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TITLE:

Visualizing and Analyzing Intracellular Transport of Organelles and Other Cargos in Astrocytes

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astrocytes, intracellular transport, live-imaging, trafficking kinetics, degradative endosomes, connexin 43, EAAT1

SUMMARY:

Here we describe an in vitro live-imaging method to visualize intracellular transport of organelles and trafficking of plasma membrane proteins in murine astrocytes. This protocol also presents an image analysis methodology to determine cargo transport itineraries and kinetics.

ABSTRACT:

Astrocytes are among the most abundant cell types in the adult brain, where they play key roles in a multiplicity of functions. As a central player in brain homeostasis, astrocytes supply neurons with vital metabolites and buffer extracellular water, ions, and glutamate. An integral component of the “tri-partite” synapse, astrocytes are also critical in the formation, pruning, maintenance, and modulation of synapses. To enable these highly interactive functions, astrocytes communicate among themselves and with other glial cells, neurons, the brain vasculature, and the extracellular environment through a multitude of specialized membrane proteins that include cell adhesion molecules, aquaporins, ion channels, neurotransmitter transporters, and gap junction molecules. To support this dynamic flux, astrocytes, like neurons, rely on tightly coordinated and efficient intracellular transport. Unlike neurons, however, where intracellular trafficking has been extensively delineated, microtubule-based transport in astrocytes has been less studied. Nonetheless, exo- and endocytic trafficking of cell membrane proteins and intracellular organelle transport orchestrates astrocytes’ normal biology, and these processes are often affected in disease or in response to injury. Here we present a straightforward protocol to culture high quality murine astrocytes, to fluorescently label astrocytic proteins and organelles of interest, and to record their intracellular transport dynamics using time-lapse confocal

microscopy. We also demonstrate how to extract and quantify relevant transport parameters from the acquired movies using available image analysis software (i.e., ImageJ/FIJI) plugins.

INTRODUCTION:

Astrocytes are the most abundant cells in the adult central nervous system, where they perform unique developmental and homeostatic functions[1]. Astrocytes modulate synaptic development through direct contact with pre- and postsynaptic terminals as part of the tri-partite synapse, which contains neurotransmitter receptors, transporters, and cell adhesion molecules that facilitate synapse formation and neuron-astrocyte communication[2]. In addition, astrocytes actively control synaptic transmission and prevent neuronal excitotoxicity by rapidly removing excitatory neurotransmitters from the synaptic cleft, recycling neurotransmitters, and participating in synaptic pruning[3-6]. To enable these highly interactive functions, astrocytes communicate among themselves, with other glial cells, and with neurons through specialized membrane proteins, including cell adhesion molecules, aquaporins, ion channels, neurotransmitter transporters, and gap junction molecules. Astrocytes actively change the surface levels of these proteins in response to fluctuations in their intra- and extracellular environment[7]. Furthermore, changes in the levels and distribution of mitochondria, lipid droplets, and degradative and recycling organelles modulate energy supply, metabolite availability, and cellular clearing processes that are essential for astrocyte function and survival.

The dynamic changes in membrane protein and organelle trafficking and positioning in astrocytes are facilitated by the concerted function of motor proteins and adaptors that promote cargo motility[8,9]. Similarly, surface levels of membrane proteins are modulated through internalization and recycling events[10]. These cargos are transported via an intricate network of actin, microtubules, and possibly intermediate filaments tracks[8]. Studies based on immunofluorescence staining of end-binding protein 1 (EB1), which accumulates at the growing microtubule plus ends, suggest that in astrocytes bundles of microtubules radiate out from the perinucleus and extend their plus end towards the periphery[11]. However, a comprehensive examination of the organization and polarity of microtubules and other cytoskeletal elements using live-cell imaging is still lacking. While many of the mechanisms underlying the dynamics of organelles and membrane proteins have been extensively studied in neurons and other cell types, cargo motility in astrocytes is less well understood. Most of our current knowledge about changes in protein and organelle distribution in astrocytes is based on traditional antibody-based labeling of fixed preparation, which precludes precise spatial and temporal examination of cargo dynamics[7,12].

Here, we describe a method to label membrane proteins and organelles for live imaging in high purity primary mouse astrocyte cultures. Using this protocol, we provide examples in which we track the dynamic localization of green fluorescent protein (GFP)-tagged membrane proteins in transfected astrocytes, including the gap junction protein connexin 43 (Cx43-GFP) and the excitatory amino acid transporter 1 (EAAT1-GFP). We also describe the use of a fluorescent acidotropic probe to visualize acidic organelles and follow their trafficking dynamics in live astrocytes. Finally, we demonstrate how to analyze the time-lapse data to extract and evaluate transport parameters for individual cargos.

PROTOCOL:

All animal procedures were performed with the approval of the University of North Carolina at Chapel Hill Animal Care and Use Committee (IACUC).

1. Brain dissection and culture of primary mouse astrocytes

NOTE: The following protocol was adapted from published methods, which follows the original procedure developed by McCarthy and deVellis[13,14,15]. Mixed cell cultures of McCarthy/deVellis (MD) astrocytes are prepared from postnatal day 2−4 (P2−P4) mouse cortices, treated with an anti-mitotic factor, and purified to yield the final enriched astrocyte culture. Four cortices from mouse pups of either sex are sufficient to generate one T75 tissue culture flask culture.

1.1. Coat the bottom of T75 flasks (100% angled neck access, 0.22 µm hydrophobic vented filter cap) with poly-D-lysine (1 mg/mL in 0.1 M Tris buffer, pH 8.5) and incubate at 37 °C for 24 h prior to start of dissection.

1.2. Before beginning the dissection procedure, warm 30 mL of astrocyte culture media (Dulbecco's modified Eagle's medium [DMEM] high glucose, 10% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin) at 37 °C and thaw a 5 mL aliquot of 2.5% trypsin on ice. Prepare two dissecting dishes (60 mm diameter) on ice filled with 6 mL of Hank's balanced salt solution (HBSS) without Ca^{2+} or Mg^{2+}.

1.3. Disinfect all surgical tools (fine tip tweezers, dissecting scissors, Vannas scissors straight, Graefe forceps with curved tips) in 70% ethanol prior to and in between each dissection.

