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Real time videomicroscopy and semi-automated analysis of brain cell culture models of trinucleotide repeat expansion diseases.

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Real-time monitoring of brain cell models of trinucleotide expansion diseases.

Abstract

Proper brain function requires the coordinated and intricate interaction between neuronal and glial cells. Like many other neurological conditions, trinucleotide repeat expansion disorders are likely initiated by the synergistic combination of abnormalities hitting different brain cell types, which ultimately disrupt brain function and lead to the onset of neurological symptoms. Understanding how trinucleotide repeat expansions affect the phenotypes and physiology of neurons and glia is fundamental to improve our understanding of disease mechanisms in the brain and shape the design of future therapeutic interventions.

Here we describe a protocol for semi-automated videomicroscopy analysis of cultured brain cells, maintained under suitable and controlled conditions. Through real-time monitoring of basic cell phenotypes (such as proliferation, cell morphology, differentiation and migration) this method provides an accurate primary assessment of the impact of the repeat expansion on the physiology of neurons and glia. The versatility of the system, the automated image acquisition and the semi-automated processing of the data collected allow rapid phenotypic analysis of individual cell types, as well as the investigation of cell-cell interactions. The stability of the acquisition system provides reproducible and robust results. The raw data can be easily exported to other software to perform more sophisticated imaging analysis and statistical tests. In summary, the methods described offer versatile, reproducible and time-effective means to dissect the impact of the repeat expansion on different brain cell types and on intercellular interactions.

Key words:

Real-time videomicroscopy; primary cell cultures; neuron; astrocyte; oligodendrocyte; trinucleotide repeat expansion; cell phenotypes; semi-automated analysis.

1. Introduction

Trinucleotide repeat expansion diseases comprise neuromuscular, neurodevelopmental and neurodegenerative disorders [1, 2], which are usually associated with cognitive deficits and behavioral changes. Today we know that repeat expansions mediate disease etiology through a variety of mechanisms, including loss of function (*e.g.* fragile X syndrome, FXS; and Friedreich ataxia, FRDA) [3, 4], toxic gain of function of polyglutamine tracts (*e.g.* Huntington disease, HD) [5] and RNA toxicity (*e.g.* myotonic dystrophy type 1, DM1) [6]. On top of these mechanisms, the accumulation of toxic homopolypeptides by repeat-associated non-AUG (RAN) translation may also play a determinant pathogenic role [7].

Mouse models have been crucial to decipher the molecular pathogenesis of trinucleotide repeat expansion diseases, notably in the brain, where they recreate the intricate and multidirectional interactions between different cell types [8]. However, the broad view revealed by the analysis of tissue samples, does not grant sufficient resolution to focus on pathological events occurring in specific cell populations. This is particularly relevant in conditions in which it is conceivable that disease susceptibility and the cascade of pathogenic events vary between different cell types and/or subpopulations. Cell type selective pathogenesis in trinucleotide repeat expansion disorders is illustrated by the preferential degeneration of medium spiny neurons in the striatum of HD patients [9] and by the pervasive loss of Purkinje cells in many spirocerebellar ataxias (SCAs) [10], while other neuronal types remain relatively spared. Rat primary cultures corroborated the increased vulnerability of striatal neurons to expanded huntingtin, relative to cortical neurons [11]. More recently, the generation of human iPSC-derived Pukinje cells confirmed the burden of SCAs to this neuronal cell type [12].

In spite of the neuronal damage that precedes the neurological manifestations of trinucleotide repeat expansion diseases, a less “neurocentric” view of brain pathology is emerging, pointing to the key role of glial cells. It has been reported that astrocyte dysfunction prevents

normal neuronal development and proper synaptogenesis in FXS [13, 14] and FRDA [15, 16]. Astrocyte deficits may also cause Purkinje cell hyperexcitability in SCA7 [17] and DM1 [18]. Signs of astrocyte pathology were also reported in HD [19], fragile X-associated tremor/ataxia syndrome (FXTAS) [20] and SCA1 [21]. In addition to astrocytes, oligodendrocytes are also affected by trinucleotide repeat expansions. The dysfunction of this glial cell type was documented in mouse models of HD and FXS, and it was characterized by delayed and reduced myelination in association with defective maturation of oligodendrocytes [22, 23]. The involvement of microglia in trinucleotide repeat expansion diseases has been less explored. Nonetheless, microglia activation was reported in HD patients, as well as in mouse models of HD [24, 25] and SCA1 [21]. In HD models, microglia activation is accompanied by abnormal cell morphology and defective migration response to chemotaxis [26].

Together these findings demonstrate the diverse response of different brain cells to disease, and the critical insight that can be gained from the analysis of individual cell types. Understanding the disease mechanisms that operate in different cell populations and underlie the complex neurological symptoms is crucial for future therapeutic developments. Cell culture techniques provide a powerful setting to address this question. While homogenous cell cultures can reveal the impact of the disease on the phenotype and physiology of different cell types, mixed cultures (established through the combination of different cells) can help untangle the mechanisms of intercellular miscommunication in the brain and their contribution to disease. These studies were traditionally performed on cultured cells fixed at different time points. Although informative, this strategy presents two main limitations: (a) the readouts represent isolated snapshots of physiological processes that are continuously ongoing and they may therefore miss relevant events; (b) the methodology is time-consuming, involving cell manipulation, cell labeling, signal detection, data collection and laborious data analysis.

The alternative is to perform prolonged recordings of live cell phenotypes, by real-time semi-automated videomicroscopy. The success of this methodology depends on the maintenance of well-defined and suitable culture conditions, which must guarantee cell function and stability over long periods of time. This requirement is particularly important for brain cells, given their high sensitivity to environmental changes: subtle uncontrolled fluctuations may have deleterious consequences to cell homeostasis and affect the quality and reproducibility of the results. The advanced Incucyte® live-cell analysis system (Essen Bioscience) reduces these technical challenges and maximizes the quality and reproducibility of the data collected. The videosystem is incorporated in a regular CO_2 incubator, allowing the cells to be kept in their optimal temperature and humidity throughout the experiment. Image acquisition is automatic: once the experimenter has defined the acquisition settings he/she can walk away, leaving the cells unperturbed. The semi-automatic analysis of the Incucyte® system is another major strength: the system offers pre-defined analysis settings that can be adapted by the scientist to better fit the type of cells and the biological process being studied. Once defined, the same settings and analysis can be immediately applied to the images as they are acquired by the video system - the computerized workflow translates into real-time results and traceability of eventual problems, therefore saving precious time.

