Video Article

Live Imaging of Mitosis in the Developing Mouse Embryonic Cortex

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Abstract

Although of short duration, mitosis is a complex and dynamic multi-step process fundamental for development of organs including the brain. In the developing cerebral cortex, abnormal mitosis of neural progenitors can cause defects in brain size and function. Hence, there is a critical need for tools to understand the mechanisms of neural progenitor mitosis. Cortical development in rodents is an outstanding model for studying this process. Neural progenitor mitosis is commonly examined in fixed brain sections. This protocol will describe in detail an approach for live imaging of mitosis in *ex vivo* embryonic brain slices. We will describe the critical steps for this procedure, which include: brain extraction, brain embedding, vibratome sectioning of brain slices, staining and culturing of slices, and time-lapse imaging. We will then demonstrate and describe in detail how to perform post-acquisition analysis of mitosis. We include representative results from this assay using the vital dye Syto11, transgenic mice (histone H2B-EGFP and centrin-EGFP), and *in utero* electroporation (mCherry-α-tubulin). We will discuss how this procedure can be best optimized and how it can be modified for study of genetic regulation of mitosis. Live imaging of mitosis in brain slices is a flexible approach to assess the impact of age, anatomy, and genetic perturbation in a controlled environment, and to generate a large amount of data with high temporal and spatial resolution. Hence this protocol will complement existing tools for analysis of neural progenitor mitosis.

Video Link

The video component of this article can be found at https://www.jove.com/video/51298/

Introduction

The overall goal of this protocol is to describe how to perform live imaging of neural progenitor mitosis in embryonic brain slices. Using live imaging of brain slices in culture, this protocol provides a simple method to assay multiple aspects of mitosis in neural progenitors in an environment highly similar to an *in vivo* setting. It can be applied to brains of mutant animals and/or brains that have been manipulated with *in utero* electroporation)[1-5]. This technique is also ideal to test the effect of pharmacological agents on neural precursors' mitosis, by simply adding an agent to the culture medium. In sum, this article will make a technically challenging protocol accessible to those studying neurogenesis.

During neurogenesis, distinct neural progenitor populations undergo precise divisions to generate neurons that eventually contribute to the six cortical layers of the adult neocortex[6-8]. Early in cortical development, the neural precursor pool expands as neuroepithelial (NE) cells divide symmetrically to self-renew. NE cells then convert into radial glial cells (RGCs). Initially RGCs divide symmetrically to produce two new RGCs, however during the bulk of neurogenesis, RGCs' main mode of division is asymmetric. In asymmetric division, 1 RGC gives rise to a new RGC and either a post-mitotic neuron, or a more specialized progenitor (either a short neural precursor (SNP), an outer radial glia (ORG), or an intermediate progenitor (INP)[2,3,7,9]. INPs, SNPs, and ORGs can then generate neurons at the sub-ventricular, ventricular, and basal regions of the cortex, respectively. Hence, cell division of progenitors is a fundamental process for generating neurons of the neocortex.

Numerous studies point to a correlation between specific mitotic traits of RGCs and the fate of daughter cells. Haydar *et al.* and Takahashi *et al.* have shown that RGC mitotic duration and cell cycle length increase as neurogenesis proceeds, a finding echoed in follow up studies[10-13]. A number of studies have suggested that mitotic spindle orientation relative to the ventricle influences aspects of neurogenesis and corticogenesis, including types of neurons generated and location of progeny in the brain, respectively[3,10,14-16]. Whether cleavage plane orientation directly influences cell fate is controversial, but the conclusion remains that this mitotic parameter impacts neurogenesis. Further underscoring the importance of mitosis is the observation that many genes involved in the mechanics of mitosis are crucial for neurogenesis and for proper brain development[17-20].

Mitosis is a dynamic process, yet to date most studies detailing neural progenitor mitosis utilize analysis of fixed tissue sections or imaging of neural progenitors via *in vitro* cell culture. Thus, the mainstream methods to evaluate mitosis only provide a snapshot of this process and fail to uncover how cells behave in a tissue. Live imaging of neural progenitor mitosis has increasingly become a critical tool for understanding neural progenitor function. For examples please see these references[4,8,10,21-25]. Several outstanding protocols have been published for preparation and imaging of brain slices[26,27]. However to date, a comprehensive protocol for imaging and analysis of mitosis has not been described nor demonstrated in video.

This technique offers several significant advantages over fixed analysis of brain sections. Time-lapse analysis of brain slices enables generation of significantly more data points that can be analyzed in a flexible fashion. First, data is gathered at individual time points over the course of several minutes or several hours. One can analyze individual time points (to create a static montage) or can combine different time points into movies. Second, confocal imaging of slices enables generation of data at different Z sections in brain slices. As a result, individual sections can be analyzed. Alternatively, stacks of individual sections can be combined into a maximum intensity projection. Third, analysis is done in the context of a tissue, revealing how cells divide relative to neighboring cells and structures. Fourth, it is ideally suited to analysis of mutants that show some evidence of mitotic defects. Together this protocol will help clarify critical steps to aid investigators who wish to carry out live imaging of neural progenitor mitosis in their own laboratories.

