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Establishing a Protocol to Culture Primary Hippocampal Neurons

by Ann Mae DiLeonardi

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14. ABSTRACT
The brain is the most complex organ of the body organized in discrete anatomical and functional regions. Cell culture systems are an efficient way to manipulate neuronal cells and evaluate their response. Cells from the hippocampus, the area of the brain associated with learning and memory, are commonly used for primary neuronal cultures. This report describes the steps necessary to establish a primary neuronal culture laboratory and evaluates various culturing techniques of primary hippocampal neuronal cultures to determine the best, most appropriate techniques for guiding current and future Army needs.

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1. Introduction

The brain is the most complex organ of the body organized in discrete anatomical and functional regions. The individual and network behaviors of these regions in the context of normal activity and disease states provide the impetus for much neuroscience research.[1] Cell culture systems are an efficient way to manipulate neuronal cells and evaluate their response. More specifically, mammalian cell culture systems provide a platform to perform repeatable, controlled experiments with widespread applications, including toxicity, accumulation of materials within cells, in situ health monitoring, incapacitation methods, traumatic injury, absorption of environment hazards and their effects, and so on.

Cell culture systems use explanted cells from the body kept alive in conditions set to mimic that of the living organism (controlled nutrients, temperature, pH and gases, etc.). Cells in culture can come from mitotic cell lines (non-neuronal) or those that continuously divide, including glia, fibroblast, epithelial, tumor, and endothelial cells; or post-mitotic cell lines that do not continuously divide, such as neurons. Culturing cells immediately following tissue explant are referred to as primary cultures.

Like any scientific technique, there are advantages and disadvantages to cell cultures. Limitations of utilizing a primary neuronal culture system include the inability to evaluate functional relationships between anatomical areas, behavior, or a whole tissue response. Only a couple different cell types can be represented at one time, and survivability is shorter making long-term analysis difficult. However, there are also numerous advantages to primary neuronal cultures that cannot be obtained in a whole animal model. Cell culture allows for the observation and manipulation at the single cell level. These experiments offer a more cost-effective means to perform repeatable, high-throughput data collection. They are precisely controlled allowing for direct study of intracellular signaling cascades, protein trafficking, and subcellular localization of constituents. One can “see” inside a cell to study cellular mechanisms without additional interfering systemic input.

Cells from the hippocampus, the area of the brain associated with learning and memory, are commonly used for primary neuronal cultures. These cultures are generated by extracting embryonic day-18 hippocampi. At this developmental stage, most of the cells are pyramidal neurons, which account for the majority of the total neuronal population. These cells also develop several phenotypic features in culture, such as well-formed dendrites with spines and functional synaptically connected networks. There are minimal glia cells present, and dentate granular neurons have not begun development at this time point, resulting in an almost

homologous cell culture population. This report describes the steps necessary to establish a primary neuronal culture laboratory and evaluates various culturing techniques of primary hippocampal neuronal cultures to determine the best, most appropriate techniques for guiding current and future Army needs.

2. Methods

The first step in establishing a new lab of primary neuronal cultures is to successfully grow uncontaminated cultures for several weeks. Adequate time should be allowed to master basic culturing technique before proceeding with experimental plans for cultures. It is safe to assume three to four separate cultures will be needed to work through the beginning stages of cultures to ensure healthy, consistent cultures.

2.1 Choosing and Preparing Workspace

There are several options of workspaces that are appropriate for cell culture, including a laminar flow hood (LFH), a Class II biosafety cabinet (BSCII), or a Class III biosafety cabinet (BSCIII). An LFH has an open front (no sash) and takes air from the environment, passes it through a high-efficiency particulate air (HEPA) filter to remove contaminants, and flows the sterile air from the back of the hood over the workspace out toward the user. These hoods are *only* to be used for cell culture work that does not contain any infectious material, presenting limitations of the type of work that can be performed in these hoods. A BSCII protects the specimen, the user, and the environment from infectious material. Air is pulled in from the cabinet (70% recycled) as well as the environment (30% room air), passed through a HEPA filter, and pushed down back into the cabinet. A BSC has a shield to further protect the specimen and the user. Contaminated air from the cabinet is also filtered through a HEPA filter before removal through exhaust back into the room. A BSCIII is similar to a class II unit with the only difference being the contaminated air is passed through the HEPA filter then released through a rooftop exhaust out of the laboratory. This is required if one desires to work with toxic fumes and gases and was not a requirement for the CCDC Army Research Laboratory (ARL) Cellular Biology and Neuroscience Facility. A BSCII was deemed most appropriate for ARL to provide adequate protection of both the user and the specimen. The BSC was cleaned prior to each use by lifting the sash to the marked height, turning on the blower, spraying with 70% EtOH, and sitting for 15–20 min. All steps using the hippocampi, culture dishes, or media were carried out in the biosafety cabinet to prevent them from getting infected and maintain a healthy culture. All items, including gloved hands, were sprayed with 70% EtOH prior to being placed inside the BSC.

2.2 Dish Preparation

Neurons must adhere to the substrate on which they will grow, either glass cover slips or silicone. Surfaces may be coated with either poly-l-lysine (PLL) or poly-d-lysine (PDL) to provide a positive charge to the surface, which improves the attachment of cells compared to the negatively charged surface of the plastic or glass.[2] To determine if there was a difference in the number of attached neurons based on substrate, PLL- and PDL-coated cover slips were used. PDL glass cover slips were obtained from BrainBits LLC while PLL cover slips were prepared in the lab as follows.

Two days prior to culturing neurons, 22×22-mm cover slips (Corning, cat. no. 2845-22) were placed in a glass Pyrex baking dish on gauze pads then covered with aluminum foil. The cover slips were sterilized in an autoclave at 138 °C for 30 min. Once the cover slips cooled, the baking dish was moved to the biosafety cabinet and a single cover slip was placed in each well of sterile Costar 6 well plates (Corning, cat. no. 3736). Stock of 1 mg/mL of PLL (Sigma, cat. no. P1524) was made, aliquoted, and stored at –20 °C for future use. Stock PLL was defrosted and diluted to 0.01 mg/mL by adding 1 mL of stock PLL to 99 mL of sterile water. Working PLL was filter-sterilized by passing the solution through a 0.22-µM filter. Then, 3 mL of 0.01 mg/mL PLL was added to each well, and plates were left in the BSC with the blower off for 20–24 h. The following day, excess PLL was removed from the plates using vacuum suction, and cover slips were washed with sterile water twice for 10 min. After the second wash, additional sterile water was put on the cover slips and plates were moved to the incubator at 37 °C and 5% CO_2 overnight until culturing the following day. On the day of the cultures, about 20–24 h after the sterile water washes, the water was removed from the wells using vacuum suction, and 1.5 mL of filter-sterilized feeding media (Neurobasal media, Gibco, cat. no. 21103049; 2% B-27, Invitrogen, cat. no. 17504044; 0.4-mM GlutaMAX, Invitrogen, cat. no. 35050061) was placed in the wells. Plates were returned to the incubator. From this point on, all solutions were filter-sterilized using a 0.22-µM filter and warmed to 37 °C using a water bath prior to use.

2.3 Neuron Isolation

Hippocampi from an embryonic day-18 pregnant rat (dam) are typically collected as described in Banker.[2] Briefly, the dam is anesthetized with CO_2, a caesarean section is performed to remove the pups, which are then placed on ice, the pups are decapitated, and the brains are extracted and dissected to isolate hippocampi. At ARL, embryonic day-18 rat hippocampi (cat. no. SDEHP) were purchased from

BrainBits LLC (Springfield, Illinois). Hippocampi arrived in BrainBits LLC Hibernate media and were stored at 4 °C for 24 h prior to culturing.*

2.4 Dissociation

Two commonly used dissociating reagents (trypsin/DNaseI and papain) were used to determine which worked best with hippocampi from BrainBits LLC. Trypsin is a member of the serine protease family and is commonly used to detach cells to create a single cell suspension. It is used in combination with DNase I, a deoxyribonuclease, which prevents clumping in concentrated cell suspensions. Papain is a sulfhydryl protease also used for cell dissociation.

When ready to culture, hippocampi were carefully removed from the BrainBits LLC vials using a glass Pasteur pipette and placed in an empty 15-mL conical tube. Once all hippocampi were in the tube, the remaining hibernate media was removed, taking care to not disturb the hippocampi at the bottom. Next, hippocampi were incubated in 5 mL of feeding media containing trypsin (0.3 mg/mL, Sigma-Aldrich, cat. no. T9935-100mg) and DNase I (0.2 mg/mL, Sigma-Aldrich, cat. no. 10104159001) or papain (2 mg/mL, BrainBits LLC) for 20 min in the incubator at 37 °C. Hippocampi were carefully removed and placed into a clean 15-mL conical tube using a glass Pasteur pipette, and the remaining feeding media with trypsin/DNaseI or papain was removed. At that point, 2 to 5 mL of plating media (feeding media containing 5% heat-inactivated fetal bovine serum; Sigma-Aldrich, cat. no. F4135) was added to the hippocampi.