1.4. Spray the head and neck of the P2−P4 mouse pups with 70% ethanol before quickly euthanizing them by decapitation with surgical scissors. Perform a posterior to anterior midline incision along the scalp to expose the skull. Carefully cut the skull from the neck to the nose.

1.5. Perform two additional lateral incisions superior to the eyes. Using fine tip tweezers gently flip the skull flaps to the side. Remove the brain and place it in the first dissecting dish filled with ice-cold HBSS without Ca^{2+} or Mg^{2+}. Keep the dish on ice and continue harvesting the rest of the brains.

NOTE: Perform the remainder of the dissection procedure under a dissecting scope.

1.6. Remove the olfactory bulbs using Vannas scissors or fine tweezers (**Figure 1Ai**). With the curved tip forceps at the intersection of the transverse and interhemispheric fissures, gently insert a second pair of curved tip forceps between the cortex and diencephalon, slowly opening the forceps to separate the cerebral hemispheres from the rest of the brain (**Figure 1Aii,Aiii**). Remove the unwanted tissue (**Figure 1Aiv**).

1.7. For each cortical hemisphere, carefully remove the meninges with fine tip tweezers. Optionally, remove the hippocampus from the cortices. Transfer the dissected mouse cortices into a second dish filled with ice-cold HBSS without Ca^{2+} or Mg^{2+} and keep it on ice while dissecting the remaining brains.

NOTE: Conduct the next steps under aseptic conditions in a laminar flow hood.

1.8. Cut the dissected cortices into small pieces (approximately 2−4 mm) using sterile sharp surgical scissors or a scalpel. Transfer the dissected tissue to a 50 mL conical tube containing enzyme solution (22.5 mL HBSS without Ca^{2+} or Mg^{2+}, 2.5 mL 2.5% trypsin). Incubate at 37 °C for 30 min with gentle inversion every 10 min.

1.9. Collect the digested tissue by centrifugation at 300 x *g* for 5 min and remove the enzyme solution by decantation (do not use a vacuum aspirator). Add 10 mL of astrocyte culture media and dissociate the tissue into single cells by pipetting the solution 20−30x using a 10 mL serological pipette.

1.10. Filter the cell suspension through a 70 µm cell strainer to minimize cell clumps and undigested tissue. Collect the filtrate in a clean 50 mL conical tube and adjust the total volume to 20 mL with astrocyte culture media. At this point, evaluate the efficiency of the cortical dissociation by mixing 10 µL of the cell suspension with 10 µL of trypan blue and counting cells with a hemocytometer.

NOTE: **One preparation from four P2−P4 mouse cortices yields 10−15 x 10^6 single cells.**

1.11. Aspirate the poly-D-lysine coating solution from the T75 culture flasks and wash twice with sterile water. Plate the 20 mL cell suspension and incubate at 37 °C and 5% CO_2.

2. Purification and maintenance of astrocyte cultures

NOTE: Perform all media changes under aseptic conditions in a laminar flow hood using sterile filtered (0.22 µm filter membrane) astrocyte media warmed to 37 °C.

2.1. Replace the cell media 48 h after plating (day in vitro 2, DIV2).

2.2. At DIV5, replace the media with fresh astrocyte culture media supplemented with 10 µM cytosine arabinoside (AraC).

NOTE: This step facilitates the removal of contaminant cells, including fibroblasts and other glial cells. **It is important to treat the cultures with AraC no longer than 48 h as longer incubation times will reduce astrocyte viability**.

2.3. At DIV7, change media to astrocyte culture media without AraC and start to check the culture confluency daily. Once cells have reached 80% confluence, coat two-T75 flasks for each

unpurified astrocyte culture flask with poly-D-lysine and incubate at 37 °C for at least 8 h to overnight.

2.4. The following day, begin the astrocyte purification by shaking the culture flasks at 180 rpm for 30 min in a 37 °C orbital shaking incubator. Remove the media and replace it with fresh astrocyte culture media. Shake cells at 240 rpm for 6 h in a 37 °C orbital shaking incubator. Prewarm 1x phosphate-buffered saline (PBS), astrocyte culture media, and 0.25% trypsin-EDTA to 37 °C 20−30 min before the end of the shaking period.

NOTE: **CO_2 incubation is not required during the shaking steps.** If desired, the media collected after the 30-min and 6-h shaking steps may be used to culture microglia and oligodendrocytes, respectively. AraC treatment of the mixed culture will, however, reduce the abundance of the other glial cells.

2.5. Remove the culture flasks from the shaker and immediately replace the media with 10 mL of warm 1x PBS per flask. Remove PBS and add 3 mL of 0.25% trypsin-EDTA per flask. Incubate at 37 °C and 5% CO_2, checking every 5 min for detachment.

2.6. Once cells have detached from the flask, stop trypsinization by adding 10 mL of astrocyte culture media. Transfer detached astrocytes to a 50 mL conical tube and pellet cells by centrifugation at 300 x *g* for 5 min. Aspirate supernatant and re-suspend the cells in 40 mL of astrocyte culture media.

2.7. Wash the poly-D-lysine-coated T75 flasks twice with sterile water and plate 20 mL of the purified astrocyte suspension. Return to the 37 °C and 5% CO_2 incubator. Change the astrocyte culture media every two days for an additional 10 days until astrocytes mature.

NOTE: Each T75 flask of purified astrocytes should be sufficient to produce 2 new T75 flasks with 3 x 10^5 cells each at plating.

2.8. Repeat steps 2.5−2.7 for subsequent passes or to plate astrocytes for microscopy.

NOTE: The purity of the astrocyte cultures after the addition of AraC and following purification can be assessed qualitatively through brightfield microscopy, or more precisely by quantifying the percentage of glial fibrillary acid protein (GFAP)-positive cells per field of view in fluorescent images (**Figure 1B,C**).

3. Transfection of fluorescently tagged plasmids

3.1. The day prior to transfection or labeling, seed astrocytes at desired density for imaging 24−48 h post-transfection. A recommended density for 24-well plates or 14 mm glass bottom wells is 2 x 10^4 cells/well.

NOTE: This protocol is optimized for transfection of astrocytes plated on 12 mm glass coverslips

on 24-well plates or 14 mm glass bottom wells using a lipofection-based method. Reagents should be scaled depending on the size of the culture dish in which the astrocytes are growing.