Protocol

1. Preparation of Media (Figure 1, Step 1)

1. Slice Culture Medium
    1. 25 ml of slice culture medium is sufficient to prepare 5 glass bottom dishes with 2 slices per well.
    2. In a 50 ml conical tube, add 250 µl of a 100x N2 solution and 500 µl of a 50x B27 solution without vitamin A. Add DMEM/F12 to a volume of 22.5 ml.
    3. Filter sterilize solution and subsequently add 1.25 ml of heat inactivated horse serum and 1.25 ml of fetal bovine serum.
    4. Incubate solution in a water bath at 37 °C for the duration of the preparation of the slices.
    5. Add growth factors (FGF and EGF, final concentration of 10 ng/ml and 20 ng/ml, respectively) immediately prior to the beginning of culture. The composition of this medium has been optimized to promote the survival of cells in culture and to increase the proliferation rate of neural progenitors. This will thus maximize the probability to observe mitotic cells in the slice.

2. Full HBSS Dissection Solution
    1. Prepare 500 ml of full HBSS by adding 50 ml of 10x HBSS, 1.25 ml of 1 M Hepes (pH 7.4, FC 2.5 mM), 6 ml of 2.5 M D-Glucose (FC 30 mM), 2.2 ml of 0.9 M $NaHCO_3$ (FC 4 mM) and autoclaved diH_2O to final volume of 500 ml.
    2. Filter sterilize solution and store at 4 °C until the collection of uterine horns from the pregnant mouse. This solution can be kept at 4 °C for weeks.
    3. Keep HBSS on ice during the whole dissection procedure.

3. Embedding solution
    1. For the embedding of 4 embryonic brains, prepare 30 ml of 3% low melting agarose in the full HBSS solution. In a 50 ml conical tube, add 0.9 g of low melting agarose and full HBSS to 30 ml.
    2. Mix the solution with a vortex mixer, and melt in a microwave with the tube standing and the cap open.
    3. While in the microwave, monitor the solution to prevent overflow due to excessive boiling. When boiling starts, stop the microwave and vortex the solution.
    4. Repeat these steps until all the agarose has melted.
    5. Store the tube in a water bath at 42 °C.

2. Dissection of the Embryos (Figure 1, Step 2)

1. After careful euthanasia of the pregnant mouse, collect the uterine horns and transfer them into a 10 cm^2 Petri dish containing cold full 1x HBSS. The age of the embryo can vary depending upon the study. This protocol has been used successfully for culturing brain slices from embryonic days E12.5 to E17.5. Due to their size, younger brains tend to be more difficult to work with.
2. Collect the embryos and dissect out the embryonic brains as described previously in the rat and mouse[26,27], until a full embryonic brain including the hindbrain and the forebrain with the two cerebral hemispheres is obtained. These can be kept at RT until all brains have been dissected.
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3. Embedding of the Embryonic Brains (Figure 1, Step 3)

1. In a bucket containing ice, create a hole in ice to insert the embedding molds into.
2. Pour the 3% agarose medium into a plastic mold and place that mold into the hole prepared in the ice.
3. Stir the agarose medium with the tip of a digital thermometer until the temperature reaches 35 °C.
4. Promptly transfer the brain into the embedding medium.
5. Critical Step: To remove the excess HBSS at the interface between the brain and the agarose, carefully and gently tumble/rotate the brain repeatedly in the embedding solution with the forceps.
6. A cushion of gelled agarose will form at the bottom of the mold in contact with the ice. Once the cushion can be felt with the tip of the forceps while stirring the brain, position the brain with the dorsal side up. The brain should not sink to the very bottom of the mold.

4. Preparation of Agarose Block (Figure 1, Step 4)

1. Let the agarose harden in the ice for at least 5 min.
2. Using a razor blade, section the corners of the mold.

3. Carefully carve an agarose block around the brain using a razor blade. Try to minimize the number of cuts to avoid perturbing the embedded brain.
4. Make sure that the block has a larger surface area in the caudal region of the brain (versus the rostral region, see **Figure 1**, Step 4) to increase the stability of the block on the sectioning pedestal.
5. The last cut should be the one on the caudal side of brain; this cut will define the correct sectioning plane with the vibratome.
6. Do so by aligning the blade perpendicular to the rostro-caudal axis of the brain and the agarose block. Slide down the blade caudally along the rostro-caudal axis and make the final cut about 5 mm away from the most caudal region of the brain.
7. Set the agarose/brain block aside and repeat embedding of other brains.

5. Transfer of the Embedded Brains into the Tray of the Vibratome (Figure 1, Step 5)

1. Add a drop of glue onto the bottom of the vibratome tray. Place glue so that the agarose blocks will be positioned within reach of the vibrating blade.
2. Place the agarose/brain block caudal side down on the edge of the spatula, with about 50% of the block tipping over the edge of the spatula.
3. Apply the caudal face of the agarose block to the glue and slide the spatula away, maintaining the agarose block to the bottom of the tray with the shoulder of a pair of tweezers. Do not let the spatula directly contact the glue.
4. Let the glue solidify at room temperature for 5 min.

6. Sectioning of the Embedded Brains (Figure 1, Step 6)

1. Fill the vibratome tray with HBSS.
2. Define the start and finish positions of the vibratome blade.
3. Generate 200 - 250 µm slices. With the VT1000s vibratome, use the following parameters: speed 2 - 4, frequency 8.
4. Carefully remove the slices from the vibratome as they are cut. Transfer the slices with a spatula and the back end of tweezers into 12 well dishes filled with full HBSS. Alternatively some researchers have used a paintbrush *in lieu* of tweezers. Key point: During transfer, take care that the brain slices remain attached to the surrounding agarose.