The methods described here provide a videomicroscopy semi-automated approach to assess the primary impact of the disease on different cell lineages of the brain (neurons, astrocytes and oligodendrocytes), using proliferation, differentiation, cell adhesion, morphological complexity and migration (among others) as basic readouts of cell dysfunction. The results generated offer meaningful clues to design future experiments and extend the analysis of the disease mechanisms operating in individual cell populations, through more refined approaches. In spite of our focus on brain cells, the protocols can be easily adapted and applied to a myriad of other cell types.

2. Materials

2.1. Specific equipment and reagents

1. IncuCyte Zoom videomicroscope equipped with IncuCyte® Scratch Wound Cell Migration Software Module (9600-0012, Essen Bioscience) and IncuCyte® NeuroTrack Software Module (9600-0010, Essen Bioscience).
2. Computer equipped with Incucyte Zoom software, FIJI-ImageJ software [27] and DiPer program [28].
3. IncuCyte® Cell Migration Kit (4493, Essen Bioscience).
4. IncuCyte® ImageLock Plates (4379, Essen Bioscience).
5. IncuCyte® NeuroLight Red Lentivirus - Synapsin Promoter (4584, Essen Bioscience).
6. GentleMACS Dissociator (130-093-235 or superior, Miltenyi).
7. Neural tissue dissociation kit (130-092-628, Miltenyi) and magnetic anti-CD140a microbeads (130-092-628, Miltenyi).
8. Equipment maintenance: 0.5% Alconox, 1% Virkon, double distilled H_2O and 70% Ethanol.

2.2. Mouse experiments

For experiments involving animal models you must follow the institutional, national and/or international guidelines for ethical conduct in the care and use of animals.

2.3. Cell culture media and coating reagents

1. Dissection medium for neurons and astrocytes: Leibovitz's L-15 Medium, 30 µM glucose.
2. Dissection medium for OPC: Hank's balanced salt solution without (HBSS) Ca^{2+} and Mg^{2+}.
3. Neuronal medium: Neurobasal-A, 1X B27 supplement, 0.5 mM L-Glutamine, 0.25 µg/mL Amphotericin B, 100 U/mL penicillin and 100 µg/mL streptomycin.
4. Astrocytes medium: DMEM low glucose, 10% fetal bovine serum (FBS) and 0.05 mg/mL Gentamycin.

5. OPC medium: DMEM F-12, 1% N2 supplement, 2% B27 supplement, 50 units/mL penicillin and 50 µg/mL streptomycin, 0.01% bovine serum albumin, 40 ng/mL FGF2 and 20 ng/mL PDGF-AA.
6. Oligodendrocytes medium: DMEM F-12, 1% N2 supplement, 2% B27 supplement, 50 units/mL penicillin and 50 µg/mL streptomycin, 50 µg/mL insulin, 40 ng/mL triiodo-thyronin and 1 ng/mL CNTF.
7. Coating: 0.1 mg/mL of poly-D-lysine (PDL) and 10 µg/mL laminin (See **Note 1**).

3. Methods

3.1. Primary mouse cell cultures

Different protocols are available in the literature and can be used to establish primary mouse neurons, astrocytes and oligodendrocytes cultures. We briefly describe below the protocols used in our laboratory to ensure healthy and reproducible cell cultures prior to cell seeding (see **Note 2**).

For primary neuron cultures:

1. Prepare primary dissociated cell cultures of cortical neurons from embryonic day (E) 16.5 mouse embryos (see **Note 3**). Dissect the brain and place it on a dish with dissociation medium for neurons, under a stereoscope.
2. Separate the cortices from the rest of the brain and carefully remove the meninges with the help of micro tweezers.
3. Maintain the cortices in dissection medium on ice until end of the dissection.
4. Wash the cortices twice with neuronal medium, previously warmed at 37˚C.
5. Incubate the cortices in neuronal medium containing 0.05% of trypsin and 10 U/mL of DNaseI 15 min at 37ºC, shaking gently the tube every 5 minutes (min).
6. Wash the cell suspension twice with neuronal medium supplemented with 5% FBS to inhibit further tissue digestion by trypsin.

7. Dissociate the cells passing them through a 21 G needle, wash twice in neuronal medium, collecting the cells at 200 g for 5 min at room temperature (RT) in between washes.
8. Count cells (see **Note 4**) before plating at appropriated cell density in PDL and laminin-coated 96 well plates (see **Note 5**) in pre-warmed neuronal medium containing 5% FBS.
9. Replace the medium 4 hours (h) after plating with neuronal medium supplemented with 100 μM AraC and withdraw the FBS to avoid astrocytes proliferation (see **Note 6**).
10. Change half of the medium every 3-4 days (see **Note 7**).

For primary astrocyte cultures:

1. Prepare primary dissociated cell cultures of cortical astrocytes from postnatal day (P) 1 pups (see **Note 3**). Dissect the cortices as described above.
2. Wash the tissue twice with 1 mL of astrocytes medium and mechanically dissociate, passing through 21 G needle twice and then three times through a 26 G needle, waiting 2-3 min for the sedimentation of the fragments in between each dissociation.
3. Centrifuge the cell suspension 200 g for 5 min at RT, remove the supernatant and resuspend the cells in 10 mL of pre-warmed astrocytes medium.
4. Plate the cells in a T75 dish at 300,000 cells/mL.
5. On the following day, change the medium (see **Note 7**) to remove the dead cells.
6. Primary astrocytes adhere, grow and proliferate on both coated and uncoated dishes (see **Note 1** and **6**).
7. Incubate the cells for 2 weeks in astrocyte medium, changing the medium every 3 days (see **Note 8**).

For primary oligodendrocyte progenitors (OPC) cell cultures:

1. Prepare dissociated cell cultures of cortical OPC from P0-P2 mice (see **Note 3**). Perform the dissection as described above using dissection medium for OPC.

2. Dissociate the tissue using the neural tissue dissociation kit and following the manufacture's protocol. Dissociate the tissue by cycles of mechanic and enzymatic dissociation to produce a single cell suspension, then filter the suspension through a 70 µm strainer and wash with HBSS with Ca^{2+} and Mg^{2+}.
3. Centrifuge the cell suspension at 300 g for 10 min at RT, remove the supernatant and resuspend the cells in a 1X PBS with Ca^{2+} and Mg^{2+}, supplemented with 5% BSA (BSA buffer).
4. Isolate the OPC using magnetic anti-CD140a (PDGFRα) labelled microbeads (protocol adapted from Dincman *et al.* 2012 [29]).
5. After blocking, incubate the cells with the magnetic microbeads to allow the binding of the $PDGFR\alpha^+$ cells, then wash the cells in BSA buffer and collect at 300 g for 10 min at RT, to eliminate cell-free microbeads.
6. To separate the OPC, pass the cell suspension trough a column attached to a magnetic field, where the $PDGFR\alpha^+$ cells are retained.
7. Remove the column from the magnetic field and flush the cells in OPC medium.
8. Count OPC and plate them at a density of 9,000-15,000 cells/cm^2 in PDL and laminin-coated 96 well plates (see **Note 5**) and keep in OPC medium to proliferate (See **Note 9**).
9. Two days later, replace the medium with oligodendrocyte medium, in order to induce the differentiation of OPC into oligodendrocytes (see **Note 6**).