3.2. Dilute the lipofection reagent (**Table of Materials**) in reduced-serum media (**Table of Materials**). To optimize the ratio of lipofection reagent to DNA, test a range of adequate dilutions. For example, if using the reagent employed in this protocol (**Table of Materials**), dilute 2, 3, 4, and 5 µL of the lipofection reagent in 50 µL of reduced-serum media.

3.3. Dilute 5 µg of high purity DNA in 250 µL of reduced-serum media. Add 5 µL of lipofection enhancer reagent (**Table of Materials**). Combine the tube containing the lipofection reagent with an equal volume of the DNA-lipofection enhancer mix. Mix by pipetting and incubate at room temperature (RT) for 15 min.

3.4. Remove astrocyte culture media and add transfection mix (step 3.3) to cells dropwise. After a 6-h incubation at 37 °C and 5% CO_2, replace the transfection complex with an appropriate volume of astrocyte culture media (2 mL for 14 mm glass bottom dishes). Incubate for an additional 24–72 h before proceeding to the image acquisition. Closely monitor the duration of the incubation step to achieve the best balance between protein expression and cell viability.

4. Labeling of late endosomes/lysosomes using fluorescent probes

NOTE: Some cargos can be labeled using fluorescent dyes with high affinity for cargo-specific proteins. The following example permits the labelling of late endosomes/lysosomes with a fluorescent acidotropic probe.

4.1. Dilute the lysosomal-labeling probe (**Table of Materials**) in 200 µL of astrocyte culture media to a working concentration of 1 µM and apply to astrocytes from step 3.1 at a density of 2 x 10^4 cells/well. Incubate for 30 min at 37 °C.

4.2. Wash the cells once with warm astrocyte culture media and replace with imaging media (**Table of Materials**). Proceed to live imaging immediately.

5. Image acquisition using a time-lapse imaging system

NOTE: Time-lapse live imaging should be done using a fluorescence microscope equipped with a high-speed camera, definite focus, incubation chamber, and a 40x oil objective with a high numeric aperture (e.g., Plan-Apochromat 1.4NA). A variety of acquisition software is available for time-lapse imaging. Selection of the microscopy system and acquisition software should be based on their availability and suitability for the goals of the particular study. Some general guidelines are provided below.

5.1. Place the culture chamber or dish in the proper adaptor on the microscope stage. Using epifluorescence light, select the cell(s) that express fluorescent proteins or probe to record. Adjust the fluorescent sample illumination to visualize the selected cell(s) using the digital

camera. Avoid using high illumination that might cause photobleaching and phototoxicity. Adjust focus and zoom.

5.2. Acquire single Z-stack time-lapse series at a frequency of 1 frame every 2 s for time intervals ranging between 300 s and 500 s using the zoom and definite focus functions.

NOTE: Trafficking dynamics (velocity, frequency of motion, etc.) will vary depending on the protein of interest, thus, the time course of acquisition may require adjustment. For instance, mitochondria are less motile than lysosomes and exhibit frequent pauses. In this instance, it is more appropriate to adjust the acquisition parameters to 1 frame every 5 s for 800 s.

5.3. Save time-lapse images and export them as AVI or TIFF stack files.

6. Image analysis

NOTE: Image analysis was performed using the KymoToolBox plugin for ImageJ/Fiji[16]. Detailed step-by-step written instructions are available online[17]. The following abbreviated steps should be sufficient to create the kymographs and extract the particle transport parameters.

6.1. Open time-lapse image sequence in ImageJ/Fiji software. Import images from the **File** menu as AVI or TIFF stack files. Convert the images to 8-bit or 16-bit by selecting one of these image types using the **Type** tool from the **Image** menu. If working with RGB files, split channels using the **Split Channel** tool available under the **Color** feature in the **Image** menu.

6.2. To generate a kymograph (representation in position and time of particle trajectories), draw each track in which particles are moving by tracing a line along the trajectories of the particles using the **segmented line** tool. To correctly assign directionality of the movement, draw the polyline using the same desired directional convention for all movies so that the polarity is consistent within and across all cells being studied. For instance, draw the polyline from the center of the cell towards the periphery or vice versa. Adjust the line width to match the thickness of the track by double clicking the **Line** tool.

6.3. Run the **Draw Kymo** macro of the KymoToolBox plugin using a line width of 10. A prompt asking to calibrate the image in time (number of frames, frame rate) and space (x,y resolution) will appear. Once calibrated, the kymographs are generated and can be saved as TIFF files for data presentation or further particle analysis.

6.4. Assign particle trajectories by manually tracking each particle in the kymograph using the **segmented line** tool for the full duration of the acquisition. Record each particle trajectory as a region of interest (ROI) using the **ROI manager** tool found in the **Analyze/Tools** menu. Save all ROIs per each time-lapse video for further analysis.

NOTE: It is critical to assign the correct trajectory for individual particles on the kymograph to achieve the most accurate calculation of transport parameters.

6.4.1. Run the **Analyze Kymo** macro of the KymoToolBox plugin. A window will open asking to define the "outward" direction of particle motion (from left to right or vice versa) from the drop-down menu. This selection depends on the position of the fluorescent cargos imaged relative to the nucleus or the cell periphery, as established in step 6.2. For instance, if the nucleus is positioned to the left of the cargos, and the polyline in step 6.2 was drawn away from the nucleus, define the outward particle motion as "from left to right".

6.4.2. Define the limit speed (minimum speed at which a cargo is considered motile) in the **Analyze Kymo** window. For cargos that move at fast intracellular transport speeds, 0.1 µm/second is an appropriate choice. Modify this value to adjust the software's sensitivity to each cargo of interest. Adjust the line width to match the thickness of each particle trajectory.

6.4.3. Select the **Log all data** and **Log extrapolated coordinates** options in the **Analyze kymo** window. This will calculate various cargo transport parameters, including mean speed, inward speed, outward speed, cumulative distance traveled, distance traveled in either the inward or outward direction, number of switches for each particle, percentage of time moving in each direction or paused, etc. Data calculated for individual tracks are then pooled per kymograph and saved in text files specific to each image.

NOTE: The **Show Colored Kymo** option of **Analyze Kymo** macro generates a color-coded overlay of the path over the original kymograph. Particles traveling outward are colored in green, inward in red, and stationary particles in blue. The **Report Coordinates on Original Stack** option of the **Analyze Kymo** macro generates a color-coded overlay of the extrapolated object's position on the original time-lapse video and kymograph.