Hippocampi were dissociated by repeatedly pipetting them up and down in progressively smaller pipettes—10-mL serological pipette for 10 reps, then a regular Pasteur pipette for 10 reps, and finished with a flame-polished Pasteur pipette for 10 reps (BrainBits LLC, cat. no. FPP).† Solution was forcefully expelled against the wall of the tube, and care was taken to not expel the last bit from the pipette to reduce bubbles. The final solution appeared cloudy and had no visible chunks of tissue.

* BrainBits LLC order notes: Orders must be placed to BrainBits LLC by the Monday the week cultures are planned. BrainBits LLC dissects on Tuesday and ships overnight. Once the ARL warehouse receives the package from BrainBits LLC Wednesday, they will call for pickup.

† Flame-polished pipettes can be made in the lab using a Bunsen burner to reduce the tip diameter of the pipette by half. Bunsen burners CANNOT be used in the biosafety cabinet; therefore, we chose to purchase prefired, sterilized pipettes.

2.5 Titration and Cell Plating

Trypan blue was used to distinguish between live cells and dead cells, as the intact membrane of a healthy cell excludes the dye, and the dead cells absorb the dye. Therefore, healthy cells appear as bright spheres with clear centers amid the blue background, whereas the cytoplasm of a dead cell will appear blue. Ten microliters of the cell suspension was added to 90 µL of trypan blue (0.04%, Sigma-Aldrich, T8154) in a 500-µL tube. The tube was gently flicked to mix the cell suspension and trypan blue before adding10 µL of this solution to the hemacytometer to determine the cell density of the cell suspension. Cells from each of the four quadrants (orange boxes, Fig. 1A) were counted and averaged. The top and right boundaries were used as exclusion lines; cells touching these lines were not counted (red X, Fig. 1B), whereas those touching the bottom and left lines were included. The following equation was used to determine the number of cells per milliliter: [(A + B + C + D) / 4] × 10 × 1000 × 10 = cells/mL. If there were many clumps, cells within each clump were counted individually and the glass Pasteur pipette was used an additional five times to further dissociate the cells.

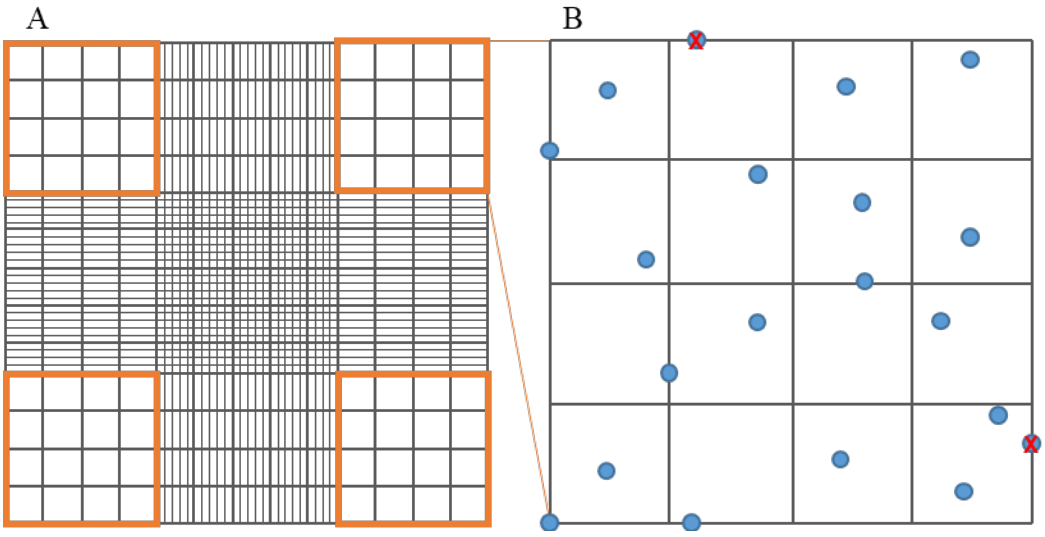

Fig. 1 Schmatic drawing of hemacytometer (A). Schematic representation of cell exclusion criteria used to perform cell count (B).

After calculating the number of cells per milliliter in the cell suspension, the following calculations were used to determine the amount of cell suspension to be added to the plating media and placed into each well:

a. Determine the volume of cell suspension (V) needed

$V = (W*X*Y) / Z$

W = No. of wells (e.g., 12 wells)

X = Desired volume to add to each well; add 1 mL per well

Y = Desired density; 100,000 cells/well

Z = cells/mL in suspension

b. Determine the amount of plating media

Volume plating media = (W*X) – V

These two volumes (cell suspension and plating media needed) were mixed gently in a conical tube by gently turning it upside-down and back a few times then pipetting up and down a few times. Then, 1 mL of titrated cell suspension was added to each well, and plates were returned to the incubator.*

Cells were then plated on PLL- or PDL-coated cover slips at densities of 50,000; 100,000; or 250,000 cells/cover slip to evaluate how different densities of cells respond to the different coating treatments. On day in vitro (DIV) 4, phase-contrast images were captured and the number of healthy cells, clumps of cells, and dead/degenerating cells were counted.

2.6 Time Course

Filter-sterilized, warmed feeding media was replaced 4 days after the initial plating, then 1–2 times per week. Cells were maintained at 37 °C, 5% CO_2 for up to 12 DIV.

An imaging study was conducted to track the progression of neuronal growth as a function of time in vitro. Images were collected using a Nikon inverted Eclipse microscope at 0 min, 1 h, DIV1, DIV4, DIV8, and DIV12 post plating. The number of heathy cells, clumps of cells, and dying cells were counted at DIV 4, 8, and 12. Coordinates were tracked to ensure the same location was used to capture an image (Appendix A).

2.7 Inhibition of Glial Growth

If the desired cultures needed to contain primary pyramidal neurons and not glia, the anti-mitotic agent Cytosine β-D-arabinofuranoside (Ara-C, 1 µM; Sigma, cat. no. C1768) was added 24 h post plating. Ara-C inhibits mitosis resulting in little to no glia being present in the culture. Twenty-five microliters of a 100-µM solution was added per well to reach a final concentration of 1 µM. In order to determine the purity of cultures, some cultures received Ara-C treatment while another group

* Total volume per well is 2.5 mL—1.5 mL of feeding media and 1 mL of cell suspension. This equation only works when adding 1 mL of cell suspension.

did not. Cells were grown and fixed at DIV12 as described above and processed for immunocytochemistry.

To prepare the cultures for immunocytochemistry, 1x PBS (recipe in Appendix B) was placed in the fridge at 4 °C. Stock solution of 16% paraformaldehyde (Sigma, cat. no. P-6148) was diluted to 4% with 1x PBS on the day of fixation. Media was removed from wells using vacuum suction, and 1 mL 4% paraformaldehyde was added to each well. Plates sat in the biosafety cabinet at room temperature for 15 min. Paraformaldehyde was removed with a vacuum syringe, and wells were washed 1x 15 min with cold 1x PBS. Fresh 1x PBS was added to the wells, and plates were stored at 4 °C until staining.*

Immunocytochemistry uses antibodies to visualize proteins within cells using fluorescence. This is a general technique that can be used to visualize a host of different proteins in/on a cell simply by using different primary antibodies. Briefly, cells were permeabilized with Triton X-100 (0.25%, Sigma-Aldrich, X100) for 10 min and blocked with bovine serum albumin (BSA, 10% in 1x PBS, Sigma-Aldrich cat. no. A4503) for 30 min. Cultures were incubated with the primary antibody for 1 h, followed by a 1-h incubation with the appropriate secondary. Antibodies were diluted in 1x PBS containing 1% BSA. Lastly, cultures were washed in 1x PBS and mounted to slides using Prolong Diamond (Life Technologies, cat no. P36971).

Antibodies for glial fibrillary acidic protein (GFAP, green), an intermediate filament found in astrocytes (a type of glia cell), and beta-III tubulin (red), building blocks of microtubules, were used to stain astrocytes and neurons, respectively. Cells were also treated with DAPI (4′,6-diamidino-2-phenylindole) a blue-fluorescent DNA stain.

3. Results

3.1 Effect of Coating on Cell Counts: PLL vs. PDL

Healthy neurons were identified as phase-bright, non-granulated somas with processes extending outward. As expected, there were more healthy cells present at the higher plating concentrations (50k<100k<250k) for both the PLL and PDL conditions (Fig. 2A–G). There were more healthy cells present on PDL cover slips compared to PLL cover slips. There were also more clumps of cells in the higher plating concentrations (50k<100k<250k). In general, there was more clumping in all the PDL cover slips regardless of plating density compared to the PLL cover

* If staining will not occur for several weeks, be sure there is adequate volume of 1x PBS per well to ensure cover slips will not dry out.

slips (Fig. 2H). There were not many dying/degenerating cells in PLL or PDL cover slips, and plating concentration had no effect on the number of dying/degenerating cells (Fig. 2I).