3.2. Cell proliferation (Astrocytes and OPC)

In this protocol, cell proliferation is estimated by the percentage of well surface area occupied by cells (i.e. confluence) over time.

1. Seed the astrocytes and OPC at 20,000 or 5,000 cells/well, respectively (see **Note 10**), on 96-well plates (see **Note 5**) and keep the cells in their corresponding culture media.

2. Set the system to acquire a phase-contrast picture (see **Note 11**) every 45 min over 50 h for astrocytes, or every 2 h during 7-8 days for oligodendrocytes, selecting the "Standard" type of scan and using the 20X objective (see **Note 12**). Change the cell medium every 2-3 days (see **Notes 6** and **13**).
3. Set the image analysis by first creating a collection of images (see **Note 14**) representative of the phenotypes to be monitored throughout the experiment. Select the first picture and click on "Create or Add to Image Collection" (**Fig. 1** option **A**), select the "Basic Analyzer" job type mode and add the image to the new image collection. Select another 4-6 new images (see **Note 15**) and add them to the same image collection.
4. Using this collection of images, define the settings to be applied to the analyses. Under the option "New Processing Definition" (**Fig. 1** option **B**) run the "Preview Current" command to automatically generate the "Confluence Mask" that will contour and cover the surface of what the software considers to be cells in the first picture selected.
5. Since the automatic mask is unlikely to fit all the cell shapes/densities through time and/or all cell conditions (**Fig. 2A)**, adjust this mask in order to fully outline the shape of the cells. To do so use the filters named "Cell/Background", "Size" and "Eccentricity", which are available on the "Segmentation Adjustment" panel (See **Note 16**).
6. Apply the defined settings to all the image collection by selecting the option "Preview All", and check if the mask fits perfectly the majority of the cells in the different images of the image collection previously chosen (**Fig. 2B**).
7. Initiate the analysis by selecting "Launch New Analysis Job" (**Fig. 1** option **C**). The software will apply the defined settings to all the pictures acquired from the plate over time (see **Note 17**).
8. The results will estimate cell proliferation, measured as the percentage of cell confluence (see **Notes 18** and **19**) or as a phase-contrast object (cell) count (see **Notes 11** and **20**) during

the time-course of the experiment. The results are plotted as the percentage of confluence over time for each well, or as the average of technical/biological replicates (**Fig. 2C**) (See **Note 21**).

3.3. Wound healing migration (Astrocytes and OPC)

This analysis evaluates the capacity on the cells to migrate towards and fill an empty space created by a wound on a cell monolayer. It estimates the percentage of the wound surface occupied by migrating cells over time.

1. Seed 20,000 astrocytes or 10,000 OPC per well (see **Note 10**), on a 96-well plate Incucyte ® Image Lock (see **Notes 1, 22** and **23**) and keep the cells in their medium. Incubate the cells for 1-2 days to obtain a confluent monolayer of astrocytes, or a 70-90% confluent layer of OPC.
2. Place the open plate in the base of the Incucyte® Wound Maker (see **Note 24**) and carefully introduce the wound maker pin block into the wells. Perform the wound on the bottom of the well by pushing and holding the lever (see **Note 25**). Wash the cells gently with pre-warm astrocyte or OPC medium twice, to remove any cell debris remaining in the wound (while avoiding to disturb the cells) and refill the wells with 100 µL of medium (See **Note 7**) using a multichannel micropipette.
3. Set the system to acquire a phase-contrast picture (see **Note 11**) every 45-60 min over 3 days (See **Note 22**), selecting the "Scratch Wound" on the "Scan Type" dropdown and using the 10x objective.
4. Set the image analysis by creating a representative set of images taking into account the phenotype to be analyzed over time (See **Note 14**). To this end, select the first picture and click on "Create or Add to Image Collection" (**Fig. 1** option **A**), select the "Scratch Wound" option on the job type mode and add the image to the new image collection. Select another

4-6 new images (see **Note 15**), including the first scan (time 0) after wounding, which will be used to define the initial mask for the surface of the wounded (cell-free) area. Add the images to the previous image collection.

5. Using this collection of images, define the settings to be applied to the analyses. Under the option "Process Definition" (**Fig. 1** option **B**) run the "Preview Current" command on the first picture to automatically generate the "Confluence Mask" (which will cover and contour the surface of what the software supposes to be a cell layer (**Fig. 3A-1**)) and the "Scratch Wound Mask" (which will cover the cell-free space created by the scratch (**Fig. 3A-3**)).
6. Since the automatic mask may not be able to perfectly detect and outlines the cell layer limits and/or some cell debris may remain on the wounded area (**Fig. 3A)**, adjust the mask so it fully covers and delineates cell shapes and the cell front on each side of the wound, using the filters named "Cell/Background", "Size" and "Eccentricity" available on the "Segmentation Adjustment" panel (See **Notes 16** and **26**).
7. Apply the defined settings to the entire image collection previously created by running "Preview All" and check if the masks provide a perfect fit for the front of migration (**Fig. 3A-2**), cell layer and wounded space for the majority of the different images previously selected (**Fig. 3B**).
8. Initiate the analysis by clicking on the "Launch New Analysis Job" (**Fig. 1** option **C**). The software will apply the settings previously defined to all the pictures acquired on the plate (see **Note 17**).
9. The results will estimate the rate of occupancy of the space left empty following the scratch. They can be represented by one of three measurements: a) wound width over time (distance between the front of migration of each side of the cell-free space); b) wound confluence over time (the percentage of the wound initial surface occupied by the cells) or c) relative wound

density (RWD, the percentage of the cell density in the wound relative to the percentage of cell density in the regions surrounding the wound) (see **Notes 21** and **27**) (**Fig. 3C**).

3.4. Single cell tracking (Astrocytes)

This analysis allows the drawing of the migration path, the measurement of speed, directionality and displacement of individual astrocytes by manually tracking the position of the nucleus in each image, using Fiji and the open-source DiPer computer software [28].