REPRESENTATIVE RESULTS:

The protocol for establishing primary mouse MD astrocytes outlined above should yield reproducible, high quality cultures. Although cultures initially contain a mix of astrocytes, fibroblasts, and other glial cells, including microglia and oligodendrocytes (**Figure 1Bi,Biv**; red arrowheads), the addition of AraC to the mixed culture between DIV5-DIV7 minimizes the proliferation of these contaminant cells. The combined AraC treatment and shaking-based purification strategy enriches the purity of the astrocyte cultures (**Figure 1Bii**) over traditional protocols that only include the purification steps (**Figure 1Bv**; blue arrowheads)[13]. The expected morphology and composition (assessed by GFAP and 4',6-diamidino-2-phenylindole [DAPI] staining) of the purified astrocyte cultures treated with AraC or left untreated is shown in **Figure 1Biii,Bvi**. Quantification of the percentage of the $GFAP^+$ cells per field of view (ratio of number of cells with GFAP staining to total number of cells identified by DAPI staining) shows an increase of over 27% in purity with AraC supplementation (**Figure 1C**). This high-purity mouse astrocyte culture is suitable for evaluation of RNA and protein expression, cell morphology, and other functional assays.

We used lipofection-based transfection to express GFP-tagged versions of the gap junction protein Cx43 and the EAAT1 transporter in murine astrocytes. This methodology allows transient

expression of proteins at levels that are optimal for live cell imaging without causing toxicity or affecting astrocyte viability (**Figure 2A,E**). Similarly, the use of a fluorescent probe permitted rapid and efficient labelling of acidic endo-lysosomal organelles (**Figure 2I**) to track their dynamics in astrocytes.

Figure 2 shows representative results of the analyses of cargo dynamics in astrocytes transiently transfected with EAAT1-GFP, Cx43-GFP, or labeled with a selective dye that recognizes acidic endolysosomal vesicles. Each of these cargos appeared as a fluorescent punctum decorating the perinuclear region, cytosol, and processes of astrocytes (**Figure 2A,E,I,** left panels). The time-lapse data was used to generate kymographs that track cargo motion in time and space (**Figure 2A,E,I, right panels**). In these kymographs, anterograde and retrograde movement of the indicated cargo is represented by trajectories with negative (green lines) and positive (red lines) slopes, respectively. Stationary vesicles are shown as vertical trajectories (blue lines). Kymograph analysis revealed that the three types of cargo analyzed undergo bidirectional transport with occasional fast, processive runs in both directions. Furthermore, quantification of the flux of a cargo through an area of the astrocyte (red box) revealed differences in the percentage of motile particles among cargos. For example, while 70% of EAAT1-GFP puncta are stationary (**Figure 2B**), less than 20% of Cx43-GFP (**Figure 2F**) and 45% of probe-labeled endolysosomal cargos (**Figure 2J**) are non-motile. These differences in motility among cargos are likely representative of their normal, baseline motility in the region of the astrocyte where the movies were acquired.

The precise mapping of the change in X-Y position along the full time scale for each particle obtained from the kymograph can also be used to evaluate other motion parameters, such as cargo velocity (**Figure 2C,G,K**) and run length (distance traveled) (**Figure 2D,H,L**). Motion parameters can be further analyzed for individual cargos to determine changes in directionality of movement (anterograde vs retrograde motion) as well as reversals in particle direction of motion, number of pauses, etc. The results from these types of analyses can provide meaningful quantitative information regarding changes in the cellular distribution of organelles and membrane proteins in astrocytes under basal or abnormal conditions in the intra- and extracellular environment.

FIGURE LEGENDS:

Figure 1: Establishment of primary mouse astrocyte cultures. (**A**) Schematic representation of steps required for dissection of P2−P4 mouse brains to collect cortical hemispheres. (**B**) (**i,iv**) Representative brightfield images of mixed glial cultures treated (**i**) and non-treated (**iv**) with AraC. Red arrowheads indicate contaminant microglia. (**ii,v**) Representative brightfield images of purified astrocyte cultures treated (**ii**) and non-treated (**v**) with AraC. Blue arrowheads indicate contaminant cells, including oligodendrocytes. (**iii,vi**) Representative confocal images of purified astrocyte cultures treated (**iii**) and non-treated (**vi**) with AraC for two days prior to purification. Green shows astrocytes labeled by GFAP staining. DAPI (blue) staining labels nuclei of all cells. (**C**) Quantification of percentage of GFAP-positive cells per culture dish treated (n = 6) or not (n = 4) with AraC. Data represents mean ± SEM. ***$p < 0.001$, unpaired t-test.

Figure 2: Quantitative analyses of cargo dynamics in astrocytes. (**A,E,I**) Left panels: Representative images of astrocytes expressing EAAT1-GFP (**A**), Cx43-GFP (**E**) or treated with a probe that labels late endosomes and lysosomes (**I**) acquired by confocal time-lapse microscopy at a rate of 1 frame every 2 s. Red boxes indicate regions used to analyze particle dynamics. Right panels: Original and color-coded kymographs showing trajectories for the indicated cargos within the area demarked by red boxes. Red lines represent retrograde-moving cargos, green lines denote anterograde-moving cargos, and blue lines show non-motile cargos. (**B,F,J**) Quantification of percentage of stationary and motile particles for each cargo. (**C,G,K**) Quantification of anterograde and retrograde velocity for each cargo. (**D,H,L**) Quantification of anterograde and retrograde run length for each cargo. For all graphs, data represents mean ± SEM.

DISCUSSION:

Here, we describe an experimental approach to express, visualize, and track fluorescently tagged organelles and membrane proteins of interest using time-lapse video microscopy in high-purity primary mouse cortical MD astrocytes. We also outline a methodology for measuring particle dynamics. Direct visualization of protein and organelle dynamics in primary astrocytes provides a powerful tool to study the regulation of intracellular transport in these cells in vitro.

The methodology for establishing mouse MD astrocyte cultures described above combines steps from other published protocols[13,14,15], which results in both a simpler method and in higher purity and quality cultures. The combination of AraC[15] treatment and shaking-based purification[13,14] can yield astrocyte cultures with a purity as high as 98% (assessed by the ratio of $GFAP^+$ cells total cells), which in our example results in a 27% increase in purity over cultures subjected to the purification steps only (**Figure 1B,C**). The high purity cultures of mouse astrocytes obtained with this method is similar to the values reported for rat astrocyte cultures (assessed by enzymatic assays and electron microscopy) by McCarthy and deVellis[14]. However, the McCarthy/deVellis method, which does not include AraC treatment, requires a longer shaking step (15−18 h) and two additional rounds of purification[14].