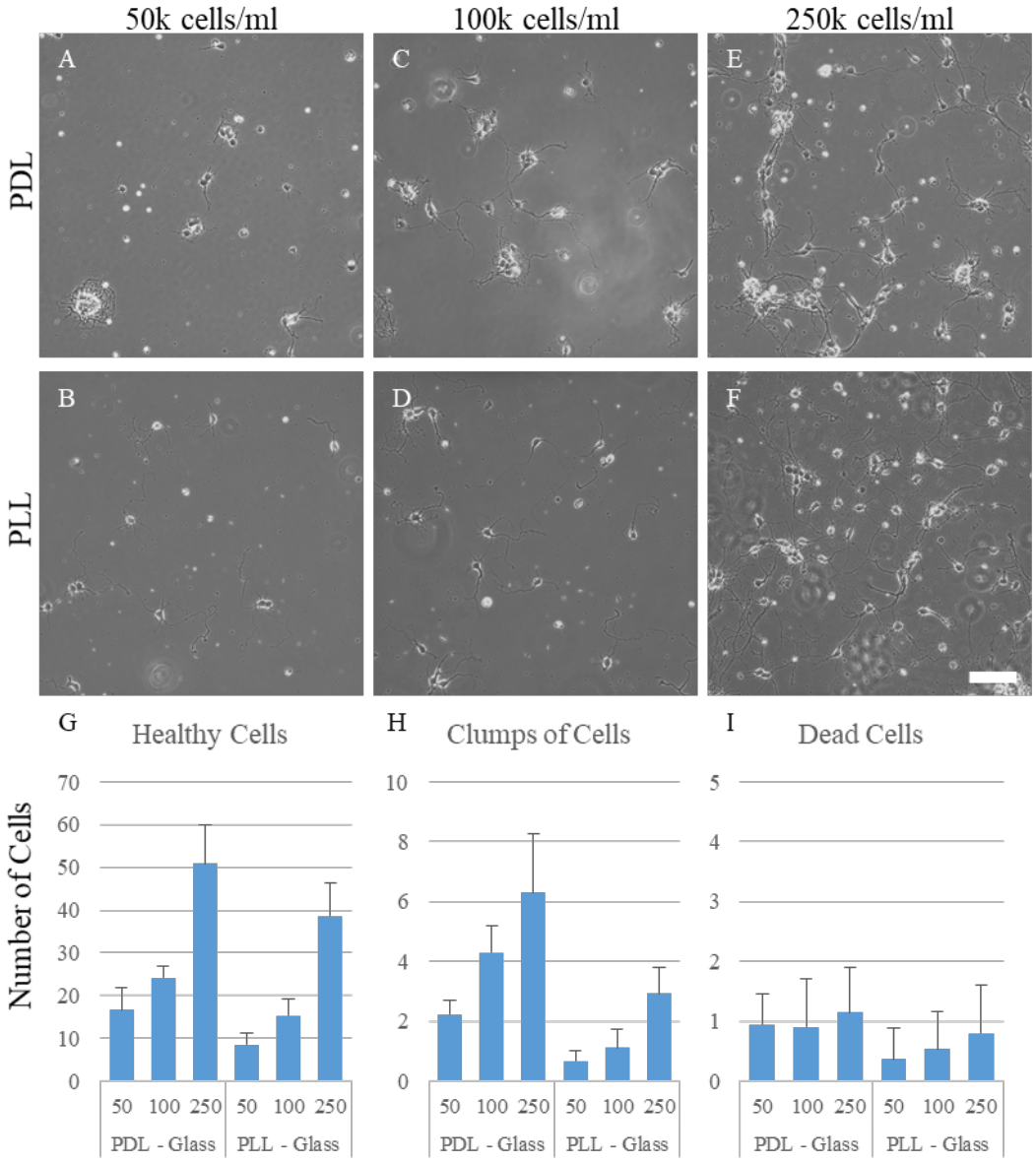

Fig. 2 Representative micrographs illustrating the number of neurons present after coating cover slips with PLL (A, C, E) or PDL (B, D, F) at densities of 50k cells/mL (A-B), 100k cells/mL (C–D), and 250k cells/mL (E–F). Quantification of the average number of cells per field of view of healthy cells (G), cell clumps (H), and dead cells (I). Scale bar = 100 µm.

3.2 Effect of Dissociation Agents: Typsin/DNaseI vs. Papain

Both cultures seeded with trypin-treated cells or papain-treated cells had cells that were healthy with long neurites protruding from the cell body and good separation between the cells at DIV4 (Fig. 3A–B). By DIV8 (Fig. 3C–D), neurites extended longer and cells still appeared healthy. By DIV13 (Fig. 3E–F), there were fewer cells present compared to earlier time points, and there was evidence of degeneration among some remaining healthy cells in both conditions. There were no qualitative differences observed between the trypsin- versus the papain-treated cultures. All subsequent cultures used trypsin because it is more commonly used in many hippocampal cultures.[3]

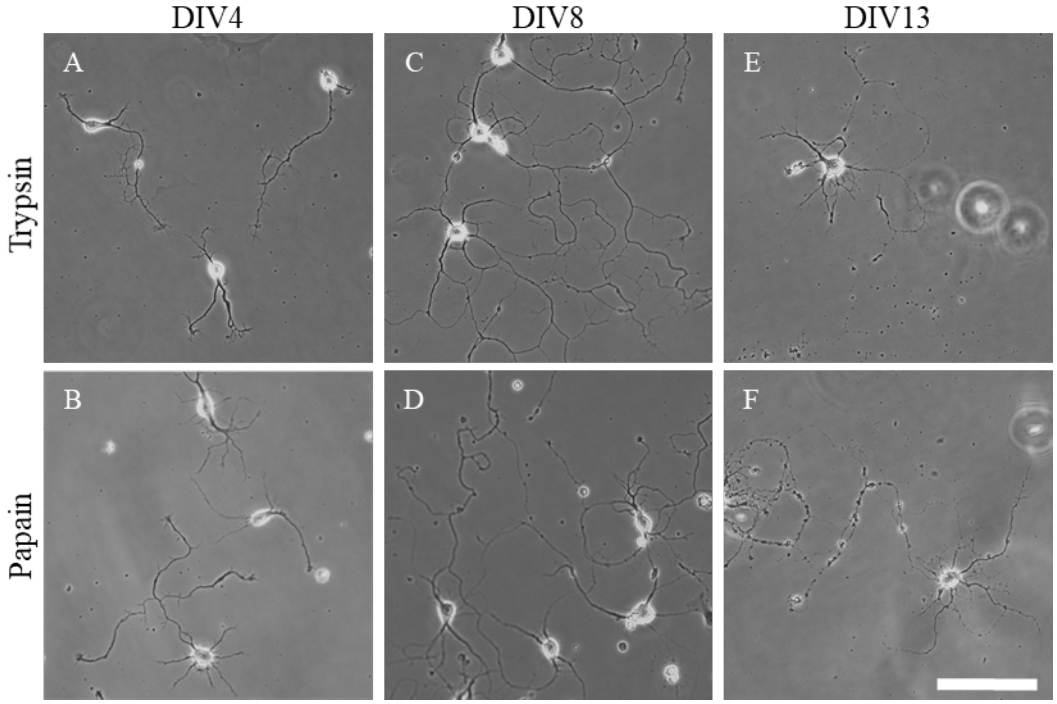

Fig. 3 Representative micrographs illustrating neurons at day in vitro (DIV) 4 (A–B), DIV8 (C–D), and DIV13 (E–F) following dissociation using trypsin (A, C, E) or papain (B, D, F). Scale = 100 µm.

3.3 Time Course: Tracking Cell Growth over Time in Culture

Neurons appeared as dark spheres surrounded by glowing halos immediately following plating. If the tray was shaken, most spheres moved but some immediately adhered to the surface (Fig. 4A). By 1 h post-plate, the cells have the same appearance, but most cells have adhered to the substrate and no longer move when the tray is shaken (Fig. 4B). Neurites were observed extending out from the soma at DIV1 (Fig. 4C) and increased in length at DIV4 (Fig. 5D). At this time, it

was sometimes possible to determine which neurite was the axon, the longest neurite, by capturing multiple images if the entire projection was not within one field of view. It was also noted that cells move around the culture and are not always in the same location between plating and DIV4, but remained in relatively the same locations at all time points beyond DIV4. At DIV8 (Fig. 4E) and DIV12 (Fig. 4F), neurons have remained in their respective locations, and neurites have increased in length and can sometimes be seen bundled together (Fig. 4F). Overall, there is a decrease in the number of healthy cells calculated as the days in vitro increase (Fig. 4G), while the days in vitro have no effect on the number of visible dead cells or cell clumps observed at any time point (Fig. 4H).