1. Following the wound healing assay, export migration videos from the IncuCyte® in uncompressed AVI format and open them using Fiji (see **Note 28**). Make sure to export at least one video (or one image) with the embedded scale, to be able to use it for setting the scale in Fiji (see **Note 29**). The image visualization of the videos can be modified to facilitate tracking (See **Note 30** and **31**).
2. After setting the scale (see **Note 28**) use the "Manual Tracking Fiji" plugin to track the nucleus of at least 40 astrocytes per video. Insert the time interval in the corresponding panel of the Tracking window, and the x/y calibration (corresponding to the pixel width, which can be found when setting the scale in Fiji). Start tracking by clicking "Add track" (**Fig. 4A**) and following the advancement of one selected cell. This will measure the position of the astrocytes on the X and Y coordinates, the distance and the velocity of the astrocytes (**Fig. 4B**).
3. The distance and velocity results can be directly analyzed using the appropriate statistical program, or they can be used to calculate more complex parameters using the DiPer open-source macros for Microsoft Excel [28]. These macros allow the calculation of cells directionality (defined as the straight line between the start and the end point of the migration trajectory divided by the total length of the trajectory), or the mean square displacement (which will assess the area explored by the migrating cells over time), as well

as plotting each cell trajectory, individually or collectively, on X/Y axes, emanating from the original starting point of displacement (**Fig. 4C**).

3.5. Morphological complexity (Neurons and Oligodendrocytes)

In the following protocols the analyses measure the changes in length and branching of neurites and oligodendrocyte processes over time.

Neurite development:

1. Seed primary neurons at 30,000 cell/well on a coated 96-well plate and keep the cells in neuronal medium (see **Notes 1**, **5** and **10**). Incubate the plates at 37ºC overnight before placing them in the Incucyte® 16 hours later (in the next morning).
2. Set the system to acquire one phase-contrast picture (see **Note 11**) every 3 h over 7 days, using the "Standard" type of scan and the 20X objective, (see **Notes 12** and **32**).
3. Set the image analysis, starting by creating a representative set of images taking into account the cell morphology to be analyzed during the course of the experiment (see **Note 14**). To this end, select the first picture and click on "Create or Add to Image Collection" (**Fig. 1** option **A**), select the mode "Neurotrack" as job type and add the image to the new image collection. Select another 4-6 new images (see **Note 15**) and add them to the previous collection.
4. Using this collection of images, define the settings to be applied to the analyses. Under the option "Process Definition" (**Fig. 1** option **B**) run the "Preview Current" on the first picture to automatically generate the "Phase Cell-Body Cluster Mask" and a the "Phase Neurite Mask" (See **Note 33**). The first will cover and contour only the cell-body surface, while the second will trace what the software assumes to be neurites (cell processes).
5. Due to the complexity of the neuronal cultures, the automatic mask may not be able to perfectly trace all neurites. In that case, select "Phase Neurites (Analyze)" and adjust the

masks in order to fully cover and delineate the cell shape. To this end, use the filters "Cell/Background", "Size" and "Eccentricity" to adjust the "Phase Cell-Body Cluster Mask" (see **Note 16**), and the neurite sensitivity parameters (see **Note 34)** for the "Neurite Mask" refinement. These option and filters are available on the "Cell-body Cluster" and "Neurite" parameter panels.

6. Apply the defined settings to all the image collection by running "Preview All" and check if the masks faithfully reproduce the complexity of the neuronal cell shape (**Fig. 5A**).
7. Initiate the analysis selecting the "Launch New Analysis Job" (**Fig. 1** option **C**) and select the "NeuroTrack" job type. The software will apply the settings previously defined to all the pictures acquired on the plate throughout the experiments (see **Note 17**).
8. The results will measure the changes in neurite length and number of branching points per well surface over time (**Fig. 5C)** (see **Note 21** and **35**).

Process extension:

1. Seed OPC at 5,000 cell/well on coated 96-well plates and keep the cells in OPC medium for two days to stabilize the culture (see **Notes 1, 5** and **10**).
2. Set the system to acquire one phase-contrast picture every 2 h over 8-10 days, selecting the "Standard" type of scan and using the 20X objective (see **Notes 12** and **32)**.
3. After 2 days, replace the medium with oligodendrocyte medium to induce OPC differentiation into mature, fully ramified oligodendrocytes. Change the medium every 2-3 days (see **Notes 7** and **13)**.
4. To set the analysis, start by creating a representative set of images taking into account the changes in cell morphology that occur over time and that you will monitor over the course of the experiment (See **Note 14**). Proceed as described in section 3.5 (Neurite development, steps 4-5). Please note that the "Neurotrack" algorithm was developed to analyze neurons,

so the automatic mask may not be able to perfectly detect the thin oligodendrocytes processes and secondary branching (See **Note 34**). To adjust the "Neurite Mask" to better fit oligodendrocyte processes and branching, set the "Neurite" parameters to maximum sensitivity (See **Note 36**) (**Fig. 6A**).

5. Launch the analyses as described in section 3.5 (Neurite development, step 7).
6. The results will show the length of the processes and the number of secondary branching per cell-body cluster, thus reflecting the cell differentiation over time (see **Note 21 and 35**) (**Fig. 6C** and **6D**).

Co-cultures analyses:

This type of analysis allows the investigation of neuro-glia interaction. In particular, this protocol describes how to assess the effect of astrocytes on neurite development by measuring the neurite length and branching of red fluorescent neurons over time, in the presence of astrocytes.

1. Seed primary neurons at 30,000 cell/well on a coated 96-well plate and keep the cells in neuronal medium supplemented with 5% of FBS (see **Notes 1**, **5** and **10**). Incubate cells at 37ºC for 4 h.
2. Remove the culture medium. Infect neurons with IncuCyte® NeuroLight Red Lentivirus with the diluted lentivirus at a multiciplity of infection (MOI) of 3 TU/cell (see **Note 37**) in 200 µL of neuronal medium. Incubate cells with the lentivirus overnight at 37ºC.
3. The next morning (after 8-10 hours of lentiviral infection), remove 190 µL of lentivirus-containing medium, wash once with neuronal medium, and plate 50,000 previously cultured primary astrocytes in 200 µL of neuronal medium per well, on top of neurons. Perform half-medium changes every 3 days (see **Notes 7** and **13**).