7. Syto11 Staining of the Slices (Figure 1, Step 7)

1. Dilute Syto11 to a final concentration of 0.5 - 1 µM in the slice culture medium supplemented with growth factors.
2. In the well of a 12-well plate, incubate slices from one brain in 2.5 ml of the staining solution for 1 hr at 37 °C.
3. Wash the slices in 2.5 ml of slice culture medium without staining solution for 20 min.

8. Mounting the Slices in a Glass Bottom Dish (Figure 1, Steps 8, 9)

1. Prepare 15 µl of embedding collagen solution per slice. Prepare a 1.5 mg/ml collagen solution by mixing 375 µl of 3 mg/ml collagen type I solution with 75 µl of 10x DMEM, 9.4 µl of 1 M NaOH, and 290 µl of H_2O. Store on ice.
2. Place a 15 µl drop of the collagen solution at the bottom of a 35 mm Glass bottom Microwell Dish. Spread it with a pipet tip to match the size of the slice.
3. Transfer the slice into the drop of collagen with a spatula and pair of tweezers. Key point: Mounting multiple slices in different dishes increases the probability to acquire slices suitable for imaging. Screen through different slices and select the ones that best meet the criteria described below in the "Representative Results" section.
4. Let the slices incubate at RT for 10 min before transferring them into a 37 °C incubator with 5% CO_2.
5. After 20 min, add 1.2 ml of slice culture medium into the Glass Bottom Microwell Dish. Add 600 µl of medium at a time and spread it with a pipette tip.
6. Capture a low magnification image of the brain slice to record the anatomical level and integrity of the slice.

9. Live Imaging of the Slices (Figure 1, Steps 10, 11)

1. Let the slices recover in the incubator for at least 1 hr and 30 min at 37 °C with 5% CO_2 before live imaging.
2. Image the slices with the microscope of choice, keeping in mind that Syto11 is very prone to photobleaching. Here an inverted spinning disk confocal microscope is used (Andor XD revolution spinning disk confocal microscope). Alternatively a laser scanning confocal microscope can be used. The following parameters are for the spinning disk microscope set-up.
3. During the entire live imaging session, maintain slices at 37 °C, 5% CO_2 in a humidified incubation chamber attached to the microscope.
4. Image cells with a 60X silicon oil objective with a working distance of 300 µm, and a numerical aperture of 1.3 (for examples see **Figures 3B, 3C, 3E, 3F, 4A, 4B**). A 100X oil objective with a working distance of 130 µm and a numerical aperture of 1.4 (for example see **Figure 4C**) can also be used. The resolution of the camera is 512 x 512.
5. Image the cells in a 30 µm z-stack, with the center of the z-stack located about 40 µm below the surface of the slice. Trying to image deeper into the tissue can cause the objective to add mechanical pressure on the slice. This can affect the integrity of the brain slice. If planning to make 3D reconstructions of the cells, use a z-interval of no more than 2 µm.
6. Adjust the laser power and exposure times to limit photobleaching. Use exposure times ranging from 30 to 200 msec, depending on the intensity of the Syto11 signal.
7. With a motorized stage, image multiple positions across multiple slices mounted in 1 dish. For example, image up to 20 positions scattered across 4 different slices in 1 single dish.
8. For live imaging, use a temporal resolution of less than 5 min to enable the identification of the different phases of mitosis. Using Syto11 and/or histone H2B-EGFP, 4 - 5 hr of live imaging are sufficient to observe a significant number of mitotic cells. However, if imaging sparsely

labeled mitotic cells (such as those achieved by *in utero* electroporation), then a longer imaging parameter (overnight) is appropriate and works well.
Note: Using this protocol, we typically observe on average 10 mitotic cells per position. As stated above, imaging multiple mounted slices increases the probability of having a successful experiment. With this approach we typically identify mitotic cells in virtually all experiments performed with Syto11 or histone H2B-EGFP.

10. Post-acquisition Analysis of Mitosis (Figure 2)

All the procedures in this section have been optimized in Fiji (ImageJ), which is advantageous because it is a free open source software. Other software solutions are available to perform the same tasks, such as Metamorph, Imaris, and Amira.

1. Identification of Mitotic Figures in Fiji
    1. After opening the dataset for one position as a "hyperstack", select one z-plane (see **Figure 2A** for the location of the scroll bar) where the tissue and cells look healthy (see criteria in the discussion section). Scroll across the temporal dimension to identify cells going through anaphase, as it is the easiest mitotic phase to identify.
    2. Scroll back in time to identify the time point where the cell enters mitosis (DNA condensation). The cell may move across z-planes, but the temporal resolution and the z-stack parameters described earlier have been optimized to enable this process.
    3. In a spreadsheet, record the 4D coordinates of the cell in the hyperstack (X,Y,Z and time, see **Figure 2A** to visualize the location of these figures in the Fiji interface) as it enters mitosis. Knowing these coordinates will prevent counting the same cell several times.
    4. Scroll forward in time and record the time when the cell progresses from one phase to another. Document the duration of each mitotic phase for one given cell.
    5. Proceed with other cells, across the full dataset (multiple cells within and across positions).

