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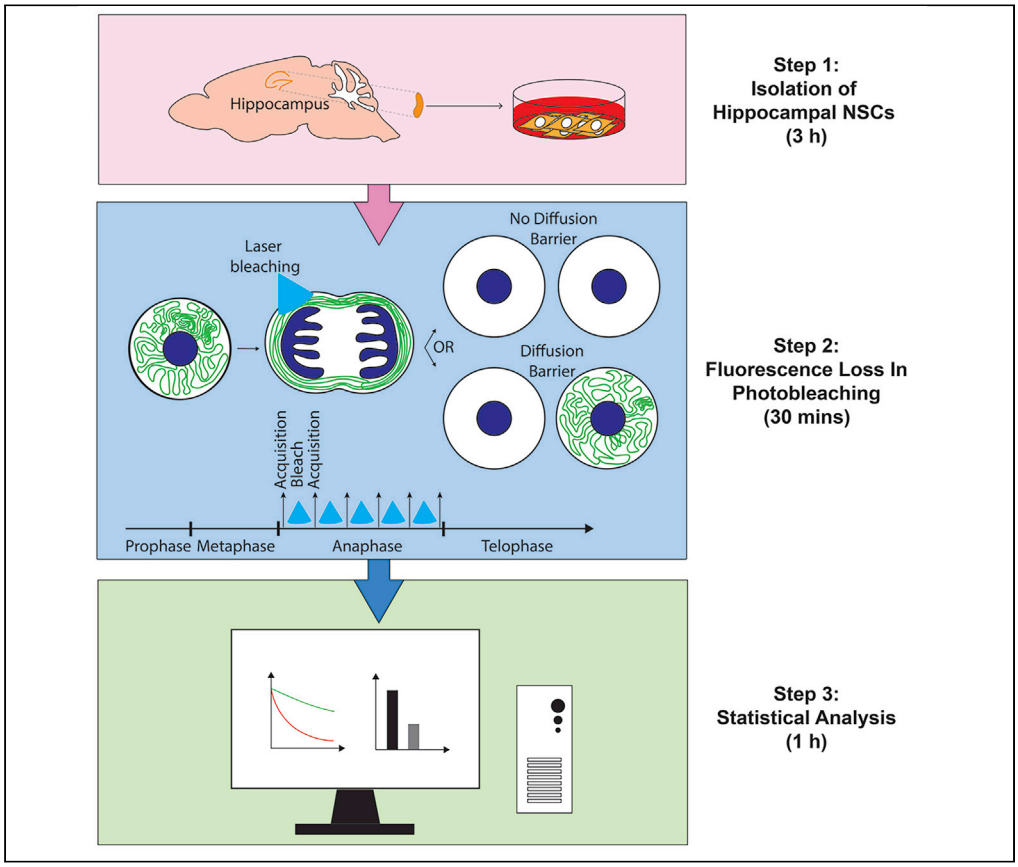
Protocol

Isolation of adult mouse hippocampal neural stem cells for fluorescence loss in photobleaching assays



This protocol describes the isolation and culturing of primary neural stem cells (NSCs) from the adult mouse hippocampus, followed by the experimental approach for fluorescence loss in photobleaching assays, previously used to characterize the presence of an endoplasmic reticulum (ER) membrane diffusion barrier. The assay described here can be used to study live asymmetry in the ER membrane or other organelles that is established in dividing NSCs.

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Highlights

Protocol describes the isolation of hippocampal neural stem cells (NSCs)

Approach to culture NSCs for fluorescence loss in photobleaching (FLIP) assays

FLIP assays of NSCs explained step by step

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Isolation of adult mouse hippocampal neural stem cells for fluorescence loss in photobleaching assays

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SUMMARY

This protocol describes the isolation and culturing of primary neural stem cells (NSCs) from the adult mouse hippocampus, followed by the experimental approach for fluorescence loss in photobleaching assays, previously used to characterize the presence of an endoplasmic reticulum (ER) membrane diffusion barrier. The assay described here can be used to study live asymmetry in the ER membrane or other organelles that is established in dividing NSCs.