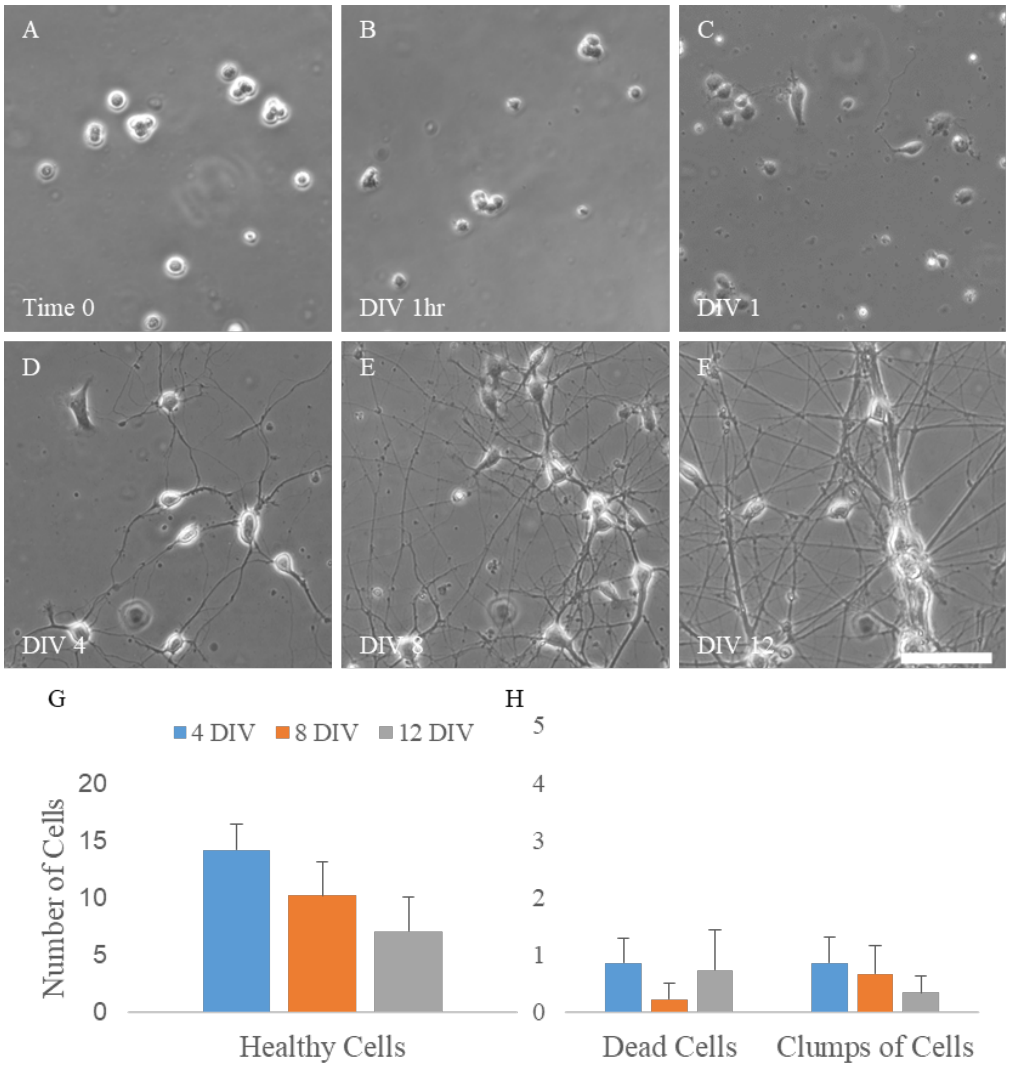

Fig. 4 Primary cultures were imaged over time starting immediately post-plating (A), 1 h post-plating (B), DIV1 (C), DIV4 (D), DIV8 (E), and DIV12 (F). Beginning at DIV4, images were captured at the same location for subsequent days. Number of healthy cells imaged per field decreased as DIV increased (G). There was no changes observed in the number of dead cells or cell clumps at any time point assessed. Scale bar = 100 µm.

3.4 Inhibition of Glial Growth

When Ara-C was excluded from the cultures, there were many GFAP reactive cells intermingled with the neurons (Fig. 5A). When cultures were treated with Ara-C, there was little to no GFAP staining, only tubulin reactivity (Fig. 5B). High magnification revealed astrocytes extended processes relatively short distances from their cell bodies, which terminated in a branched segment (Fig. 5C). Qualitatively, neurites in astrocyte-containing cultures appeared thicker with a less punctate appearance compared to their glia-free counterparts (Fig. 5C-D). However, the presence of astroctyes did not affect the number of healthy cells (Fig. 5E). Typically, there was an increase in the number of dead cells in the absence of astrocytes (Fig. 5F) as well as more clumps of cells when astrocytes were present (Fig. 5G).

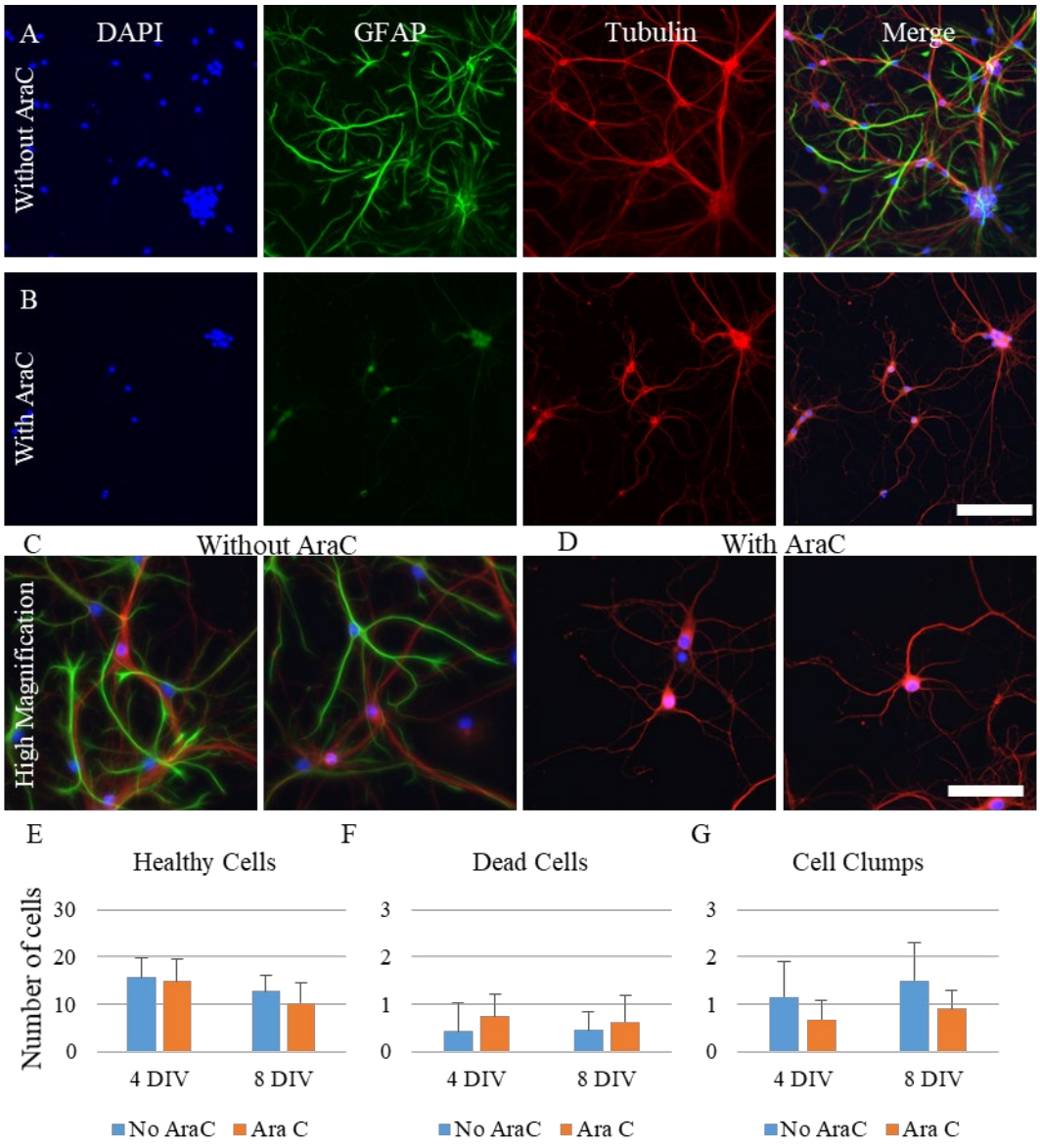

Fig. 5 Cultures not treated with Ara-C (A) contain astrocytes as evidenced by GFAP immunoreactivity. Neurons were visualized using an antibody for tubulin, and nuclei from both neurons and astrocytes were stained with DAPI. Cultures treated with Ara-C (B) did not have immunoreactivity for GFAP. Higher-magnification images reveal astrocytes growing along qualitatively thicker, contiguous neurites in cultures not treated with Ara-C (C). Neurites grown in the absence of astrocytes have a more punctate appearance (D). Cell counts of healthy cells (E), dead cells (F), and cell clumps (G) with or without Ara-C treatment. Scale bars = 100 µm.