2. 3D Reconstruction of the Cells and Quantitation of Rotation During Metaphase (Adapted from Haydar *et al*, 2003) (**Figure 2B**).
    1. After having identified multiple mitotic cells, use the coordinates of a cell and scroll forward in time until the beginning of metaphase.
    2. Rotate the entire "hyperstack" so that the ventricular border is planar (using the "Image/Transform/Rotate…" command). Use the "angle" tool to determine the angle to apply for this rotation.
    3. Identify the z-plane where the cell of interest is the most visible. In that plane, use the "rectangle selection" tool to draw a selection that includes the whole cell.
    4. Identify the z-planes that include the cell of interest. Use the "Image/Duplicate" command to create a "sub-stack". In the dialog box, leave "Duplicate hyperstack" checked. The "Slices (z)" parameter corresponds to the z-planes including the whole cell, and the "Frames (t)" parameter corresponds to the current time point.
    5. If the resolution is poor, increase the size of the "sub-stack" using the "Image/Adjust/Size…" command.
    6. Generate a 3D reconstruction of the cell at the current time point using the "Image/Stacks/3D project…" command. The parameters for this command are displayed in **Figure 2B**. The "Slice spacing" corresponds to the interval between each of the z-plane images (µm). Important: make sure that the physical dimension (µm) of the sub-stack has been conserved. If not, the interpolation of the pixels in the z dimension will be incorrect, and the 3D reconstruction will be inaccurate.
    7. Using the scrollbar, rotate the resulting 3D reconstruction so that the edge of the metaphase plate is visible. The number of the frame corresponds to the beta angle described in **Figure 3B**, record this number. Using the "angle" tool and the "Analyze/Measure…" command, measure the angle between the horizontal and a line bisecting the metaphase plate perpendicularly (**Figure 2B**). This is the angle alpha.
    8. Record these angles for all the metaphase time points of the cell of interest. The angle of rotation between time points (t) and (t-1) equals to the absolute value of the difference between an angle at (t) and this angle at (t-1).

3. Measuring the Orientation of the Cleavage Plane
    1. 3D reconstruct a cell of interest at one time point during anaphase following the instructions described for the 3D reconstruction of metaphase plates.
    2. Rotate the reconstruction until the edges of the two plates formed by the segregating chromosomes are visible.
    3. Use the angle tool to measure the angle between the horizontal (the ventricular edge) and a line parallel to the two plates formed by the segregating chromosomes. This is the angle of the cleavage plane (**Figure 2C**).

4. Generation of Movies
    1. As the cells often move across different z-planes, identify the z-planes occupied by the cell during the live imaging session. Generate a "Maximum intensity projection" of these z-planes using the "Image/Stacks/Z project…" command. Save the resulting stack as a ".TIF" file.
    2. Open this file in the 1.45 version of ImageJ as Fiji does not include a stable plugin for the generation of ".mov" or ".avi" files.
    3. Use the "File/Save as/…" command of ImageJ to generate a movie in a format compatible with your down-stream applications.

Representative Results

The success of this assay and the observation of multiple mitotic cells during one live imaging session largely depend upon both the integrity and anatomical level of the slice where acquisitions are made. As discussed below, the anatomical level of a slice is an important factor. **Figure 3A** shows rostro-caudal and medial-lateral locations where we have been most successful. For additional discussion of this subject please see Noctor[26]. The integrity of the slice can vary in different regions of the slice. This can be determined prior to the beginning of the time-lapse (using imaging of the entire slice as in Step 7.6). In a good region of an E13.5 brain the following observations can be made: many mitotic cells will be found at the ventricle border, the interphase nuclei will be shaped elliptical rather than round, and interphase nuclei will appear columnar relative

to the ventricle border. In a less desirable region of the brain, the nuclei will look rounded and somewhat disorganized (Compare **Figures 3B** and **3C**, and see **Figure 3D** for a picture of a well-mounted slice).

When ideal conditions are achieved, this assay will enable the qualitative identification of different phases of mitosis. These can be detected by analysis of the DNA pattern (see **Figure 3E** for examples of apically dividing cells in each phase of mitosis). During prophase, DNA condensation will have the appearance of sparsely labeled DNA (DNA-rich and DNA-poor regions in the nucleus). During metaphase, the chromosomes form a plane at the equator of the cell body. Depending on the orientation of the plane, the DNA pattern is either (i) a thick line (**Figure 3E,** view 1) or (ii) a "rosette" with a DNA-poor region in the middle (**Figure 3E,** view 2). During anaphase when chromosomes separate, they look like "hands" that migrate in opposite directions. This phase can be hard to identify when the cleavage plane is in the X - Y plane of acquisition. During telophase, DNA relaxes and acquires an ellipsoid shape as the nuclear envelope reforms. Individual time points can be collected to visualize mitosis over time (see **Figure 3F**).

In addition to imaging mitosis of apically dividing progenitors, this assay can be used to image basally dividing cells such as ORGs or INPs, the latter of which are most likely represented in **Figure 3G.** Such cells are identifiable by their location for division in the SVZ layer. However to truly mark these cells it is best to couple the analysis with expression of a fluorescent marker that labels the cell process or fate (see **Figures 4B-D)**. INPs will have neither an apical nor basal process, ORGs will have a basal process with their cell body located in the outer layers, and RGCs will have a basal process with their cell body located in the VZ layer.