For complete details on the use and execution of this protocol, please refer to Clay et al. (2014); bin Imtiaz et al. (2021); Lee et al. (2016); Luedeke et al. (2005); Moore et al. (2015); Shcheprova et al. (2008).

BEFORE YOU BEGIN

The protocol describes the experimental steps to isolate mouse hippocampal NSCs and to perform fluorescence loss in photobleaching (FLIP) assays (Walker and Kempermann, 2014). A comparable protocol has been used to perform FLIP assays on different cell types ranging from mouse embryonic fibroblasts to human neural stem cells. With some alterations, this protocol has been used for additional cell types in *C. elegans* and budding yeast (Lee et al., 2016; Luedeke et al., 2005; Shcheprova et al., 2008).

Media and reagent preparation for isolation of mouse hippocampal NSCs

Timing: 2h

1. Depending on the specific ages and genotype needed, order or plan for the animals to be used.
   a. In case of young animals (<6 weeks), 3 mice should be used. In case of old (>6 months), at least 5 mice should be used. For this protocol, only male mice were used.
2. For isolating neural stem cells (NSCs), MACS neural tissue dissociation kit (P) will be used. Other similar protocols can also be used to isolate hippocampal NSCs (Babu et al., 2007; Guo et al., 2012; Leone et al., 2019). For the protocol described here, which uses the MACS Neural tissue dissociation kit (P), prepare the following solutions (see tables in Materials and Equipment)
   a. 2 x 50mL tubes of HBSS without Ca^{+2} and Mg^{+2} (hereafter called HBSS w/o). Add 500µL of Penicillin/Streptomycin/Amphotericin-B (PSF) in each HBSS w/o tube. Keep these tubes on ice.
   b. 2 x 50mL tubes of HBSS with Ca^{+2} and Mg^{+2} (hereafter called HBSS w/). Add 500µL of PSF in each HBSS w/ tube. Keep these tubes in a 37°C water-bath.

Table 1. Enzyme mixture 1

Reagent	Final concentration	Amount
Buffer X	N/A	1.9 mL
β-mercaptoethanol	67.5μM	2.6 μL
Enzyme P	N/A	50 μL

c. 7.2% sucrose solution. Add 15.4g of sucrose in 25mL HBSS w/. Dissolve and then fill up to the 50mL mark. Sterile filter the solution before adding 500μL PSF. Keep this solution on ice.
d. Enzyme-mix 1 (see Table 1); add 6.75μL of 50mM β-mercaptoethanol in 5mL of Buffer X from the MACS neural tissue dissociation kit (P). Then, add 50μL of enzyme P from the kit to 1900μL of Buffer X with β-mercaptoethanol. Keep this solution in ice until needed. 10 min before its use, pre-warm it by placing it in a 37°C water-bath.
e. Enzyme-mix 2 (see Table 2); add 20μL of Buffer Y to 10μL of Enzyme A from the MACS neural tissue dissociation kit (P).
f. Culturing media (see Table 3).
   i. In a 500mL of DMEM/F-12 Glutamax Media, add 10mL of B27 and 5mL of PSF.
   ii. Dilute the EGF and FGF in this media by adding 45μL of media to 5μL of each growth factor.
   iii. Then, in 50mL of Glutamax Media with B27 and PSF, add 10μL of EGF and FGF to the final concentration of 20ng/mL. Keep this solution in a 37°C water-bath.

3. Plug in the gentleMACS Dissociator and set it to the program "m_brain_01.01".
4. Place the charged MACSmix Tube Rotator in a 37°C incubator.

Reagent preparation for culturing and plating NSCs for FLIP assays

Timing: 1h

Here, NSCs will be plated for FLIP assays. First, the isolated NSCs should form neurospheres in culture within 10 days. Neurospheres should be passaged twice before plating them for FLIP. For the passaging, prepare the following solutions.