4. Place the plate on the Incucyte® imaging system and set the scan to acquire one image every 3 h with the 20X objective for 10 days, selecting both phase contrast and red fluorescence channels and using the "Standard" type of scan (see **Notes 10** and **12**).
5. Two days after the beginning of the acquisition (when the Neurolight red signal in the neurites is sufficiently bright) optimize the "z" focus of the Incucyte ® objective manually, by moving its scale ring (usually 1.2 it is a good starting point). Test the "z" focus by clicking on "Scan on Demand" and scan some wells of the plate each time you move the ring, until you get a good image resolution of the red neurites across the entire plate.
6. Set the image analysis. Start by creating a representative set of red fluorescent images taking into account the cell morphology and cell structures to be monitored over time (see **Note 14**). To this end, select the first picture and click on "Create an Image Collection" (**Fig. 1** option **A**), select the mode "Neurotrack" as job type and add the image to the new image collection. Select another 4-6 new images (see **Note 15**) and add them to the previous collection.
7. Using this collection of images, define the settings to be applied to the analyses. Under the option "Process Definition" (**Fig. 1** option **B**) run the "Preview Current" on the first picture to automatically generate the "Cell-Body Cluster Mask" and a the "Color Neurites Mask". The first will cover and contour only the cell-body surface, while the second will trace what the software presumes to be neurites.
8. If the automatic mask is not able to perfectly trace all neurites, adjust it in the "Color Neurites (Analyze)" panel in order to fully cover and delineate the cell shape using the filters "Cell-Body Cluster Parameters", "Cleanup", "Cell-Body Cluster Filters" and "Neurite Parameters" for the "Color Neurites Mask" (see **Note 34**) (Fig. 7A).
9. Launch the analysis as described in section 3.5 (Neurite development, steps 6-7).

10. The results will measure the changes in the neurite length and number of branching points per well surface of the red fluorescent neurons over time (See **Note 35**) in the presence of unmarked co-cultured astrocytes (See **Note 38)** (Fig. 7B).

4. Notes

1. Different extracellular matrix coating protocols and reagents can be used according with the experiment (*e.g.* to study cell-to-matrix interactions) and cell type used. First, coat plates with PDL for at least 20 min at room temperature and wash three times with double distilled sterile H_2O. Second, coat plates with laminin at least 3h at 37ºC and wash the plates twice with 1X PBS.
2. The described cell culture protocols can be used to culture cells from either one single embryo/pup or pooled animals.
3. To obtain mouse embryos at E16.5 plan the mice breeding in advance. For efficient mouse mating, we recommend to cross in the same cage one male with one/two females in proestrus/estrus phase in the evening, and check for vaginal plugs in the morning of the next day. Since the coupling is not 100% efficient you may want to scale up the number of breeding couples. Also some strains/transgenic mice have lower breeding rates, which decrease the number of embryos. For the P0-P2 mice pups, one male and two females were maintained continuously in the same cage.
4. For the cell counting dilute the cells in trypan blue and count them using a neubauer chamber. Other counting methods/instruments can also be used.
5. For the protocols described, in our laboratory we use 96 well plates (unless stated otherwise), since this type of microplate allows the use of a lower cell number and volume of reagents and a high number of biological and experimental replicates per plate. However the Incucyte® imaging system supports a high variety of common cell vessels (listed in the manufacturer datasheets) that can be used accordingly with the cell type/experiment need. In addition the system can accommodate and read six microplates simultaneously, allowing high throughput processing of samples and easy reproducibility of the results.

6. The purity of the primary cultures can be verified by immunostaining detection of cell type-specific markers, such as NeuN for neurons, GFAP for astrocytes and Olig2 for oligodendrocytes.
7. Medium should be changed with fresh pre-warmed medium at 37ºC. Cell culture medium should be replaced carefully avoiding to touch or disturb the cell layer at the bottom of the plate.
8. In most experiments described experiments we used astrocytes cultured on uncoated plates during 2 weeks *in vitro*, unless stated otherwise.
9. The cell density is a critical issue for the OPC culture: suboptimal densities can lead to cell death (if cell density is too low) or undesired cell differentiation (if cell density is too high). The OPC and oligodendrocytes are loosely attached to the surface of the dishes, for this reason medium changes should be performed with extra care, using pre-warmed medium and gentle handling of the plates.
10. The cell densities indicated are suitable for the cell types mentioned in the protocol. However for other cell types, optimal seeding densities should be established prior to the experiment. After seeding, incubate the plates 15 min at room temperature to allow the cells to distribute evenly across the bottom of the plate, before placing the cells in the Incucyte® incubator. Wait 15 min before starting the scan, to allow the dissipation of eventual condensation accumulated on the plate lid.
11. In addition to the acquisition of images in a stain-free manner, the system is also able to detect fluorescence, which diversifies the array of possible experiments. Notably, the fluorescent labeling of nuclear DNA is an alternative method to precisely count the number of cells and monitor cell proliferation, or the fluorescent labeling of neurites for the length and branching measurements (as described in section 3.4).

12. To avoid variability resulting from scanned images collected from non-representative areas of the well (in case of uneven cell distribution), it is recommended to select multiple pictures per well by editing the “Scan Pattern”.
13. To change the cell medium while the experiment is ongoing, remove the plate from the machine in between two scans, change the medium and reintroduce the plate in the machine before the next scan begins.
14. The aim of creating an image collection is to have a set of representative images to train and test the settings that will be subsequently used for the analysis.
15. Although the number of pictures in the image collection is not limited, adding too many pictures slows down the process of setting definition. The selected images should represent all the possible cell variations in shape, density or other relevant parameters over time and/or across all the conditions tested. Pictures with bizarre cell density or poor image quality should not be included in the image collection and they should be excluded from later data analyses. For example, pictures taken close to the border of the well may show non-homogeneous cell density, due to uneven seeding.
16. The “cells/background” sliding cursor is useful to distinguish more or less cells from the background. If the culture is clean from cell debris and/or the cells are flat/low contrasted, the cursors should be shifted towards the cell side to filter in the entire cell. In contrast, if the culture contains cell debris and the cell have a good phase-contrast, the cursor should be placed towards the background. Additionally, as the cell debris is normally round and of smaller size than cells, it can be filtered out by the “size” and “shape” filters - increasing the minimum “size” and “eccentricity” of the object to be considered as cells.
17. For the same group of acquisitions (“Vessel Scans”) we can generate as many image collections and process definitions as we feel necessary to better adapt the analysis to the biological process being studied. Those can be applied to all the scans at any time of the

experiment while they are acquired by the imaging system,, using the option “Open Ended”. The good fitting of the mask can be checked for each individual image once the analysis is launched and finished.

18. Using the “Cell Confluence Mask” as a measurement on cells with high cytoplasm spreading and low contrast can be difficult, because different biological processes, such as cell adhesion/spreading and proliferation, cannot be easily distinguished. To overcome this potential problem cell adhesion/spreading can be discriminated from proliferation by focusing the analysis on the initial 45 min after cell seeding. Additionally it is possible to export single images from the Incucyte® software and manually analyse the cell surface with another software (*e.g.* freehand selection tool available in ImageJ or FIJI).