4. Discussion

There are many common mistakes that may happen during the first few cultures regardless of the specific laboratory or research question. To reduce these mistakes, several helpful practices should be followed. Always be sure to filter all solutions before adding to the cells. Minimize the amount of talking done while working with cells or wear a mask. Practice good biosafety cabinet techniques: set up the space with all needed items prior to starting work, try to leave hands inside the cabinet and avoid moving in and out of the cabinet frequently, wear a mask if needed, minimize the motion of others behind the person working in the cabinet, and so on. Good biosafety cabinet techniques will reduce the likelihood of the cells becoming infected. Infections become apparent when the color of the media changes from pink to yellow, indicating a change in the pH of the media brought on by an infection. Sometimes, infected wells will appear cloudy. If a well becomes infected, use a clean Pasteur pipette to suction out all media from that well and leave it empty for the duration of the experiment. Typically, neighboring wells will remain uninfected.

Healthy neurons (Fig. 6A) will be visible using phase contrast microscopy as phase-bright, non-granulated soma (cell body) with neurites (axons and dendrites) extending from the soma. In the early days of culture, dendrites and axons are more easily identified—dendrites are shorter and taper at the ends, whereas the axon is longer and has a consistent thickness along the length of the process. This is readily apparent around 4DIV. However, as the neurons mature, the axon may circle around the soma, making it more difficult to differentiate between the two cellular constituents. For this reason, processes in culture are typically referred to as neurites.[2]

Dead or degenerating cells are not always easily detected and are more likely found in low-density cultures, pure neuronal cultures, or DIV greater than 2 weeks. Typically, the cell body will still have a halo; however, the middle will not be a solid gray circle and the neurites will appear as dotted lines (Fig. 6B).

Even if good biosafety cabinet techniques are followed, and the cultures are free of infection, other phenomena can lead to imperfect cultures. For example, bubbles may become trapped between the plastic plate and the glass cover slip during the coating and washing steps. These bubbles can negatively affect neuronal health. One way, in particular, these bubbles have been shown to affect the quality of the culture was by allowing a local area for PLL to crystalize on the cover slip (Fig. 6C). To reduce the likelihood of these bubbles, the water should be added directly to the top of the cover slip. Should bubbles still be present, tapping the tray gently

on the counter may help them to escape. To reduce the likelihood of crystallization, it is important that the PLL incubates for no longer than 24 h and is adequately washed. Following the protocol of washing PLL two times for 10 min followed by an overnight incubation with sterile water has eliminated the crystallization of PLL.

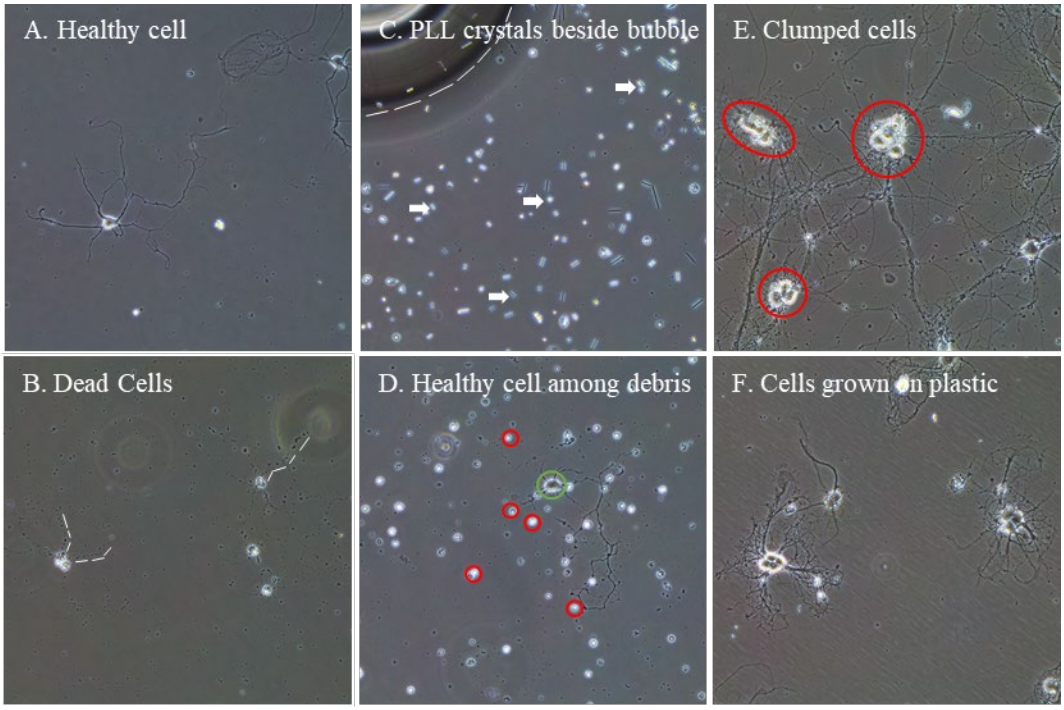

Fig. 6 Examples of initial cultures illustrate phase bright soma and neurites of a healthy cell (A) and fragmentation of degenerating cells (B). Other phenomena include dried PLL crystals on cover slips (C), random debris floating alongside healthy cells (D), clumps of cells (E), and cells growing under the cover slip on the plastic (F). All images were captured using a 20× objective.

Once cells are healthy, other factors can affect the quality of cultures for analysis. Floating debris among healthy cells may also occur in the cultures (Fig. 6D) but usually does not affect the health of the cells. The biggest influence of the debris is on degrading the quality of the images taken for analysis. Additional washing protocols can be implemented to the cover slips to reduce the likelihood of floating debris.[2]

Neurons can also aggregate into clumps, hindering the ability to analyze individual neurons (Fig. 6E). To reduce this aggregation, ensure that sufficient pipetting occurs during the dissociation step. The hemacytometer can be used to gauge the level of dissociation: if there are many clumps, pipette the suspension more. If you notice the cell walls are jagged and not intact, that is an indication that there was too much pipetting.

When cells are grown on glass cover slips in six well plates, some cells can grow on the plastic underneath the cover slip (Fig. 6F). For this reason, it is important to be sure the microscope is focusing on the glass and not the bottom of the well. If the microscope is focused on the plastic, there will be diagonal lines throughout the visual field; this is the grain of the plastic and an indication that the objective needs to move closer to the sample. Being mindful of these potential pitfalls will allow scientists to better answer research questions.

Along with the general practices of maintaining high-quality healthy cultures, specific protocols were determined and established for the needs of the research program investigating the effect of strains on neurons at ARL. Successful adhesion to the membrane/glass cover slip is crucial to cell survival and becomes especially important for experiments utilizing physical manipulation of the membrane where one operates under the assumption that if the membrane were to stretch by 30%, then the adhered cells were also being stretched by 30%. Neuronal cell membranes are negatively charged, and therefore a positively charged coating is applied to the growing substrate (i.e., cover slip or membrane).[2] For these experiments, either PDL or PLL was applied to the substrate prior to the plating of cells. Similar to others, we observed no major differences between the number of cells per unit area when comparing PLL to PDL with a trend of more cells in the PDL condition.[4] As anticipated, the number of cells per unit area increased as a function of increasing plating density. Qualitatively, we observed more clumping of neurons in the PDL condition compared to PLL, which was positively correlated to the plating density. Finally, there was no difference in the number of dead/dying cells present in either coating condition or plating density. There were not many noticeable differences between the PDL and PLL groups; however, clumping is not desirable for cell culture analysis, therefore all subsequent cultures will utilize PLL as the substrate. Also, having fewer cells makes it easier for single cell analysis, supporting the choice to use PLL for future cultures. It is important to understand how cells grow at different densities for future analysis. It is better to use a lower cell density for morphological and structural analysis because there is less clumping/overlap of the cells. However, if one were examining network function, then a denser culture of neurons would be necessary.

Cells must be dissociated prior to plating by using either trypsin or papain. Trypsin is a serine protease with specific cleaving activity of the C-terminal end of positively charged side chains, such as proteins that facilitate adhesion to the container as well as each other.[5] Due to its specificity, it is commonly used in conjunction with other proteolytic enzymes—in our case, we used DNase I.[6] Papain is a cysteine peptidase with broader specificity, which has less damaging effects on tissues and is therefore commonly used for neuronal cultures.[6] In our hands,

qualitative analysis revealed that neither neuron morphology nor cell counts were affected by the dissociation reagent. Regardless of the dissociation reagent used, cultures had similar numbers of viable cells as well as normal morphology with neurites extending similar lengths from the soma.

Cultures were observed frequently to document the morphological development of neurons in culture. In our hands, neurons adhered to the substrate (within the first hour) and extended neurites (by DIV4) on a time scale similar to what others have previously reported.[3] We did observe a decrease in the number of healthy cells as a function of number of days in vitro; however, there was no concomitant evidence of an increase in the number of degenerating cells present. There was no difference in the number of dead cells or clumps of cells on any of the days evaluated, suggesting that cells could have detached and been washed away during a feeding, leading to the reduction in cell number. The reduction in healthy cells is not surprising considering the low plating density. As discussed, low density is required for structural/morphological analysis of a single cell; however, in general, neurons have better survival at higher densities in the presence of glial support, which was not provided in these cultures.