5. Prepare culturing media as described above.
6. Prepare 0.05% Trypsin by mixing the following (see Table 4). Once prepared, this solution can be stored in -20°C for up to 1 year.
   a. 200μL of 2.5% Trypsin (Thermo Fisher Cat#15090046)
   b. 9.8mL Versene (Gibco Cat#15040066)
7. Prepare Trypsin Inhibitor (see Table 5). This can be stored in -20°C for up to 1 year.
   a. Dissolve 46.7mg of BSA (Sigma Cat#A4161-5G) in 86.7mL of L-15 medium (Gibco Cat#21083-027). Filter the final solution.
   b. Prepare 4mg/mL DNAse solution using Tris-EDTA.
   c. Add the 4mg/mL DNAse solution to the solution in step a.
   d. Add 100mg of trypsin inhibitor (Sigma Cat#T6522-100MG) to the solution prepared in step c above.
8. Coat the wells in the Nunc Lab-Tek 4-well chambered coverglass as follows. The coated plates can be stored in 4°C for 3 days.

Table 2. Enzyme mixture 2

Reagent	Final concentration	Amount
Buffer Y	N/A	20 μL
Enzyme A	N/A	10 μL

These enzyme mixtures should be freshly prepared.

Table 3. Culturing media for NSCs

Reagent	Final concentration	Amount
DMEM/F-12 Glutamax Media	N/A	500 mL
B-27	1x	10 mL
PSF	1x	5 mL
FGF	20 ng/μL	1:5000 of total volume
EGF	20 ng/μL	1:5000 of total volume

This can be stored at 4°C for up to 1 month.

a. Poly-L-Ornithine (Sigma Aldrich Cat# P3655) diluted 1:200 in distilled water for 1 h at 37°C.
b. Wash thrice with distilled water.
c. Laminin (Sigma Aldrich Cat# L2020) diluted 1:200 in DPBS for at least 3 h at 37°C or for 16 h at 4°C.

Reagent preparation for electroporating NSCs with recombinant DNA

Timing: 1h

The cells need to be transfected with a fluorescent marker for either the endoplasmic reticulum (ER) membrane or ER lumen. To achieve this, NSCs should be electroporated (see Passaging NSCs and electroporating them for FLIP) with fluorescent markers for the ER membrane and/or ER lumen (Cui et al., 2011). Sec61-α and Sel1L have been previously tagged with GFP and used as markers for ER membrane while KDEL tagged with GFP has been used as a marker for ER lumen (bin Imtiaz et al., 2021; Moore et al., 2015; Shcheprova et al., 2008). Amaxa Nucleofactor Kit from Lonza was used for the following electroporations (key resources table).

9. Add the supplement provided in the kit to the Nucleofector Solution. This mixture is stable at 4°C for 3 months.
10. Set the program in the Nucleofector Device to A033.
11. Add ~7μg of DNA to 100μL of the supplemented Nucleofector Solution.

Preparation for FLIP assay

Timing: 1h

Before starting the FLIP assay, set up the microscope as follows. The following set-up is specifically explained for Zeiss LSM 800 confocal microscope with a heating stage.

12. Pre-warm the stage to 37°C and the CO_2 levels to 5%.
13. Using the objective you intend to use, ideally 63x, set up the bleaching and the time-lapse as follows.
    a. Draw a region of interest that you will bleach.
    b. Perform trial bleaches to gauge how long each bleach will take. The settings for the bleach should be set at 80 iterations and 3% laser power.
    c. Depending on the time it takes for each bleach, set up the time lapse. The interval between images should be set at 3 s longer than the bleach time at least.