In our example, we show that this protocol is sufficiently sensitive to capture differences in motility among various membrane proteins and organelles in astrocytes, which are likely representative of their individual intracellular itineraries and cellular function. Although our example demonstrates the utility of this protocol at basal conditions, this methodology can be easily adapted to measure transport parameters within a broad range of research questions. For example, similar protocols with minor modifications could be used to characterize transport events in astrocytes in vitro in response to intra- or extracellular environment, such as cellular damage, toxicity, pathogenic mutations, synaptic activity (in mixed cultures of neurons and astrocytes), etc. Likewise, co-expression and visualization of multiple cargos or organelles, each individually tagged with different fluorophores, can also be used to assess dynamic changes in co-localization and complex formation, transient interactions between organelles, and endocytic and membrane recycling transport events.

The success of the methodology presented here primarily relies on the ability to obtain high-quality astrocyte cultures and in the efficient labeling of cargo(s) of interest. When utilizing this

procedure for live imaging, it is important to closely monitor fluorescent expression to determine the optimal post-transfection incubation time. On average, we found that for the cargos analyzed a 24-h incubation period results in good signal intensity for live imaging acquisition. Prolonged incubation of transfected astrocytes can result in high expression of fluorescently tagged proteins, which can induce protein aggregation, thus changing cargo localization and dynamics, and diminishing the health of the cultures. Astrocytes are very vulnerable to physical damage and changes in the culture environment, which can lead to induction of transcriptional and cellular responses, including reactivity. Therefore, it is critical to reduce the time intervals between washing steps, and to minimize exposure of the cultures to air and to significant changes in temperature or light intensity over the incubation and acquisition periods.

In summary, the methods presented here can be used to evaluate and quantify dynamic changes in protein and organelle localization in astrocytes. They provide valuable tools that permit the examination of changes in astrocytic responses under physiological and pathological conditions.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

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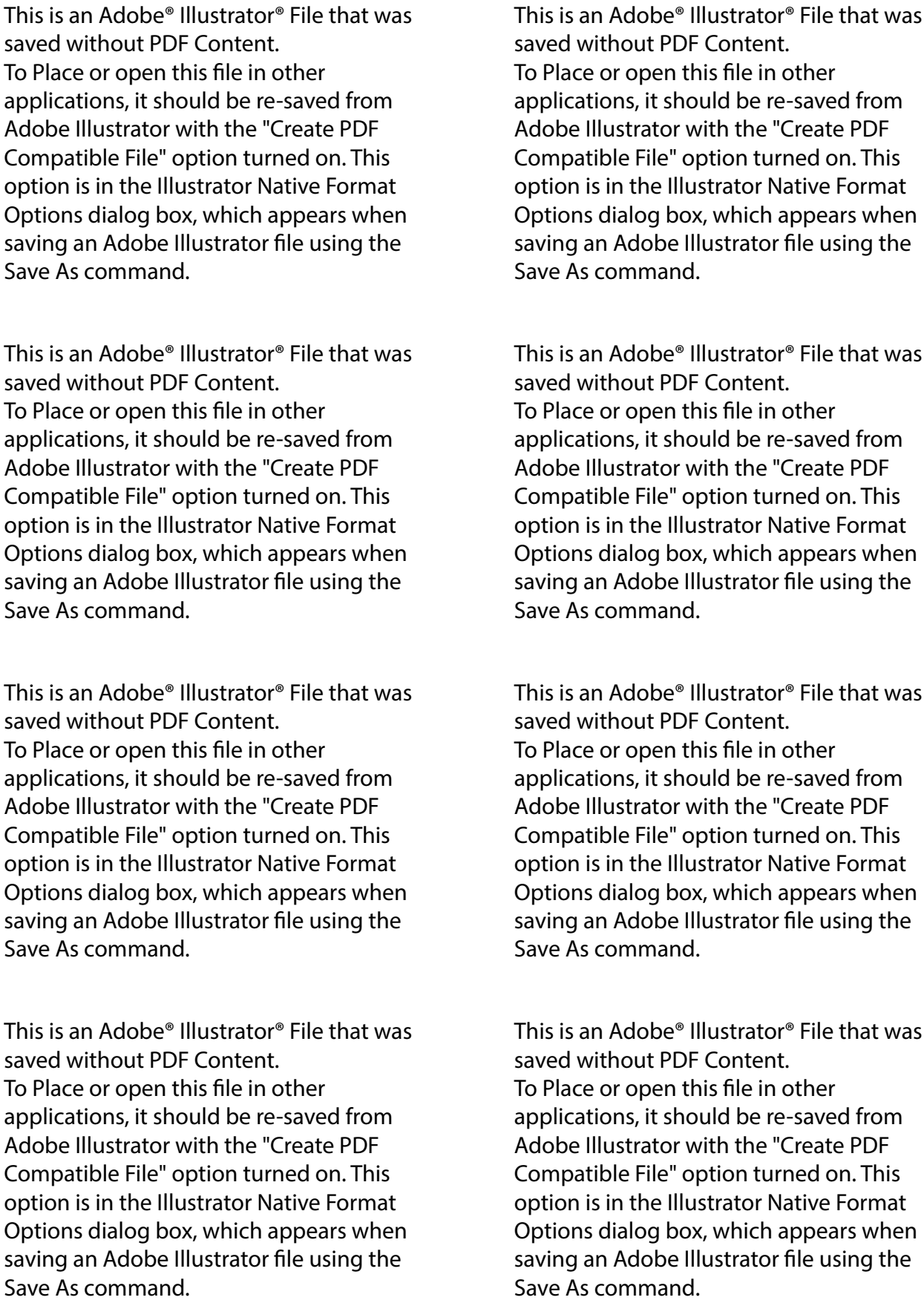