Our original intention of examining glia in our cultures was to determine how prevalent they were in the cultures and if their presence altered the neuronal population. Ara-C is a post-mitotic inhibitor commonly used to remove glia from culture by inhibiting their proliferation, thereby leaving only those glia that were present during the initial plating[7] which is minimal at the time point from which the embryos were extracted.[3] Our data reveal numerous GFAP positive cells in the absence of Ara-C and minimal reactivity when the reagent is present. Interestingly, the neurites appear more robust and thicker when glia are present in the culture. There is no noticeable change to the number of dead cells or cell clumps. Many in vitro models include only neurons in culture, but neurons are surrounded by glial cells in the brain. Including other cell types in co-culture can dramatically change the response of those cells.[8] Glia offer support to neurons via release of neurotransmitters, secretion of trophic factors, and synthesis and release of molecules to shape the extracellular matrix, among other functions.[9] In culture, neurons prefer softer substrates, which glia can provide as evidenced by other in vitro models growing neurons on top of a layer of astrocytes.[10,11] The soft, compliant environment of the glia[11] and the increased compliance[12] may positively influence glia on neurons in culture.

5. Conclusions

In conclusion, we have established a successful protocol to culture primary hippocampal neurons purchased from a commercial source. Our goal is to determine functional and structural thresholds of injury following mechanical loading at different strains and strain rates. Initial studies will utilize the Cell Injury Controller II (VCU, Richmond, Virginia) to evaluate the dose-response effect of loading on structural changes. Furthermore, it is clear that the presence of glia in the cultures has the potential to alter their response. Future studies will evaluate structural alterations following injury under different glia conditions.

6. References

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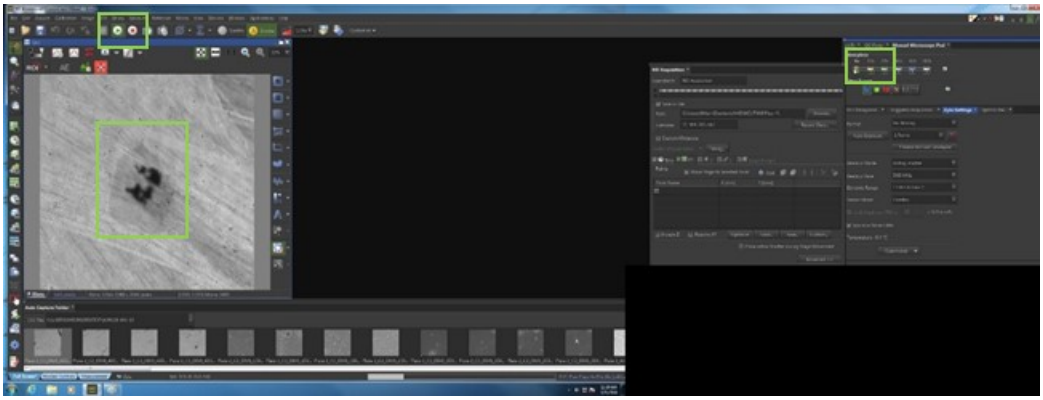
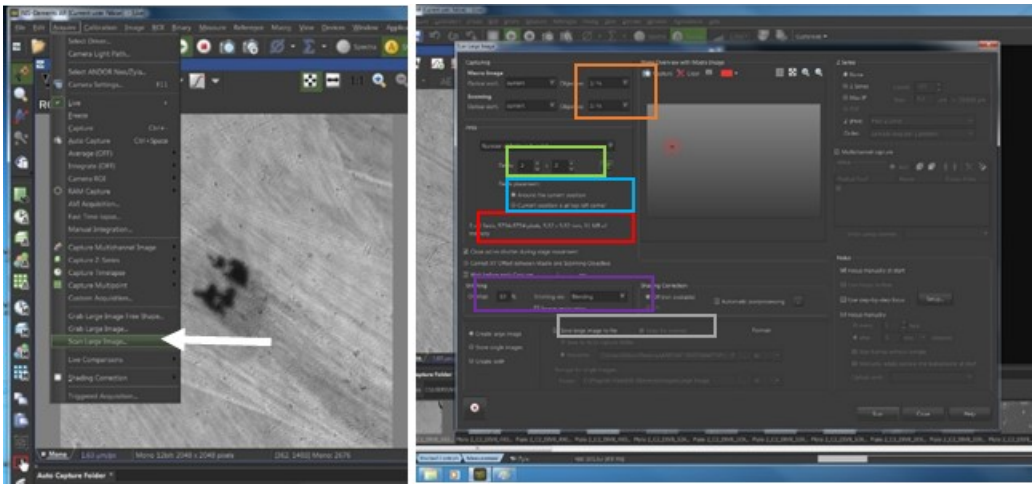
Appendix A. Coordinate Tracking on Nikon Elements

This appendix demonstrates phase imaging to track a single cell across multiple imaging sessions. (This will work on silicone wells [glass can move within the well so additional calculations would be required for each imaging session].)

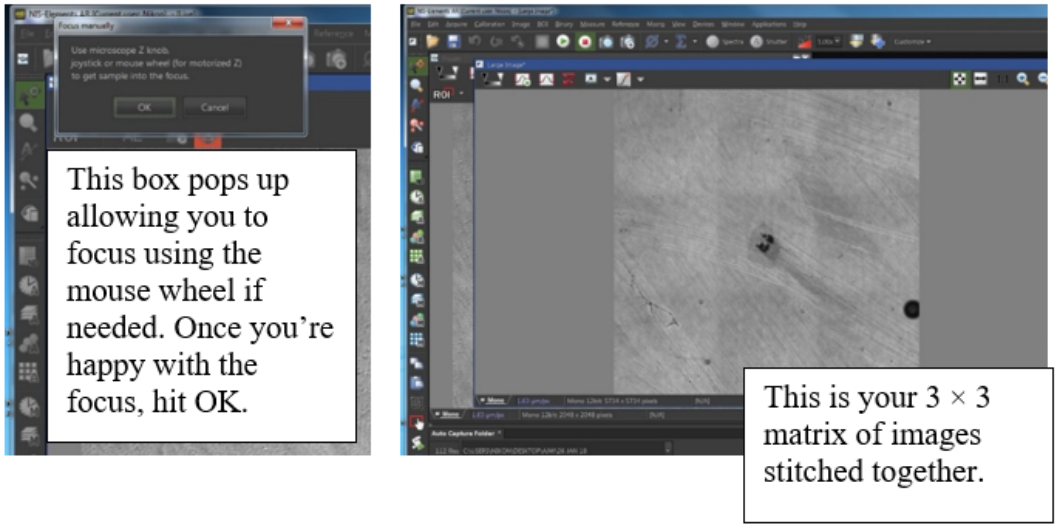
1. Before culturing, mark the center of each well (origin) with a Sharpie using the four plastic lines around the perimeter of the well on the underside of the plate to determine the middle.
2. On the day of imaging, turn on the microscope and open Elements. Be sure that the x and y coordinates are functioning properly and changing as the stage moves. If they do not, turn off the program and restart the computer. If this does not solve the problem, please contact the author of this report—the stage may need to be reinitiated or recalibrated.
3. To capture images of the same location across multiple days, you will need the coordinates of your selected location.
4. To start, you may want to capture a low magnification image to help you decide where you should capture your images.
5. Set objective to 4x on the objective wheel and computer.
6. Center the origin in the middle of the field of view.