19. This is a limitation common to other methods to measure cell growth, such as the impedance-based monitoring system xCELLigence® RTCA MP system (ACEA Biosciences Inc). Although the xCELLigence® is able to detect in real-time very fast changes in cell index (based on the measurement of electrical impedance caused by the cell growth and attachment of the cells to the plate), the results are more difficult to interpret due to the lack of visual readout.

20. The software also counts the number of “objects” (cells) over time, as a measurement of cell proliferation. However, using phase-contrast pictures for this measurement can be misleading. As a result of proliferation, the cells become, naturally more confluent, and the individual cells previously identified as isolated “objects” become adjacent and make physical contact. They are no longer distinguished, as two distinct objects, but as a single one. Consequently the “cell count” provided by the software is underestimated, and it may even decrease over time, while the real cell number on the plate goes up. A proposed solution to overcome this problem is the use of fluorescent live-cell nuclear markers.

21. The results can also be illustrated by an overview of the global population dynamics in the entire place, and individual plots can be drawn for each well of the microplate. They can also be exported as a numerical data file for subsequent statistical treatment and graphical representation by specialized software.

22. For astrocytes, uncoated plates work well and the wound takes about 50 h to close. However, the coating accelerates cell adhesion and migration, and it can help studying interactions between the cells and the extracellular matrix. For OPC the coating is necessary for cell adhesion. Although single coating with PDL can be used, allowing the wound to close over 72 h, PDL+laminin coating increases the speed of wound healing, which is completed after 48 h. Hence, the duration of the experiment will depend on the type of coating used.

23. The use of "Incucyte ® Image lock" special plate is crucial to assure that all the scans will be performed at exactly the same position throughout the experiment, since each well is marked with a cross that the IncuCyte® will use as a reference point for the positioning of the picture within the well. This will avoid variability in the scan position and therefore in the analysis.

24. Remember to clean the Incucyte® wound maker before using it with successive 5 min washes with 45 mL of double distilled H_2O and 45 mL of 70% Ethanol, using the reservoirs from the kit. While wounding the plates, all wells of the plate should contain 100 µL of medium or PBS 1X. After finishing wounding your plates, clean again the wound maker with successive 5 min washes of 45 mL of 0.5% Alconox, 1% Virkon, double distilled H_2O and 70% Ethanol. To maintain the good working conditions of the instrument it's extremely important to never turn the pin block of Incucyte® wound maker upside down, or the solutions/media will crystalize inside the pins and impair the scratching.

25. After the wound is made, lift the pin block straight up to avoid damaging the front of migration. This step will create a precise and reproducible wound throughout the 96-well plate. Using the wound maker instead of the classical pipette tip presents a great advantage since it maximises the homogeneity of the wound between wells, it guarantees the regularity of the fronts of migration and it reduces cell lifting. Overall the process is time saving: the 96 wells of a plate can be scratched in parallel in one single passage of the wound maker.

26. Changes the filter parameters in the "Segmentation Adjustment" panel will change both "Confluence Mask" and "Scratch Wound Mask". It is not possible to apply different parameters to the different masks.

27. Although this system, like the classical wound healing assays [30], does not avoid the damage-induced cell proliferation, the analysis with RWD algorithm automatically normalizes the confluence in the wound space to the confluence in the cell layer, excluding the contribution of factors other than migration (*e.g.* proliferation or cell death) towards the filling of the wound.

28. To export the videos from the IncuCyte® go to "Utilities>Export Movie or Image set" and then select the right time range (from the beginning of the experiment until the moment when the wound is closed for all conditions) and the right wells. At least one video should have the scale ("Legend") and Timestamp should be included in all videos, as a reminder for further analysis. Use "Windows AVI video (uncompressed)" format to ensure compatibility with Fiji.

29. Set the scale in Fiji by using the line tool to measure the scale on one Incucyte® image and then select "Set Scale" in the Fiji "Analyze" menu. Remember to check the "Global" box in order to apply the scale to all further images.

30. To better visualize the nucleus of the cells the stack can be flipped on the Z scale (time scale) to start the analysis from the end of the video, when the cell cytoplasm is widely spread. Once delineated, the tracking position data can be flipped back using Microsoft Excel.
31. The color of the migration images can also be processed to facilitate the visual detection of the nucleus. We recommend converting the images to 8-bit and applying the "HiLo LUT" palette.
32. Depending on the experiment the acquisition can be done over different time periods.
33. If you label neurites fluorescently, use the "Color Cell-Body Cluster Mask" and the "Color Neurite Mask" to define the settings for analysis.
34. Use the "Filtering" parameter to filter out small imperfections (*e.g.* due to irregular coating) or cell debris that can be mislabelled as neurites. This parameter can also be used to filter in thinner neurites/cell processes, through the selection of the "Better" or "Best" options in addition to the "Neurite Sensivity" slider. Define the pixel size at which the cell bodies transit into neurites/cell processes using the "Neurite Width" dropdown.
35. The neurite length and branching results can be quantified relative to the area of the well, to the cell-body cluster number or area. For neurons, the quantification of the neurite length per area is normally used, when the physiological neurite dynamics is being tested, since the number of neurons and cell body area will not change over time. However, when compounds are tested, the normalizations to cell body should be considered to exclude confounding effects, such as cell death. For oligodendrocytes, it is more accurate to normalize the measurements of cell processes relative to the cell body clusters, in an attempt to minimise the influence of possible alterations in cell number. The cell-body dynamics is estimated by measuring the percentage of confluence.

36. We adapt the "Neurotrack" algorithm to measure the process extension and branching of oligodendrocytes. However neurites and axons are bigger and thicker than the oligodendrocytes processes. As a result the settings must be adjusted to the highest sensitivity to maximise the detection of truly thin oligodendrocytes processes and branching, while distinguishing them from cell bodies.

37. A MOI of 3 TU/cell was optimized in our laboratory to infect primary cortical neurons. For other neuronal types the optimal MOI must be determined.

38. The described protocol is useful to test, for instance, the beneficial/deleterious effect of an astrocyte monolayer of a given phenotype on neuronal differentiation, and the response to environmental stress, such as the addition of a neurotoxic compound (*e.g.* excess of glutamate).

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Figure legends

Figure 1. Incucyte® Zoom software interface.

Software interface screenshot showing the panel for the definition of the semi-automated analysis. The process starts with the creation of an image collection (option **A**), followed by the definition of the analysis settings that will be applied to all the images subsequently acquired (option **B**). Finally the analysis in launched (option **C**).

Figure 2. Live-cell monitoring of OPC proliferation.

(**a**) Phase contrast image showing a merged a poor "Confluence Mask" (yellow) fit on OPC imaged 1 day after plating. The confluence mask is inadequately counting small cell debris and plate/coating imperfections as objects (cells), also including some space between adjacent cells. (**b**) Phase contrast images of OPC on day 1 and 3 after plating. Adequate confluence masks (yellow) faithfully delineate the shape of living cells, excluding cell debris, imperfections and space between cells. (**c**) The quantitative analysis of cell proliferation is plotted as the percentage of confluence (surface of the well occupied by cells) over 8 h.