Table 4. 0.05% Trypsin

Reagent	Final concentration	Amount
2.5% Trypsin	0.05%	200 μL
Versene	N/A	9.8 mL

Table 5. Trypsin inhibitor

Reagent	Final concentration	Amount
L-15 media	N/A	86.7 mL
BSA	0.053%	46.7 mg
4mg/mL DNAse	0.04 mg/mL	866 µL

KEY RESOURCES TABLE

REAGENT/RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
100x15mm Dish, Nunclon™ Delta	Thermo Fisher Scientific	Cat# 150350
2.5% Trypsin	Thermo Fisher Scientific	Cat# 15090046
B27-Supplement (50 X)	Thermo Fisher Scientific	Cat# 17504044
BSA	Sigma-Aldrich	Cat# A4161-5G
DMEM/F-12 GlutaMAX	Thermo Fisher Scientific	Cat# 31331028
DNAse I	Sigma-Aldrich	Cat# DN25
DPBS	Thermo Fisher Scientific	Cat# 14190-094
EDTA	Sigma-Aldrich	Cat# EDS
EGF	PeproTech	Cat# AF-100-15
FGF-2	PeproTech	Cat# 100-18B
HBSS with Ca and Mg	Thermo Fisher Scientific	Cat# 14025092
HBSS without Ca and Mg	Thermo Fisher Scientific	Cat# 14175095
Laminin	Sigma-Aldrich	Cat# L2020
PBS	Thermo Fisher Scientific	Cat# 10010-015
Penicillin/Streptomycin/Amphotericin-B (PSF) (100x)	Thermo Fisher Scientific	Cat# 15240062
Poly-L-ornithine	Sigma-Aldrich	Cat# P3655
Tris/EDTA	Thermo Fisher Scientific	Cat# 17890
Trypsin inhibitor	Sigma-Aldrich	Cat# T6522-100MG
Versene	Gibco	Cat# 15040066
β-Mercaptoethanol 50mM	Thermo Fisher Scientific	Cat# 31350010
L-15 medium	Gibco	Cat# 21083-027
Critical commercial assays		
Amaxa Mouse NSC Nucleofector Kit	Lonza	Cat# VPG-1004
Neural Tissue Dissociation Kit (P)	Miltenyl Biotec	Cat# 130-092-628
Experimental models: Organisms/strains		
Conventional/wild-type mice	The Jackson Laboratory	C57BL/6
Recombinant DNA		
Sel1L-sfGFP	A gift from M. Molinari	N/A
Sec61-GFP	A gift from A. Helenius	N/A
KDEL-sfGFP	A gift from A. Helenius	N/A
Software and algorithms		
Fiji/ImageJ	Fiji	http://fiji.sc RRID:SCR_002285
GraphPad Prism	GraphPad	http://graphpad.com/ RRID:SCR_002798
ZEN Blue	Carl Zeiss	http://zeiss.com RRID: SCR_013672
Other		
gentleMACS Dissociator	Miltenyl Biotec	Cat# 130-093-235
LSM 800 Confocal Microscope	Carl Zeiss	N/A
MACSmix Tube Rotator	Miltenyl Biotec	Cat# 130-090-753
Nunc Lab-Tek 4-well chambered coverglass	Thermo Fisher Scientific	Cat# 155382