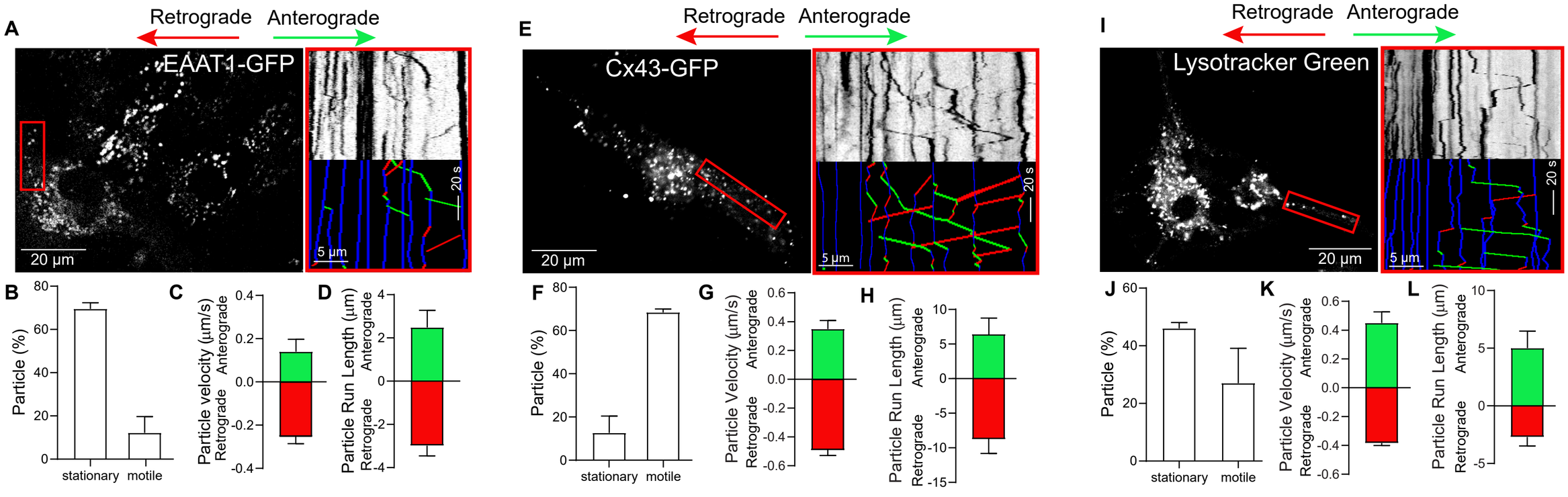

Table of Materials

Name of Material/Equipment	Company	Catalog Number	Comments/Description
2.5% Trypsin (10x)	Gibco	15090-046	
Benchtop Centrifuge	Thermo Scientific	75-203-637	Sorvall ST8 Centrifuge
Cell Culture Grade Water	Gen Clone	25-511	
Cell Culture Microscope	Zeiss	WSN-AXIOVERT A1	Vert.A1 inverted scope, Inverted tissue culture microscope with fluorescent capabilities
Cytosine Arabinoside	Sigma	C1768-100MG	(AraC) Dissolve lyophilized powder in sterile cell culture grade water to make a 10mM stock, aliquot and freeze for long term storage
DAPI	Sigma	D9542-5MG	Dissolve lyophilized powder in deionized water to a maximum concentration of 20 mg/ml, heat or sonicate as necessary. Use at 300 nM for counterstaining.
Dissecting Microscope	Zeiss		Stemi 305
Dissecting Scissors	F.S.T	14558-09	
Dulbecco's Modified Eagle Medium	Gen Clone	25-500	
Fetal Bovine Serum	Gemini	100-106	Heat-Inactivated
FIJI (Fiji is Just Image J)	NIH	Version 1.52i	
Fine Tip Tweezers	F.S.T	11254-20	Style #5
Fluorescence light source	Excelitas	012-63000	X-Cite 120Q
GFAP antibody	Cell Signaling	3670S	GFAP (GA5) mouse monoclonal antibody
Glass Bottom Dishes	Mattek corporation	P35G-1.5-14-C	35 mm Dish | No. 1.5 Coverslip | 14 mm Glass Diameter | Uncoated
Graefe Forceps	F.S.T	11054-10	Graefe Iris Forceps with curved tips
Green fluorescent dye that stains acidic compartments (late endosomes and lysosomes)	Life Technologies	L7526	LysoTracker Green DND 26. Pre-dissolved in DMSOS to a 1mM stock solution. Dilute to the final working concentration in the growth medium or buffer of choice.
Hank's Balanced Salt Solution (10x)	Gibco	14065-056	Magnesium and calcium free
Imaging Media	Life Technologies	A14291DJ	Live Cell Imaging Solution
Inverted Confocal Microscope	Zeiss		LSM 780
KymoToolBox			https://github.com/fabricecordelieres/IJ_KymoToolBox
Lipofection Enhancer Reagent	Life Technologies	11514015	Plus Reagent
Lipofection Reagent	Life Technologies	15338100	Lipofectamine LTX reagent
Orbital shaking incubator	New Brunswick Scientific	8261-30-1008	Innova 4230 , orbital shaking incubator with temperature and speed control
Penicillin/Strepomycin solution (100x)	Gen Clone	25-512	
Phosphate Buffered Saline (10x)	Gen Clone	25-507x	
Poly-D-Lysine Hydrobromide	Sigma	P7405	Dissolve in Tris buffer, pH 8.5, at 1mg/mL. Freeze for long term storage. Avoid cycles of freezing and thawing
Reduced serum medium	Gibco	31985-062	OPTI-MEM
Tissue Culture Flasks	Olympus Plastics	25-209	75 cm^2. 100% angled neck access, 0.22um hydrophobic vented filter cap
Tissue culture incubator	Thermo Scientific	51030285	HERAcell VIOS 160i, tissue culture incubator with temperature, humidity, and CO2 control
Tris-Base	Sigma	T1503	8.402 g dissolved to one liter in water with 4.829 g Tris HCl to make 0.1 M Tris buffer, pH 8.5
Tris-HCl	Sigma	T3253	4.829 g dissolved to one liter in water with 8.402 g Tris Base to make 0.1 M Tris buffer, pH 8.5
Trypsin-EDTA (0.25%), phenol red	Gibco	25200072	
Vacuum-Driven Filter Systems	Olympus Plastics	25-227	500 ml, PES membrane, 0.22 µm
Vannas scissors straight	Roboz	RS-5620	

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Author(s):	Blake A. Creighton, Theodore W. Ruffins, Damaris N. Lorenzo

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CORRESPONDING AUTHOR

Name:	Damaris N. Lorenzo
Department:	Cell Biology and Physiology
Institution:	University of North Carolina at Chapel Hill
Title:	Assistant Professor
Signature:	
Date:	05/06/2019

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June 7, 2019

Journal of Visualized Experiments

Re: Revised manuscript, JoVE60230

Dear editor:

Thank you for considering our manuscript **"Visualizing and Analyzing Intracellular Transport of Organelles and other Cargo in Astrocytes"** for publication in JoVE and for your guidance on how to revise our manuscript. We appreciate the positive feedback and useful recommendations to improve the manuscript by the reviewers. Below we address the reviewers' comments point-by-point by incorporating new figures and by modifying the text in the revised manuscript.