Figure 3. Astrocyte migration capacity tested by an automated wound healing assay.

(**a**) Phase contrast image of a cell monolayer after scratching, using the Incucyte® Wound Maker. The picture shows the cell layer (1), front of migration (2) and wounded space (3) on the left. The image on the right shows a merged bad fitting "Confluence mask" (yellow), covering only part of the front of migration and inadequately including empty wounded space without cells. (**b**) Phase contrast image of a scratched astrocyte monolayer and the fitting "Confluence Masks" (yellow) over time. The merged images show the "Confluence Mask" overlaying the cell layer and outlining some isolated cells left in the wounded space, while excluding cell debris. The "Scratch Wound

Mask" (blue) is confined to the wounded space, free of cells. The wound width (white double arrowhead) decreases as the wound confluence increase over 40 hours following the initial scratch. (**c**) The speed of collective migration of the astrocytes is estimated by the increase in wound confluence over time. Several biological replicates of astrocytes of individual mice (pink and black) are plotted.

Figure 4. Individual cell tracking procedure to study the speed, directionality and displacement of individual astrocytes.

(**a**) Screen shot of the "Manual Tracking" plugin window showing the tracking options and parameters to set up prior to the analysis. (**b**) Example of one astrocyte path tracked throughout 50 h of migration and the raw data retrieved by the plugin. (**c**) Example of the projection of the migration trajectory of 20 astrocytes on the X/Y coordinates (in μm) obtained using the DiPer macros in Microsoft Excel.

Figure 5. Semi-automated and label-free measurement of neuritogenesis.

(**a**) Phase contrast (top) and respective blended mask (bottom) images of neurons at 3 and 7 days of *in vitro* (DIV) development. The mask images show the perfect fitting of the "Phase Cell-Body Cluster Mask" (green) on neuronal cell bodies. The appropriate "Phase Neurite Mask" (pink) traces neurite development and growth over time. (**b**) The length of neurites (mm per mm^2 of plate surface) is automatically measured and plotted over time, during the entire course of the experiment. The number of branch points over time is counted and normalized relative to the area occupied by the cell bodies. Neurons derived from two different mice are plotted. Inflection points illustrate the moments when the medium of the cells was changed.

Figure 6. Analysis of oligodendrocyte differentiation by semi-automated measurements of process extension and branching.

(**a**) Phase contrast (top) and respective blended mask (bottom) images of oligodendrocytes at different stages of *in vitro* differentiation. The mask images show the perfect contour of the cell bodies by the "Phase Cell-Body Cluster Mask" (yellow) and the tracing of the oligodendrocyte processes and secondary branching by "Phase Neurite Mask" (pink). (**b**) The imaging system measures and plots the length of cell processes per cell body cluster over 7 days of differentiation. (**c**) The number of branch points relatively to cell body clusters is plotted over the course of the experiment.

Figure 7. Monitoring of neuronal neuritogenesis on a monolayer of astrocytes.

(**a**) Screen shot of the "Color Neurites (Analyze)" panel of filters used to define and fit the settings to identify and measure correctly red-labeled neurites. The settings will be applied to the entire analysis. (**b**) Neurons imaged at 7 days *in vitro* (DIV) transduced with IncuCyte® NeuroLight Red Lentivirus (left) and the blended mask (right). The "Cell-Body Cluster Mask" (green) contours the cell bodies, while the "Color Neurites Mask" (blue) delineates red fluorescent neurites. (**c**) Neurite length is measured during the course of the experiment and plotted in mm per mm^2 of plate surface over time. Two different combinations of neurons and astrocytes co-cultures are plotted. Inflection points illustrate the moments when the medium of the cells was changed.

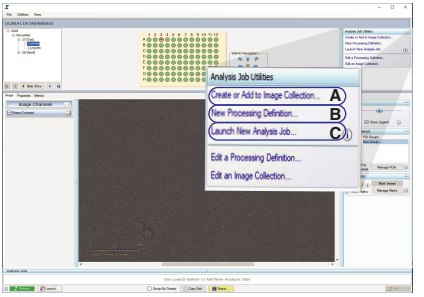

Figure 1
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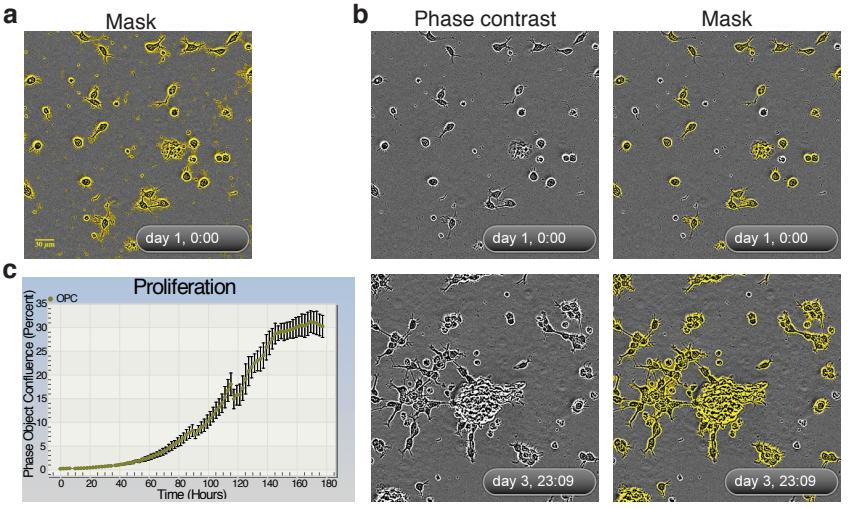