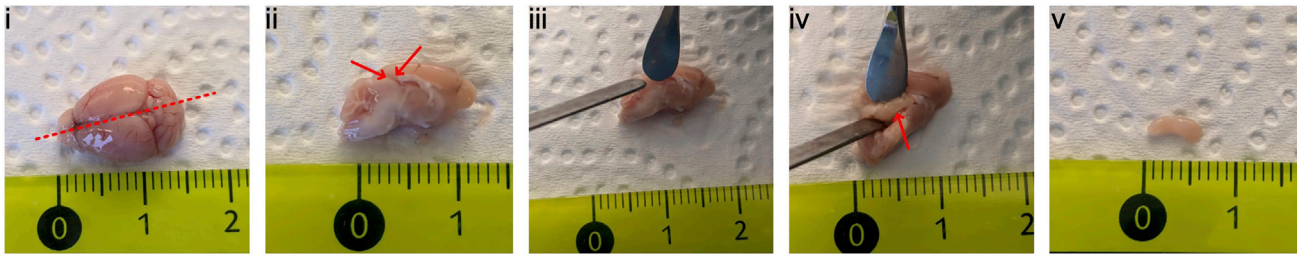

Figure 1. Dissecting hippocampi

A full brain shown along with a dotted line illustrating where the brain should be cut (i). A half brain with the arrows showing where to dissect with the tools to isolate the hippocampus (ii & iii). The hippocampus being isolated can be seen (red arrow; iv). An isolated hippocampus (v).

MATERIALS AND EQUIPMENT

The plasmids used to label the ER are available upon request.

For enzyme mixtures used in dissociation of the brain tissue, refer to the following tables. The reagent names appear as they are listed on the MACS Neural Dissociation kit.

For culturing media for NSCs, following reagents are needed. FGF and EGF are added before warming the media and hence the amount added depends on the total volume of media needed.

For passaging NSCs, prepare the trypsin and trypsin inhibitor as follows.

The prepared Trypsin and the Trypsin Inhibitor can be stored in -20°C for up to 1 year.

FLIP experiments were performed using a Zeiss LSM800 confocal microscope and ZEN Blue software. This protocol may need to be adapted for use with a different confocal microscope and software setup.

STEP-BY-STEP METHOD DETAILS

Isolation of hippocampal NSCs

Timing: 3h

Here, hippocampal NSCs will be isolated that will then be used for FLIP assays.

1. Euthanize the mice and isolate the brain.
   a. The olfactory bulbs can be discarded.
2. As shown in Figure 1, use two spatulas, one preferably curved, to carve just underneath the cerebrum and the cerebellum. The dissection should be done on ice.
   a. Place the two spatulas at the indicated arrows.
   b. The top spatula should be held straight and forced down into the tissue.
   c. Pull the other spatula that is in contact with the cerebellum back, opening up the tissue at the point where cerebellum and cerebrum meet.
   d. Once the cerebellum is pulled back, hippocampus should be easily detectable.
   e. With the curved spatula, lift the hippocampus from the tissue and cut it with the edges of the spatula from the rest of the brain
3. After isolating the hippocampus, put all of them in cold HBSS w/o prepared earlier.
   a. At this point, separate and collect the hippocampi in different 15 mL tubes depending on the age and/or the genotypes you have.
   b. Keep the tubes with the hippocampi in the HBSS w/o on ice.

