes position or detaches completely. However, too little plasma in the clot may lead to slice loss during the first few days of rotation in the incubator. In a recent experiment involving 39 slices, three were lost but some of those lost may have resulted from slice damage occurring during the slicing process. Nevertheless, we usually prepare about 50% more slices than the estimated number needed for the experiment. The second leading cause of culture problems is leakage of medium around the coverslip seal. This problem worsens when coverslips are not held firmly in position for at least 1 min after affixing them to the seal. Heat from the thumb used to apply pressure most likely helps complete the adhesion. Leakage that does occur is often through tiny air channels under the coverslip that can be observed with a dissection microscope. These usually disappear upon using prolonged thumb pressure. Loss of about 2% of cultures due to slow leakage can be expected and thus it is recommended to wait 10 days after setting up the cultures before performing viral infections. Excessive thumb pressure, especially if produced unevenly across the coverslip, can also cause the coverslip to crack. If breakage is an issue, pressing the tubes down flat onto a rubber mouse pad warmed in an incubator might help to provide more even pressure across the coverslip.

Previously described methods for brain slice culture on membranes at the air-liquid interface (open system) or on a glass coverslip inside of a sealed plastic tube (closed system) are very effective for long-term slice survival, but each method has its strengths and weaknesses. Slice cultures on membranes at the air liquid interface are advantageous for combined electrophysiological studies with immersion objectives for high-resolution imaging[7], but have drawbacks with regard to finding the exact field of cells for reimaging over time and potential user exposure and objective contamination when using viral-mediated gene expression. Use of viruses for expression of transgenes is safer and easier to perform in a closed system where contamination of microscope objectives is not an issue. Our modified roller tube method gives access of the slice for high-resolution imaging, although it is not amenable to electrophysiological studies.

Slice culture conditions have been established for many regions of the rodent brain[2], but here we utilize only hippocampus because it is one of the most widely studied brain regions and changes that occur in the hippocampus are of great interest in studies of cognitive impairment. The pyramidal cell layers of the CA and DG regions maintain their organization over several weeks in culture and can be readily observed morphologically. We have utilized a newly developed fluorescent neuronal viability marker[25], which has fluorescence properties that allow it to be used to monitor neuronal viability and organization within hippocampal slices over periods of days to months but also is compatible with the use of many other fluorescent proteins and reporters. Although not optimal for NeuO fluorescence[25], we can excite at NeuO at 488 nm and measure emission at >617 nm. Fiducial marks on the photoetched coverslips helped locate the same cells repetitively over many days of culture and allowed us to image identical regions of the slices over many weeks. Virtually no significant thinning of the slices occurred on the modified glass coverslips during 5 weeks in culture, the longest time point for which we obtained slice thickness measurements.

AV, AAV, and recombinant lentivirus vectors work well for expressing exogenous genes in slices. Lentivirus with a neuronal specific promoter is particularly useful for obtaining expression in a very high percentage (> 85%) of neurons within 8 days post-infection. Furthermore, we show that the cofilin-actin rod pathology associated with development of cognitive deficits in human AD[10,11] and Aβ overexpressing mouse AD models[32] can be monitored in slice cultures treated with relatively low concentrations (100 nM) of synthetic human $A\beta_o$. We envision that future applications of this method will include characterizing new therapeutics to reverse cofilin-actin rod pathology and/or correct dendritic spine abnormalities that occur in many neurological disorders[33].

References

1. Brewer, G. J., Torricelli, J. R., Evage, E. K., Price, P. J. Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. *J Neurosci Res.* **35**(5), 567-576 (1993).