We hope that the revised manuscript will now be acceptable for publication in the JoVE.

Sincerely,

Damaris Lorenzo

Editorial comments:

Changes to be made by the author(s):

1. ***Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.***

   The document has been proofread.

2. ***Please revise lines 124-133, 226-228 and 234-235, and 239-241 to avoid textual overlap with previously published work.***

   The language has been modified.

3. ***Please abbreviate liters to L (L, mL, µL) to avoid confusion.***

   The suggested abbreviations have been incorporated.

4. ***Please revise the Protocol text to avoid the use of personal pronouns (e.g., I, you, your, we, our) or colloquial phrases.***

   Personal pronouns have been removed from the protocol text.

5. ***Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. Step 1 followed by 1.1, followed by 1.1.1, etc. Please refrain from using bullets, dashes, or indentations.***

The appropriate numbering has been incorporated.

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   The commercial names have been removed from the protocol and the reader has been referred to the Table of Materials when appropriate.

7. ***Please include single line spacing between each numbered step or note in the protocol.***

   The suggested spacing has been incorporated.

8. ***Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.***

9. ***Line 103: Please specify the age, gender and type of the animal.***

10. ***Please specify all surgical tools used throughout the protocol.***

11. ***Line 122: How small are the pieces?***

12. ***Line 165, etc.: Please list all centrifugation speeds in terms of centrifugal g-force instead of rpm: 100 x g.***

13. ***Line 182: Please provide some guidance on desired density.***

14. ***Line 191: Please specify incubation temperature.***

15. ***Section 5: For actions involving software usage, please provide all specific details (e.g., button clicks, software commands, any user inputs, etc.) needed to execute the actions.***

16. ***Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.***

    The suggested clarifications and/or additions (points 8-16 above) have been incorporated.

17. ***After you have made all the recommended changes to your protocol section (listed above), please highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video. Bear in mind the goal of the protocol and highlight the critical steps to be filmed. Our scriptwriters***

will derive the video script directly from the highlighted text.

18. ***Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. The highlighted text must include at least one action that is written in the imperative voice per step. Notes cannot usually be filmed and should be excluded from the highlighting.***

19. ***Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.***

20. ***Figure 2: Please include a space between number and its corresponding unit (20 µm).***

    This has been fixed.

21. ***References: Please do not abbreviate journal titles; use full journal name.***

    Full names of journal titles have been incorporated in the references.

Reviewer #1:

Manuscript Summary:
Creighton et al is a very detailed and timely manuscript on live-cell imaging of cargos in astrocytes. It is very refreshing to see this topic being addressed, because as the authors state, the transport field has mostly focused on neurons. The text of the protocol is easy to follow and clear and the primary data are of very high quality and clearly presented. I very much look forward to future work from this lab on transport in astrocytes.

Major Concerns:
None.

Minor Concerns:

The manuscript is already very polished and should be accepted without additional experiments and only text changes. I would like to re-review it, because I am curious and really want to read the revised version before it is published! I have very few minor comments and they are:

1. ***In the intro, please discuss whether the polarity of microtubules in astrocytes is known, either through classic EM experiments or through live-cell imaging. If it is not known, please just state that; the authors do not need to do any additional experiments.***

   The limited literature addressing the question of microtubule polarity in astrocytes suggests that bundles of microtubules radiate out from the surrounding area of the nucleus, extending their plus end towards the periphery, as shown by Chiu *et al.* using immunofluorescence staining of EB1. We have included a brief discussion addressing this point in the introduction.

2. ***The sentence on Lines 69-72 needs a reference. I assumed it refers to the references cited earlier in the paragraph, but please re-cite them if that is the case.***

   The appropriate references have been added.

3. ***On Line 89, please reference the original 1980 JCB McCarthy and deVellis paper that this JoVE citation is adapted from. It is important to also state in the text here that these are McCarthy/deVellis (or commonly referred to in the glial field as MD) astrocytes.***

   The McCarthy and deVellis reference has been added, along with a reference to 1986 JCB Tedeschi *et al.*, as one of the original papers using the antimitotic treatment in astrocytes cultures.

As a piece of unsolicited advice (and not a point to address for the revision) the authors may consider switching to the immunopanning method for purifying astrocytes in the absence of serum; under these conditions, astrocytes extend processes that can be hundreds of ums long and would be a better model for transport.

Thank you for the advice. We are aware the methodology developed by Foo et al., which we may use in future experiments to address some specific questions in our studies.

4. ***Line 215, acquisition. The authors should discuss if they imaged 1 z-stack or multiple.***

   We have added additional details to the acquisition protocol including the specification of the number of z-stacks imaged.

5. ***The KymoToolBox plugin is not one that I've used before so I tried it, but received many error messages. Thus, I think it would useful to add the following details:***

a. How to open files initially (as a TIFF stack I presume).
b. How to set the parameters and a brief description of what they mean.
c. What line width value was entered to generate the kymograph.

We have added additional details and specification to software analysis portion of the protocol.

Completely optional comments that the authors can choose to address or ignore:

1. ***Line 274 refers to these 3 cargos are being transported "bidirectionally". I typically think of bidirectional transport as cargos simultaneously engaged with plus-end and minus-end directed motors with jiggly back-and-forth movement (fast switches and low net displacement). I would describe what the authors observe as "bidirectional transport with occasional fast, processive runs in both directions".***

   This is a subtle but important distinction. To avoid confusion, we have modified our description, as suggested by the reviewer.

2. ***The intro and/or discussion could benefit from a few sentences talking about the function of Cx43 and EAAT1 and why they are important for overall astrocyte***

maintenance.