Mitosis of progenitors can be visualized with alternative methods. **Figure 4A** demonstrates use of the histone H2B-EGFP transgenic mouse to label chromosomes of all dividing cells. This marks cells in a pattern similar to Syto11. **Figure 4B** depicts imaging performed with brains *in utero* electroporated with EGFP-α-tubulin and histone H2B-mCherry. As demonstrated, this enables one to visualize both microtubules and chromosome, and additionally the cell body and basal process. **Figure 4C** depicts imaging performed with brains from a Centrin-EGFP mouse, *in utero* electroporated with mCherry-a-tubulin and histone H2B-EGFP. As demonstrated, this enables one to image chromosomes and centrioles in the green channel and the mitotic spindle and cell body/process in the red channel. **Figure 4D** depicts imaging performed with brains *in utero* electroporated with CMV-Cre sparsely and CALNL-EGFP[28]. This technique enables one to sparsely label dividing progenitors, making analysis of individual dividing cells easier. When using electroporation however, keep in mind that this method targets a fairly synchronized cohort of cycling cells[2,12]. This means that the timing of live imaging relative to the electroporation needs to be adjusted, so that the cohort of electroporated cells approaches mitosis at the beginning of the imaging session. If not, success of the experiment in identifying any mitotic cells may be compromised in a short term imaging session.

When ideal conditions are achieved, key features of mitosis can be quantitatively measured including overall mitosis duration, duration of individual phases, rotation of the metaphase plane and cleavage plane orientation. In the described conditions, we have observed an average overall mitotic duration of 1hr 30 min at embryonic days (E) 13.5 and 14.5. As described previously, the rotation of the metaphase DNA plane can be measured at an individual time point or cumulatively over the duration of an entire metaphase[2,6,9,10]. This can be achieved using 3D reconstruction of the metaphase plane of a cell at each time point and measuring angles of DNA relative to the ventricle. We have successfully applied the method in this paper to follow cells for at least 3 hr.

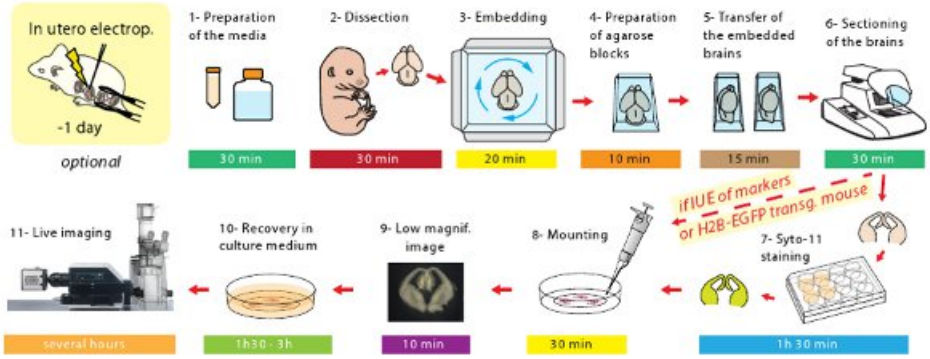

Figure 1. Schematic representation of steps in protocol. Time needed for each step is indicated under each of the steps. Details for each of these steps are described in the "protocol" section. Please note that Step 7 can be bypassed when imaging slices from histone H2B-EGFP embryos, or slices from brains that were electroporated with plasmids expressing markers for mitosis.