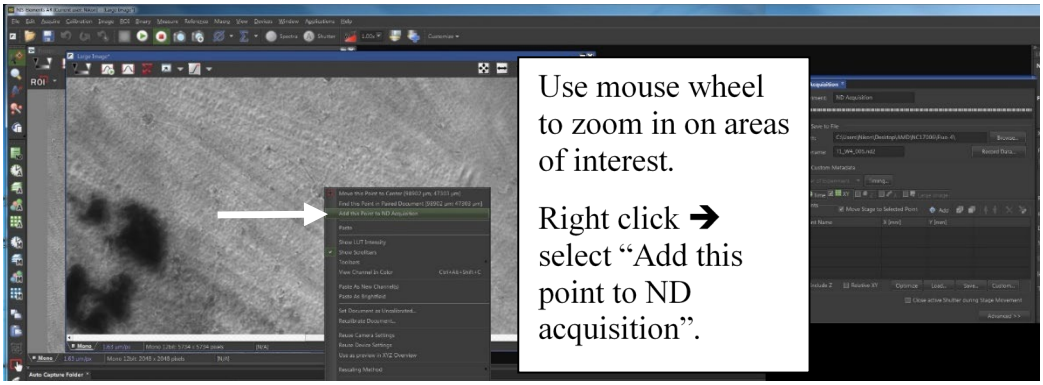
Acquire ➔scan large image

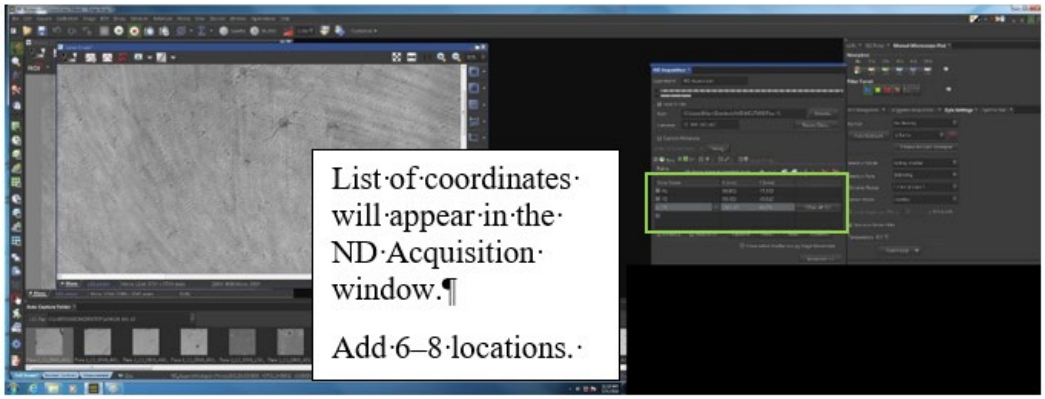


7. Set parameters (colors in the list below correspond to text boxes in the image above).
   a. Choose the appropriate objective.
   b. Set the number of tile images you want taken.
   c. Choose whether you want the current location to be the center of your grid or the top left
      (1) If you have the current location as the origin, you want center.
      (2) This will tell you how many millimeters are included in your image (this is convenient if you are interested in only looking at the center 10 mm of the well).
   d. Select the amount of desired overlap and how you want the tiles to be blended.
   e. Change the save destination.
   f. Once all parameters are set, hit scan.

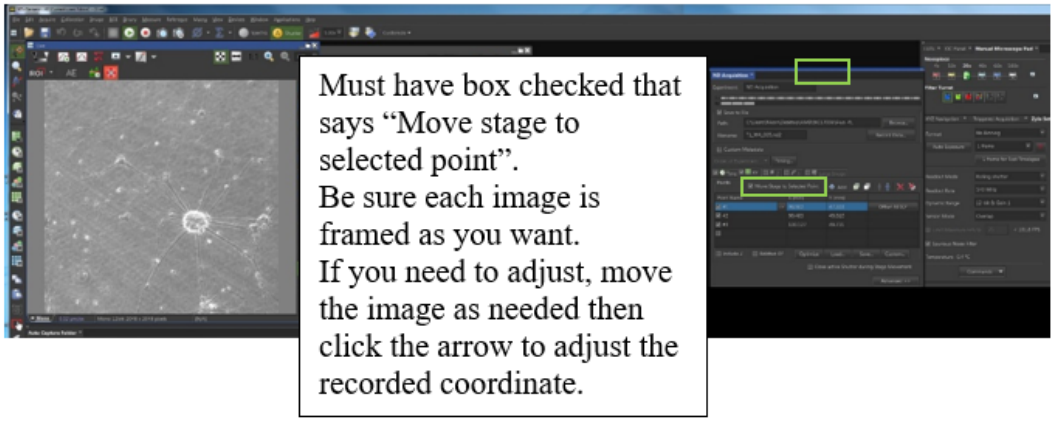


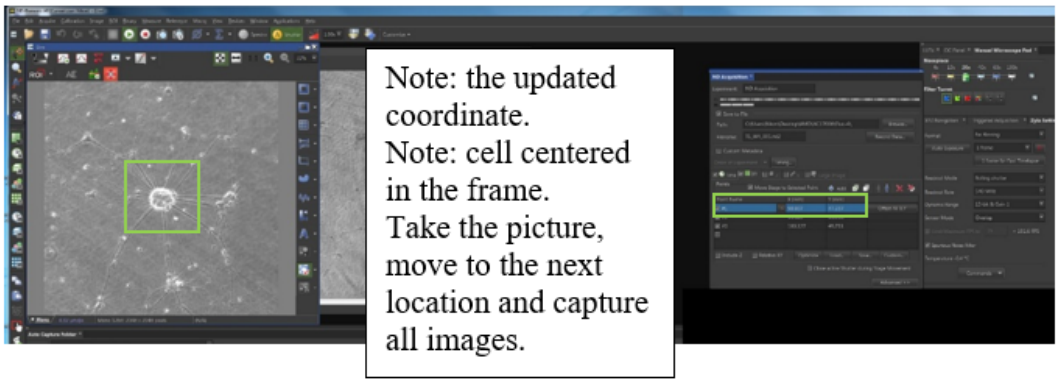
8. Find your points of interest and record coordinates. Be sure the first location includes the origin (this will serve as a check point for alignment in future imaging sessions.





9. Change to the 20x (or desired objective) to capture images. Click on each point to capture your images. (Be sure to save next as to change the file name for each well.)





10. When done, save the list of coordinates from the ND acquisition window. You can close and reopen the program and the points should still be there, unless someone else has used the scope and removed them.
11. Repeat image acquisition again by clicking the points on the next imaging day.

Appendix B. Final Protocol for Culturing Hippocampal Neurons

DISH PREPARATION (72 h minimum before dissection)

- Autoclave
  - Water
  - Pipette tips
- Determine number of dishes needed

DAY 1: Poly-l-lysine (PLL) Incubation (1 mg/100 mL final concentration)

- Make working solution
  1. Defrost stock PLL: 1 mg/mL in –20 °C freezer.
  2. Add 1 mL of 1 mg/mL stock PLL to 99 mL of ddH_2O = 0.01 mg/mL.
  3. Filter 0.01 mg/mL solution through 0.22-µM filter using syringe.
- Volume guidelines
  - For eight well plates: 1.5 mL/well
  - For 22-µm glass cover slips: 0.5 mL/cover slip
  - For six well stretch plates: 3.0 mL/well
- Leave PLL solution in dishes for 24 h in hood at room temperature

DAY 2: Water

1. Remove PLL solution from dishes – use vacuum suction.
2. Wash dishes with sterile ddH_2O – two times, 10 min each.
3. Add sterile ddH_2O to dishes.
4. Leave overnight in incubator.

DAY 3: Day of Plating

1. Remove ddH_2O from dishes – use vacuum suction.
2. Add 1.5 mL of feeding media to each well.
3. Put in incubator to equilibrate temperature.

PREPARE SOLUTIONS (All solutions are made aseptically under the hood)

- Feeding Media
  - 500 mL Neural Basal Media (NBM) (Life Technologies)
  - 10 mL B27 (–20 °C box – Life Technologies)
  - 1 mL Glutamax (4 °C or can be stored in –20 °C); take up 1 mL first and then B27 in same pipette
  - Sterile filter

- Plating Media
  - Make day of culture
  - Figure out total amount needed (4 mL per culture)
  - Feeding media + 5%–10% fetal bovine serum (FBS) (in –20 °C aliquoted)
  - For 5 mL:
    - 4.5 mL feeding media
    - 500 µL FBS

- Sterile filter

- Trypsin (0.3 mg/mL) DNase (0.2 mg/mL); day of culture for hippocampal
  - 150 µL of trypsin DNase stock (–20 °C)
  - 5 mL of feeding media and keep on 37 °C water bath
  - Sterile filter place solution in a 37 °C water bath until ready to use

CELL ISOLATION

Trypsinizing

1. Take trypsin solution out of the water bath to the hood.
2. Using a sterile 9-inch Pasteur pipette, remove tissue from Hibernate EB (HEB) and put in new tube.
3. Carefully, without removing any hippocampi, take off as much HEB as possible.
4. Add 5 mL trypsin/DNase to tube.
5. Incubate hippocampi at 37 °C, 5% CO_2 for 20 min (no longer).
6. During 20-min incubation, collect Pasteur pipettes:
   Pasteur Pipette 1: 10 mL sterile pipette.
   Pasteur Pipette 2: Sterile Pasteur pipette – do nothing to it.
   Pasteur Pipette 3: Sterile Pasteur pipette with fire-polished tip to constrict opening
7. After 20 min, return trypsinized hippocampi to the hood.
8. Place hippocampi in a new tube.
9. Add 5 mL of plating media to hippocampi.
10. Using a 10-mL sterile pipette, break up the hippocampi.
11. Using a sterile Pasteur pipette, break up the hippocampi more.
12. Using a flame-polished Pasteur pipette, break up tissue until all hippocampal material is broken up into individual cells and solution appears cloudy without any material floating in it.
13. Avoid bubbles.