For the sake of brevity, we did not discuss the function of the selected molecules in our examples. A few of the cited reference do a good job at covering the function of some of these molecules and organelles in astrocytes.

Reviewer #2:

In this manuscript, Creighton and co-authors provided a protocol for culturing murine astrocytes and investigating intracellular transport dynamics. This protocol focuses on cell culture, novel astrocyte purification method using AraC, transfection, labeling of late endosome/lysosome, and time-lapse imaging, and data analysis. Overall, the protocol provide enough information on the experiments.

The major concern on this protocol is whether this novel astrocyte purification protocol using AraC is as efficient as the protocol described previously (Schildge et al. 2013 J Vis Exp. 71, pii: 50079). The immunocyte chemistry data (GFAP/DAPI) of purified astrocytes presented in Fig 1Biii includes GFAP negative (DAPI positive) cells in the center of this image, causing doubts about the purity of this culture. The method to characterize the purity of astrocyte should be described.

This is an important point and certainly relevant to users of the protocol. As we now show, with the modified protocol we describe here, the purity of our astrocytes culture can be as high as 98%. In contrast, the purity we obtain using the protocol outlined by Schildge et al. result in purities below 70%, new Fig 1B and C.

Following are concerns/suggestions regarding this manuscript:

1. ***In Table of Materials, some material and equipment are provided by commercial name, which some readers cannot access (e.g. HERAcell VIOS 160i , Innova 4230, Mattek glass bottom dishes, Sorvall ST8 Centrifuge, LSM 780 Inverted Confocal Microscope). Please provide generic name.***

   The suggested changes have been incorporated.

2. ***Surgical instruments should be fully described in Table of Materials (Comments/Description section).***

   The new Table of Materials includes a better description of the surgical instruments.

3. ***Please provide detailed specification for culture flask, if it is critical. Should the cap of the flask be with filters? canted neck flasks or straight neck flask? The reviewer think the cap and neck is critical, as this protocol includes long (6 h) incubation in the shaking incubator.***

   All the relevant information regarding the suggested culture flasks is now included in the protocol.

4. ***Please describe the preparation protocol for the stock solution of AraC, LysoTracker Green DND 26, Trizma Base, Poly-D-Lysine Hydrobromide, etc... in Table of***

Materials (Comments/Description section).

Details about the preparation of the stock solutions have been added to the Table of Materials.

5. ***P3. L137: "PDL" should be poly-D-lysine or defined at the first appearance (P2 L94).***

   PDL is now spelled out when first mentioned.

6. ***Step 2-2) and 6) (P3): Is it necessary to control the CO2 concentration during the incubation in the shaking incubator? Only temperature control is sufficient? Please specify.***

   No. We have now made it clear in the document.

7. ***The AraC incubation is the novelty of this protocol, therefore readers may require more information. Please provide the phase contrast microscopic images (like the one in Fig 1Bi, ii) of cells before (step 2-2) and after (step3) AraC application in the REPRESENTATIVE RESULTS section, because it would be very informative to readers to confirm whether the experiment is going well or not.***

   This is indeed an important point. Please read our first comment above.

8. ***"Step 4. Image acquisition using a time-lapse imaging system" should be described step by step. At least, imaging step should include 1) placing of the specimen on the microscopic table, 2) adjustment of focus, 3) finding the cell to record, 4) recording, and 5) saving of recorded data.***

   We have added additional relevant steps to the image acquisition portion of the protocol.

9. ***The authors mention that "the time course of acquisition may require adjustment" (P5 L215). It would be informative if the authors describe more details on recording parameter adjustment.***

   We have added more details on recording parameter adjustment.

10. ***The file format acceptable for the KymoToolBox should be described in "Step 4. Image acquisition using a time-lapse imaging system", so that the readers can save the recording data in appropriate format.***

    We have now specified the file format acceptable for the KymoToolBox.

11. ***The representative single astrocyte morphology will be informative. Immunocytochemistry with GFAP labeling with lower cell density would help the authors to present nicer data.***

    We have incorporated examples of representative single astrocyte morphologies (Fig. 2Biii and vi).

12. ***FIGURE LEGENDS for figure 2: Please provide the recording frequency (0.5 Hz?).***

We have indicated the recording frequency in frames/second in the legend of Fig. 2.

Reviewer #3:

Manuscript Summary:

The present manuscript describes a protocol to obtain primary cultured murine astrocytes and to analyze trafficking of fluorescently-labelled transmembrane proteins and intracellular organelles within these cells by using time-lapse confocal microscopy. A method to extrapolate and quantify transport parameters from the records is also reported.

Major Concerns:

Advantages and weaknesses of the protocol described in the manuscript in comparison to the alternative approaches actually available should be discussed in the manuscript.

The methodology we described here combines different methodologies to streamline the production of high-quality mouse MD astrocytes cultures and the acquisition and extraction of intracellular transport data, all in one protocol. For example, we adopted the cell culture method of Schildge et al. because of its simplicity compared to other alternative protocols. However, as we now discussed, the purities we obtained using that method without the addition of antimitotic agents is below 70% (new Fig 1B and C). In contrast, addition of AraC, a commonly used antimitotic agent in the field improves the astrocytes culture purity to levels as high as 98% (new Fig 1B and C). We have added comments to note those points, along with cautionary notes about potential limitations of the method.

Minor Concerns:

1. ***Please, specify if HBSS is with or w/o Ca and Mg every time it is mentioned.***

   The suggested specification has been added.

2. ***Check centrifugation speed in section 2.10 (rpm or g).***

   All centrifugation speeds are now expressed in g.

3. ***In section 3.1, indicate which astrocyte density is better or describe how this parameter could affect the quality of the results.***

   The astrocyte density is now specified.

4. ***Applicability of the present protocol to other cell types (i.e. oligodendrocytes) should be discussed.***

   We have added some comments about the potential use of the protocol to culture other glial cells at the appropriate steps, including the avoidance of antimitotic agents, which might compromise the health of those cultures.

5. ***When necessary, for each experimental step, underline critical points and propose a***

possible contingency plan. This would greatly help researchers in the set-up of the procedure.

Critical points in selected steps are now bolded.

6. ***Authors should also carefully proof-read the manuscript to minimize typographical errors.***

   The manuscript has been carefully proofread.