2. Humpel, C. Organotypic brain slice cultures: a review. *Neuroscience.* **305**, 86-98 (2015).
3. Stoppini, L., Buchs, P. A., Muller, D. A simple method for organotypic cultures of nervous tissue. *J Neurosci Methods.* **37**(2), 173-182 (1991).
4. Organotypic monolayer cultures of nervous tissue. *J Neurosci Methods.* **4**(4), 329-342 (1981).
5. Gogolla, N., Galimberti, I., DePaola, V., Caroni, P. Long-term imaging of neuronal circuits in organotypic hippocampal slice cultures. *Nat Protoc.* **1**(3), 1223-1226 (2006).
6. Roo, M. D., Ribic, A. Analyzing structural plasticity of dendritic spines in organotypic slice culture. *Methods Mol Biol.* **1538**, 277-289 (2017).
7. Lee, K. F. H., Soares, C., Thivierge, J-P., Béïque, J-C. Correlated synaptic inputs drive dendritic calcium amplification and cooperative plasticity during clustered synapse development. *Neuron.* **89**(4), 784-799 (2016).
8. Davis, R.C., Maloney, M.T., Minamide, L.S., Flynn, K.C., Stonebraker, M.A., Bamburg, J. R. Mapping cofilin-actin rods in stressed hippocampal slices and the role of cdc42 in amyloid-beta-induced rods. *J Alzheimers Dis.* **18**(1), 35-50 (2009).
9. Clark, R.E., Squire, L.R. Similarity in form and function of the hippocampus in rodents, monkeys, and humans. *Proc Natl Acad Sci U S A.* **110** (Suppl 2), 10365-10370 (2013).
10. Minamide, L. S., Striegl, A. M., Boyle, J.A., Meberg, P. J., Bamburg, J. R. Neurodegenerative stimuli induce persistent ADF/cofilin-actin rods that disrupt distal neurite function. *Nature Cell Biol.* **2**(9), 628-636 (2000).
11. Rahman, T., *et al.* Cofilin rods and aggregates concur with tau pathology and the development of Alzheimer's disease. *J Alzheimers Dis.* **42**(4), 1443-1460 (2014).
12. Bamburg, J. R., Bernstein, B. W. Actin dynamics and cofilin-actin rods in Alzheimer disease. *Cytoskeleton(Hoboken).* **73**(9), 477-497 (2016).
13. He, T. C., Zhou, S., da Costa, L. T., Yu, J., Kinzler, K. W., Vogelstein, B. A simplified system for generating recombinant adenoviruses. *Proc Natl Acad Sci U S A.* **95**(5), 2509-2514 (1998).
14. Minamide, L.S., *et al.* Production and use of replication-deficient adenovirus for transgene expression in neurons. *Methods Cell Biol.* **71**, 387-416 (2003).
15. Kilic, E., Bähr, M. Human synapsin 1 gene promoter confers highly neuron-specific long-term transgene expression from an adenoviral vector in the adult rat brain depending on the transduced area. *Gene Ther.* **10**(4), 337-347, DOI:10.1038/sj.gt.3301905 (2003).
16. Mi, J., *et al.* A genetically encoded reporter for real-time imaging of cofilin-actin rods in living neurons. *PLOS ONE.* **8**(12): e83609 (2013).
17. Wang, L., Blouin, V., Brument, N., Bello-Roufal, M., Francois, A. Production and purification of recombinant adeno-associated vectors. *Methods Mol Biol.* **807**, 361-404 (2011).
18. Matsushita, T., *et al.* Adeno-associated virus vectors can be efficiently produced without helper virus. *Gene Therapy.* **5**(7), 938-945 (1998).
19. Ward, P., Walsh, C. E. Targeted integration of rAAV vector into the AAVS1 region. *Virology.* **433**(2), 356-366 (2012).
20. Akerboom, J., *et al.* Optimization of a GCaMP calcium indicator for neural activity imaging. *J Neurosci.* **32**(40), 13819-13840 (2012).
21. Kim, J. H., *et al.* High cleavage efficiency of a 2A peptide derived from porcine teschovirus-1 in human cell lines, zebrafish and mice. *PLOS ONE.* **6**(4): e18556 (2011).
22. Benskey, M. J., Manfredsson, F. P. Lentivirus production and purification. *Methods Mol Biol.* **1382**, 107-114 (2016).
23. Huang, L., Chen, C. Autoprocessing of human immunodeficiency virus type 1 protease miniprecursor fusions in mammalian cells. *AIDS Res Ther.* **7**, 27 (2010).
24. Bordat, A., Houvenaghel, M. C., German-Retana, S. Gibson assembly: an easy way to clone polyviral full-length infectious cDNA clones expressing an ectopic VPg. *Virol J.* **12**, 89 (2015).
25. Er, J. C., *et al.* NeuO: a fluorescent chemical probe for live neuron labeling. *Angew Chem Int Ed Engl.* **54**(8), 2242-2246 (2015).
26. Bernstein, B. W., Chen, H., Boyle, J. A., Bamburg, J. R. Formation of actin-ADF/cofilin rods transiently retards decline of mitochondrial potential and ATP in stressed neurons. *Am J Physiol Cell Physiol.* **291**(5), C828-C839 (2006).
27. Cichon, J., *et al.* Cofilin aggregation blocks intracellular trafficking and induces synaptic loss in hippocampal neurons. *J Biol Chem.* **287**(6), 3939-3929 (2012).
28. Stine, W. B. Jr, Dahlgren, K. N., Krafft, G. A., LaDu, M. J. *In vitro.* characterization of conditions for amyloid-beta peptide oligomerization and fibrillogenesis. *J Biol Chem.* **278**(13), 11612-11622 (2003).
29. Maloney, M.T., Minamide, L.S., Kinley, A.W., Boyle, J. A., Bamburg, J.R. Beta-secretase-cleaved amyloid precursor protein accumulates at actin inclusions induced in neurons by stress or amyloid beta: a feedforward mechanisms for Alzheimer's disease. *J Neurosci.* **25**(49), 11313-11321 (2005).
30. Davis, R. C. et al. Amyloid beta dimers/trimers potently induce cofilin-actin rods that are inhibited by maintaining cofilin phosphorylation. *Mol Neurodegener.* **6**:10 (2011).
31. Walsh, K. P., *et al.* Amyloid-β and proinflammatory cytokines utilize a prion protein-dependent pathway to activate NADPH oxidase and induce cofilin-actin rods in hippocampal neurons. *PLOS ONE.* **9**(4): e95995 (2014).
32. Woo, J. A., *et al.* RanBP9 at the intersection between cofilin and Aβ pathologies: rescue of neurodegenerative changes by RanBP9 reduction. *Cell Death Disease.* **6**,.1676 (2015).
33. Shaw, A. E., Bamburg, J. R. Peptide regulation of cofilin activity in the CNS: a novel therapeutic approach for treatment of multiple neurological disorders. *Pharmacol Ther.* **175**, 17-27 (2017).

Copyright © 2017 Journal of Visualized Experiments