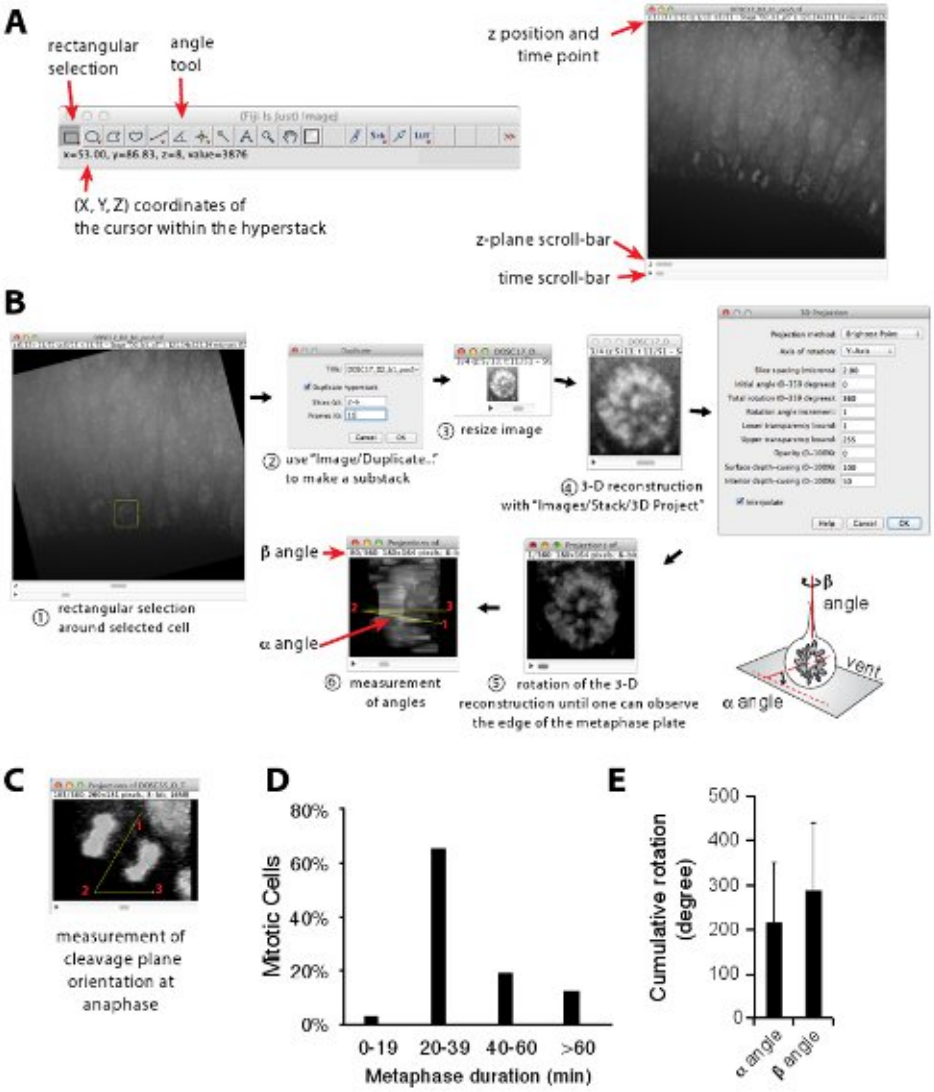

Figure 2. Post-acquisition analysis of time-lapse video microscopy datasets in Fiji (ImageJ). **A)** Location of the different tools utilized for the analysis in Fiji. **B)** Different steps involved in the 3D reconstruction of a cell in metaphase. Details for each of these steps are described in the "protocol" section. **C)** Measurement of cleavage plane orientation in a 3D reconstructed cell at anaphase. Please note that numbers in B Step 6 and C show the sequence followed to measure the angle. **D)** Representative data showing the distribution of metaphase duration in a group of mitotic cells observed at the ventricular border in E13.5 brain slices (n = 35). **E)** Representative data showing the cumulative rotation of metaphase plates in E13.5 mitotic cells at the border of the ventricle. Click here to view larger image.