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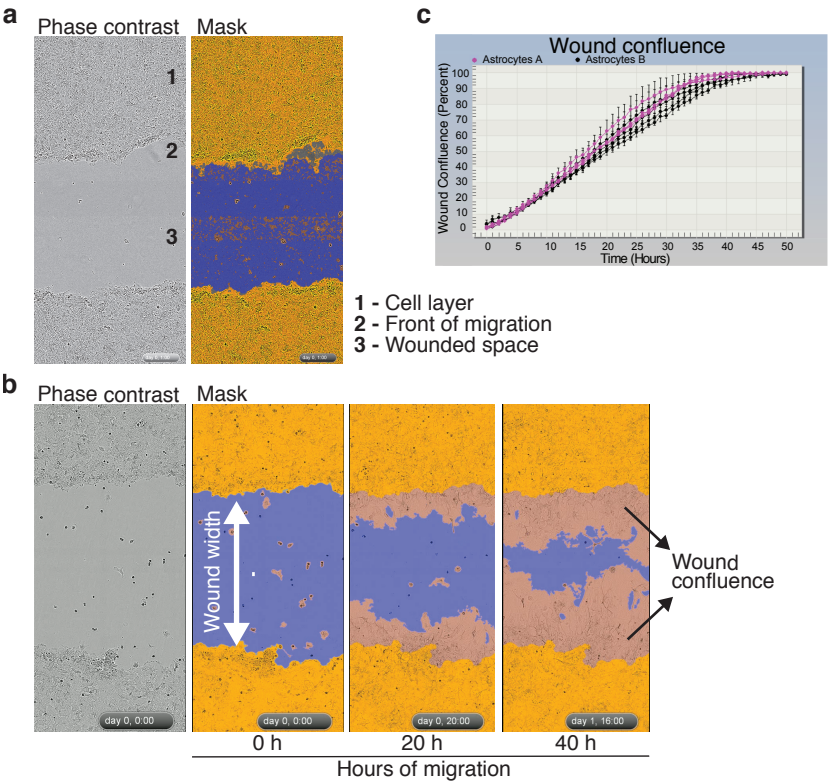

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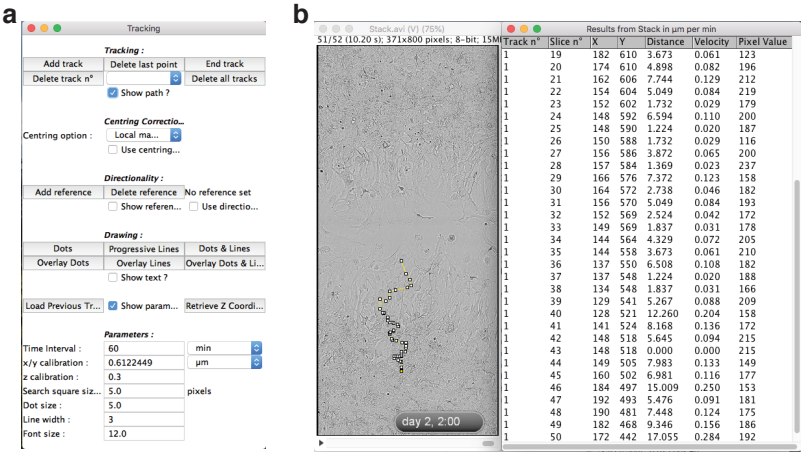

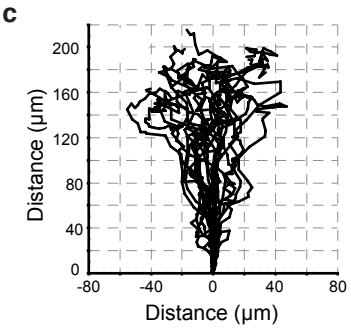

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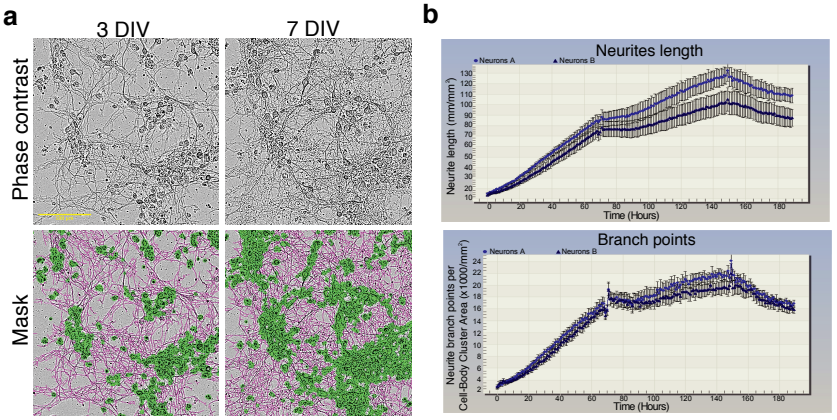

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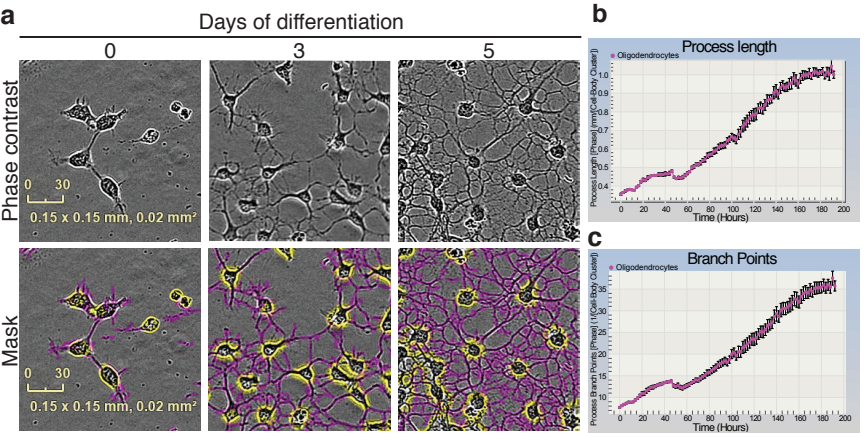

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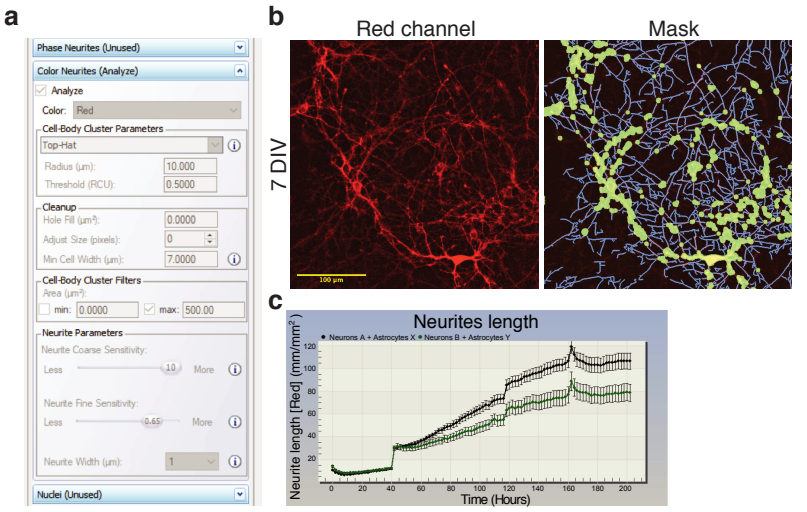

Figure 7
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