CELL PLATING

General Equipment and Solutions

- 10-mL pipette
- Hemacytometer
- Trypan blue solution (0.4%)

Procedure

- Calculations – rough estimate
  - Approximately 1 million hippocampal neurons/pup brain (1×10^6 cells)
  - Pellet is re-suspended in 5 mL plating media

- Calculations (counts)
  - Calculate the no. of cells needed per cell density.
  - Petri dishes
    - Low density = LD = 60,000 to 100,000 cells/mL
    - Example
      - (No. of dishes)(1 mL/dish)(400,000) = total amt of cells needed = *Hd*
      - (*Hd*) / (total no. of viable cells/mL) = amount of suspension needed
  - [(A+B+C+D) / 4] × 10 × 1000 × 10 = cells/mL.
  - 10-µL cell suspension + 90-µL trypan blue (0.04%) = 1:10 dilution → mix well.
  - Place 10 µL of cells/trypan blue in hemocytometer.
  - Count the no. of viable cells in each of the four (A, B, C, D) 4 × 4 corner grids → average.
  - 1000: mm^3 → mL
- Plating
  1. Draw up double-filtered cell solution.
  2. Dispense in plates or wells.

CELL FEEDING

- The first feeding should be done 3 days after cell plating.
- Subsequent feedings should be once per week.

General Equipment and Solutions

- Water bath
- Feeding media
- Glass Pasteur pipettes
- Vacuum setup
- Sterile pipettes: 1, 5, 10 mL
- Drummond pipetter

Procedure

- Feeding Media
  - Aliquot desired feeding media volume into centrifuge tubes.
  - Heat in water bath set at 37 °C.
- Removing Plating Media
  - Turn on vacuum and attach glass Pasteur pipette to vacuum tubing.
    - Uncover dish and place pipette tip just inside insert; this should aspirate plating media without disrupting surface with attached cells.
- Adding Feeding Media

- Pipette 2 mL of feeding solution into each well.
- Return wells to incubator.

CELL IMAGING (To be completed after every feeding)

1. Turn on microscope and all components (via power strip behind and left of computer monitor).
2. Turn on the computer (via power strip behind microscope boxes).
3. Turn on Nikon software. Pick a camera (Zyla is the better camera for phase).
4. Put well plate holder onto microscope stage.
5. Check filter cube (should be on 5 or 6).
6. Check objective correction collar (should be set to 1.2, not .7).
7. Start with 20× objective; click "20×" button on the *Manual Microscope Pad* tab.
8. Knob on the right of scope determines where the image is going; eye for eyepiece, L for Zyla camera, and R for color camera.
9. Focus on the cells. Be sure to focus on the cover slip and NOT the cell well (well will have striation-like appearance and be the first plane to come into focus when focusing from the bottom). Move objective closer to sample and it should focus on the glass.
10. Switch to the computer if using the eyepiece.
11. Typical exposure time for 20× objective is 15 ms; 10× objective is 1 ms.
12. Take seven to eight images of each well, capturing the various phenotypes of cells.
13. Set up auto file naming:
    - Click file
    - Save next image as
    - Fill in appropriate file name
    - Culture number_tray number_well number_image number
        - Eg. NC17001_T1_W1_01, NC17001_T1_W1_02, etc.
14. To take an image, click the camera button across the top menu with the plus sign. This will put the image in the reel at the bottom. At the bottom there is a drop-down bar to pick the folder.

SOLUTIONS

PLL

Stock solution (1 mg/mL) →

- 25 mg PLL – HBr Sigma 81356 (in –20 °C) + 25 mL ddH20. Aliquoted in 1-mL centrifuge tubes and stored in –20 °C box

Working Solution →

- 1 mL PLL stock solution + 99 mL sterile ddH_2O (in nonhazardous solutions cabinet). Filter into 100-mL flask and store in 4 °C until ready for use.

Feeding Media: Hippocampal and Cortical Cultures

- 500 mL NBM
- 10 mL B27 (–20 °C box)
- 1 mL Glutamax (stored at room temp)
- Store in 4 °C up to 4 weeks

Plating Media (Inhibits Trypsin): Hippocampal Cultures Only

- Make day of culture
- Figure out total amount needed (4 mL per culture)
- Feeding media + 5%–10% FBS (in –20 °C aliquoted)
  For 5 mL:
  - 4.5 mL feeding media
  - 500 µL FBS
- Sterile filter

Trypsin Stock (10 mg/mL)

- 50 mg of trypsin (T-9935 Sigma)
- 5 mL of 1X PBS without Mg and Cl
- Aliquot and store in –20 °C

Trypsin (10 mg/mL) DNase (6.6 mg/mL) Stock

- 5 mL of 10 mg/mL trypsin stock
- 33.3 mg of DNase
- Aliquot in 1.7 mL centrifuge tubes and store in –20 °C

Trypsin (.3 mg/mL) DNase (.2 mg/mL): Day of culture for hippocampal and neuronal

- 150 µL of trypsin DNase stock (–20 °C)
- 5 mL of feeding media and keep on 37 °C water bath
- Sterile filter

ARA C

- Stock
  - Dilute 100 mg in 6 mL dH_2O ➔ 68.525 mM (I aliquoted in 300 µL aliquots)
  - 291.86 µL in 20 mL ➔ 1 mM (aliquoted 300 µL, n = 60)
  - Add 300 µL to 2700 µL feeding media – filter and warm (100 µM)
  - Add 25 µL 100 µM to each well
  - Added the day after plated

- Final concentration per well is 1 µM

Propidium Iodide

1. Put 40 µL per plate into a small tube.
2. Pipette 5 µL per well.
3. Gently swirl plate.
4. Place plate back into incubator for 30 min.
5. Follow with paraformaldehyde (PFA) fixation.

Cell Type:	Hippocampal Neurons					
Culture ID:						
Date:						
Days BEFORE culture						
Prep Day 1:	Incubate In Poly-L-Lysine overnight					
	1:100 (PLL:sterile ddH2O)					
	Plate Type:Coverslip	Stretch and Coverslip				
	Autoclave Instuments & Water					
Prep Day 2:	Aspirate PLL off and wash with autoclaved ddH2O					
	wash 2x10min					
Prep Day 3:	Aspirate off water and add 1.5ml feeding media					
	Make Plating media: NBM + (5%) FBS					
Day OF culture						
Prep Solutions:	Thaw Trypsin/DNAse stock & FBS					
	Warm Plating media & Feeding Media					
Prep Hood:	scope, instruments, ice block with four 35mm petri dishes					
	Two (2) 15mL conical tubes					
	# of embryos isolated					
	# of heads isolated					
	# of brains isolated					
	# of hippocampi isolated					
Trypsinize:	Trypsin	10 mg/ml trypsin	150 uL			
		Feeding Media	5mL			
	Incubation		20 min			
1st Dissociation:	Remove trypsin/FM					
	Replace with Plating media:		3	mL		
Tituration:	Glass Pateur Pipette			10 times		
	Flame polished pasteur pipette (small diameter)			10 times		
	Flame polished pasteur pipette (smaller diameter)			10 times		
Cell Counting:	**Trypan blue**	90ul				
	Cell suspension	10ul				
	Add 1 drop to hemocytometer					
	Corners	**A**	**B**	**C**	**D**	
Calculate:	**AVG Cells on Hemocyt**	(A+B+C+D)/4	0	CELLS		
Calculate:	**Cells/mL in suspension**	#1*(10)(1000)(10)	0	CELLS / ML	**Z**	
Record:	# dishes (coverslips)	38	Dishes	**W**		
	Volume to add	1	mL/dish	**X**		
	Desired Density	100,000	cells/mL	**Y**		
Calculate:	**Amount of Cell Suspension**	(W*X*Y) / Z	#DIV/0!	mL	V	
Calculate:	**Amount of Plating Media:**	(W*X) - V	#DIV/0!	mL		
	Add calculated amount of cell suspension to calculated amount of plating media					
	Put 1mL in each well					

List of Symbols, Abbreviations, and Acronyms

ARL	Army Research Laboratory
BSA	bovine serum albumin
BSCII	Class II biosafety cabinet
BSCIII	Class III biosafety cabinet
CCDC	US Army Combat Capabilities Development Command
DAPI	4′,6-diamidino-2-phenylindole
DIV	day in vitro
DNA	deoxyribonucleic acid
FBS	fetal bovine serum
GFAP	glial fibrillary acidic protein
HEPA	high-efficiency particulate air
LFH	laminar flow hood
NBM	Neurobasal Media
PBS	Phospho-buffered saline
PDL	poly-d-lysine
PLL	poly-l-lysine

1 (PDF) DEFENSE TECHNICAL INFORMATION CTR DTIC OCA

1 (PDF) CCDC ARL FCDD RLD CL TECH LIB

1 (PDF) NORTHSHORE UNIVERSITY HEALTH SYSTEM J FINAN

1 (PDF) UNIFORMED SERVICES UNIVERSITY M SHAUGHNESS

4 (PDF) MRMC DOD BLAST INJURY RSRCH PROG COORDINATING OFC R GUPTA M LEGGIERI R SHOGE T PIEHLER

15 (PDF) CCDC ARL FCDD RLW L A M DILEONARDI T SHEPPARD T THOMAS C GOOD A WEGENER A DAGRO A EIDSMORE FCDD RLW A RAWLETT S SCHOENFELD J ZABINSKI A WEST FCDD RLW PB C HOPPEL M KLEINBERGER E MATHEIS K RAFAELS