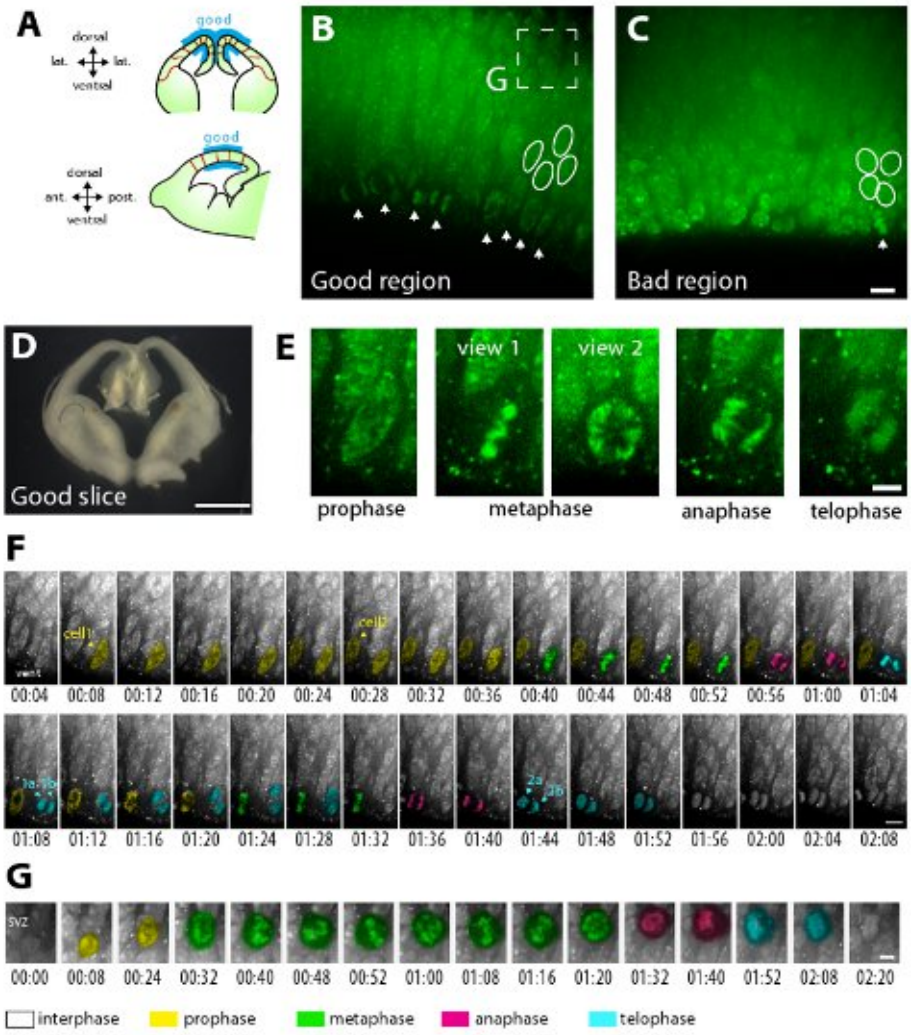

Figure 3. Examples of representative data from live imaging analysis. A) Along the dorso-ventral axis, mitosis is most easily imaged in dorsal regions of brain slices, because the basal process of RGCs is shorter and thus less likely to be severed by vibratome. Along the rostro-caudal axis, slices in the caudal regions of the dorsal cortex give most consistent results in our hands. **B and C)** Examples are shown of a good region (B) and a bad region (C) for imaging. In a good region, nuclei are elliptically shaped (circled regions) and many mitotic cells are observed at the border of the ventricle prior to time-lapse imaging (arrows). In a bad region, very few mitotic cells are observed at the border of the ventricle (single arrow) and nuclei are round (circled regions). **D)** Example of a good slice observed with a low magnification microscope prior to live imaging. **E)** Examples of Syto11 labeled cells in different phases of mitosis, as noted. **F)** Montage of a region at the border of the ventricle where two neural progenitors go through the different phases of mitosis, marked in different colors (time, hh:mm). **G)** Montage of a region highlighted in (B) where a neural progenitor divides in away from the ventricular border. In montages (D) and (E), cells were pseudo colored using Photoshop to highlight the different mitotic phases. (Scale bars: B, C, F: 10 µm; D: 1 mm, E, G: 5 µm).

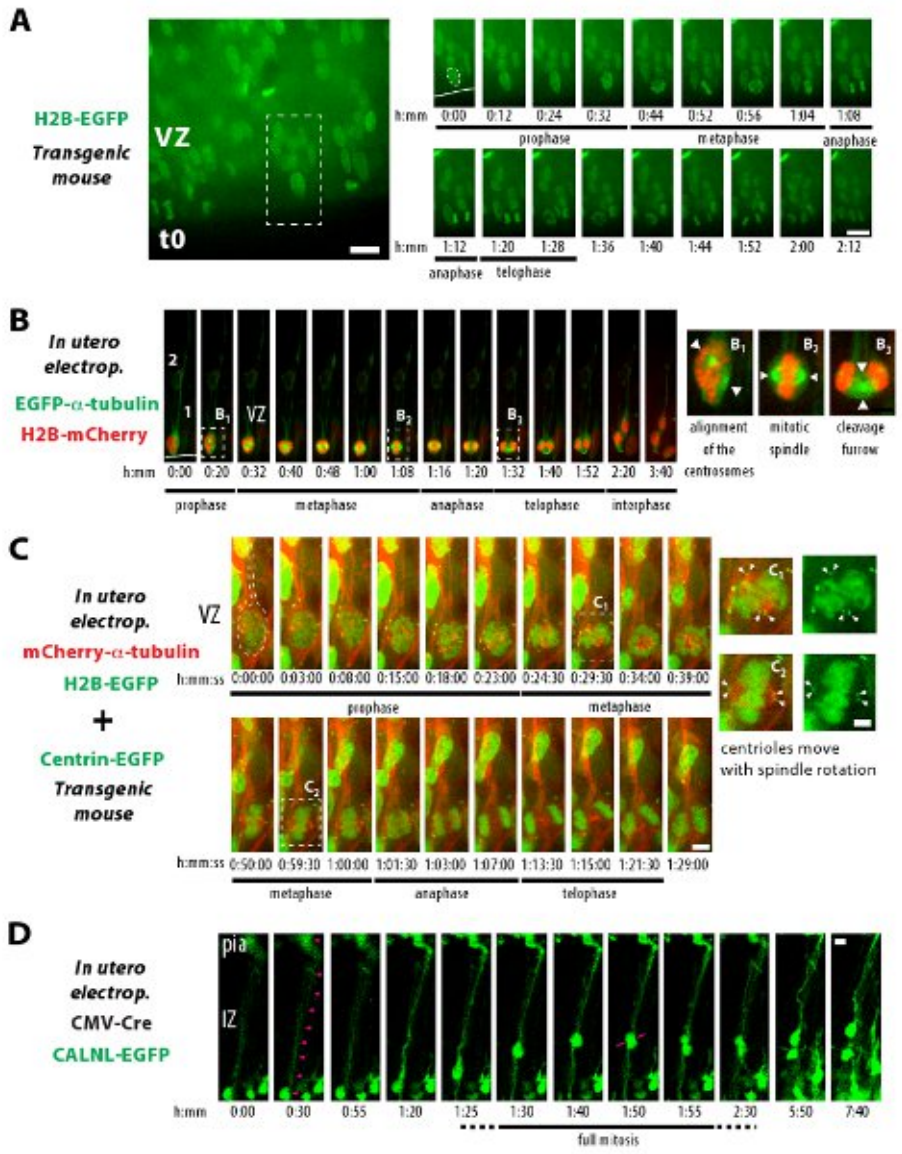

Figure 4. Examples of alternative tools to visualize mitosis of neural progenitors. A) Montage of a region at the border of the ventricle in a brain slice from a histone H2B-EGFP transgenic embryo at E13.5. One progenitor is observed as it proceeds through the different phases of mitosis. **B)** Montage of a VZ at E14.5 in the slice of a brain that was electroporated at E13.5 with vectors expressing EGFP-a-tubulin and H2B-mCherry. EGFP-a-tubulin allows the visualization of microtubule dynamics during mitosis (B_1-B_2) **C)** Montage of a ventricular region in an E15.5 brain from a Centrin-EGFP transgenic embryo that was electroporated at E14.5 with plasmids expressing mCherry-a-tubulin and H2B-EGFP. Centrin-EGFP allows the visualization of centrioles dynamics during mitosis (C_1 and C_2). **D)** Montage of a region showing the IZ of an E14.5 brain slice where cells were sparsely labeled by the electroporation at E13.5 with a low concentration of a plasmid expressing Cre and a standard concentration of a plasmid expressing EGFP after Cre recombination[28]. The dividing cell in this montage has the morphology of an RGosvz with a long basal process (pink arrows) and no apical process[9]. The objective used for live imaging of the (A) and (B) examples is a 60X silicon oil objective, 100X oil objective for example (C), and 10X air objective for example D. The temporal resolution used to image the (A), (B), (C), and (D) examples were 4 min, 4 min, 30 sec, and 5 min, respectively. VZ: Ventricular Zone, IZ: intermediate Zone, RGosvz: outer subventricular zone radial glia-like cell. (Scale bars: A: 15 µm, B, C, D: 5 µm, C_1, C_2: 2.5 µm) Click here to view larger image.

Discussion

The major advantage of the protocol we have described is that it provides a dynamic temporal resolution of mitosis of neural progenitors. Typically, assays to visualize mitosis in the developing brain are performed using immunofluorescence of fixed tissue sections. But this approach only provides a snapshot of mitosis at one time point.

There are several steps that are most critical for imaging mitosis in brain slices: 1) The brain slices should remain attached to the agarose, to preserve the integrity of brain sections during transfer and mounting. 2) For the generation and imaging of slices, control of rostro-caudal levels is important. Caudal brain slices are typically best, because in these slices, the basal processes of the RGC will be kept intact, which is healthier for the cells. 3) For live imaging, use of a confocal spinning-disk microscope with a 60X objective is best for both high resolution and to avoid photobleaching of samples, which can occur with Syto11 dye. 4) For live imaging, the temporal parameters of acquisition are critical. Depending upon the microscope equipment used, time-lapse can be performed ranging from every min to every 10 min. Greater than 10 min is typically too long to achieve high-resolution dynamics of mitosis.

This protocol does have certain limitations. The experiment is performed with *ex vivo* tissue. It is conceivable that observations made in brain slices could be different *in vivo*. Hence, when making comparisons amongst experiments performed on different days or using different samples, the following parameters should be kept constant: use of similar rostro-caudal and dorso-lateral levels, contents of media, use of microscope and acquisition parameters. In addition, phototoxicity is possible with long-term imaging. Therefore multiple experiments should be carried out before making conclusions. Moreover, Syto11 itself could potentially influence mitotic events, especially in a mutant background. Therefore it is recommended that when one makes conclusions about mitosis based on Syto11, they also confirm findings using independent assays, such as markers described in **Figure 4**.

As described in the protocol, mitosis can be imaged after marking chromosomes and the cytoskeleton in various ways. Other Syto dyes can be used to visualize mitosis in other fluorescent channels. *In lieu* of using Syto11 staining to visualize DNA, slices can be generated from fluorescent transgenic lines. For example, as demonstrated, histone H2B-EGFP transgenic lines can be used[29]. Use of histone H2B-EGFP is particularly useful and recommended for confirming any phenotype observed with Syto11 treatment. In addition it could also be useful for overcoming challenges associated with photobleaching with Syto11. Use of other transgenic lines, such as centrin-EGFP[30], can be used to visualize centrioles, the components of centrosomes. While these alone would not suffice for visualizing mitosis, they work well in combination with other markers of chromosomes or microtubules. Such markers can be introduced by *in utero* electroporation[4,5,10,31,32]. As demonstrated, expression of fusion proteins one day prior to dissection can be useful for this approach. In our experience, expressing constructs under a CAG promoter can often yield robust expression within one day. For example we introduced α-Tubulin and histone H2B-EGFP into embryonic brains using this approach.

Once this technique is mastered there are additional modifications that can be made for future experiments. *In utero* electroporation can also be used to manipulate gene expression. For example, introduction of shRNA or cDNAs can be useful to determine the impact of loss-of-function or over-expression of a gene of interest upon neural progenitor mitosis[33]. In addition, slices can be treated with chemical drugs or small molecules to measure the impact of inhibiting signaling pathways or molecules upon mitosis[34,35].

In our procedure we describe imaging performed using a spinning disk confocal. The major advantage of a spinning disk confocal microscope is the speed of acquisition. This enables the imaging of multiple positions from different brain slices in one live imaging session with a high temporal resolution, and a reasonable image quality. Other setups such as a multi-photon laser scanning confocal microscope afford the possibility to acquire high quality images deep into the tissue, where the radial glial cells are less likely to be severed at the level of the basal process. This will therefore increase the probability to image healthy neural progenitors. However, this is usually associated with a sacrifice of the speed of acquisition. This in turn will limit the number of positions that can be imaged during one live imaging session, as well as the temporal resolution. Imaging of mitosis can also be performed on a wide field microscope. However in this case it is best to image sparsely labeled cells, as opposed to histone H2B-EGFP or Syto11, as the overall brightness of these reagents would make imaging much more difficult. Also, the protocol described here utilizes an inverted microscope. The main advantages of an inverted microscope are the possibility to use high-resolution oil objectives and the freedom of movement when the microscope is coupled with a motorized stage. As a conclusion, the experimenter needs to find a balance between the advantages and disadvantages of a particular set-up and the purpose of an experiment.

Disclosures

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