4. Using a pipette, carefully remove the HBSS w/o from the tubes.
5. Add the prewarmed Enzyme Mix 1 gently into the tube, lifting the tissue from the bottom of the tube. The total volume prepared for Enzyme Mix 1 is ample for up to 10 hippocampi.
    a. Using a 2mL pipette, transfer the Enzyme Mix 1 with the hippocampi to a labeled C tube.
6. Close the C tube and flick it upside down to push the hippocampi to the top of the tube.
7. Attach the C tube upside down onto the gentleMACS Dissociator.
8. Run the program "m_brain_01.01".
9. After the program is complete, take off the C tube and flick the tube so that the hippocampi are on the bottom of the tube now.
10. Place the tube in the MACSmix Tube Rotator and allow it to rotate slowly for 15 min at 37°C.
11. After the run is over, take the tube off and flip the tissue to the lid again.
12. Place the C tube on the gentleMACS dissociator and run the program "m_brain_02.01".
13. After the program is run, add Enzyme Mix 2 to the tube.
14. Put the tube onto the MACSmix Tube Rotator again and allow it to rotate slowly for 10 min at 37°C.
15. Once the 10 min are complete, flick the C tube again to flip the tissue to the lid.
16. Place the C tube on the gentleMACS Dissociator and run program "m_brain_03.01".
17. After the program is run, flip the tissue to the bottom again.
18. Place the C tube in the MACSmix Tube Rotator and allow it to rotate slowly for 10 min at 37°C.
19. After the run, centrifuge the C tube at 300g for 2 min at 20°C–25°C. This will bring all the tissue to the bottom of the tube.
20. Place a 70μm cell strainer on top of a 50 mL tube.
21. Pre-wet a 70μm cell strainer with 1m L of warm HBSS w/.
22. After centrifugation, gently resuspend the pellet in its own supernatant using a 1mL pipette.
23. Add all of the cell suspension to the strainer.
24. Add 1mL of warm HBSS w/ to the C tube to rinse it and then apply it onto the cell strainer.
25. Wash the cell strainer with preheated HBSS w/.
26. Take the cell strainer off carefully so as not to spill any liquid hanging from the bottom of the strainer.
27. Transfer the liquid to a labeled 15 mL tube.
28. Centrifuge the tube at 300g for 10 min at 20°C–25°C.
29. After centrifugation, discard the supernatant by aspirating it.
    a. The cell pellet at the bottom of the tube will be easily evident at this stage.
30. Resuspend the pellet in 10mL of the cold 7.2% sucrose solution prepared earlier.
31. Centrifuge this tube at 850g for 10 min at 20°C–25°C.
32. After the centrifugation, aspirate the supernatant carefully as the pellet would be small.
    a. To be cautious, aspirate the last few μls with a 200 μL pipette.
33. Resuspend the pellet again in 10mL warm HBSS w/.
34. Centrifuge this for 10 min at 300g at 20°C–25°C.
35. Discard the supernatant after the spin and resuspend the pellet in 1mL preheated culturing media prepared earlier.
36. Put 10mL warm media in a 100x15mm dish (Thermo Fisher Cat# 150350).
37. Add the 1mL media with the cells to the dish.
38. The NSCs should be first fed 2 days after the isolation. For feeding the NSCs, follow the steps below;
    a. Take out 7mL of media and spin it at 120g for 5 min at 20°C–25°C.
    b. Replace the media in the plate with 8mL of freshly prepared culturing media. Some media will evaporate in the incubator, hence more media is added. Depending on the total volume in the dish, lower volume can be added.
    c. Carefully aspirate the supernatant.
    d. Resuspend the pellet in 1mL of warm media.
    e. Add the resuspended cells to the dish.

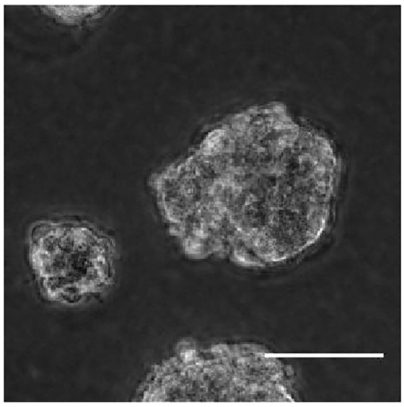

Figure 2. Neurospheres formed by NSCs are shown
Scale bar represents 50μm

Feed the NSCs with fresh culturing media prepared as outlined above every 2–3 days. The typical schedule is Monday, Wednesday and Friday. Wait for the isolated NSCs to form neurospheres and then passage them as described below. NSCs should make neurospheres twice before proceeding with the FLIP assay.

The risk of cultured NSCs differentiating is negligible in these conditions. They will only differentiate upon withdrawal of mitogens(Walker and Kempermann, 2014). However, to make certain that the NSCs are still pluripotent, they can be stained against Nestin.

Pause point: At this stage, cells need to be cultured *in vitro*, providing the opportunity to pause before proceeding with the protocol below.

⚠ CRITICAL: Putting the brain in cold HBSS w/o for a few minutes ideally helps with the dissection as the tissue becomes more rigid. Be careful when dissecting not to crush the hippocampus.

Passaging and electroporating NSCs for FLIP

Timing: 1h

Here, NSCs will be passaged and electroporated with the recombinant DNA before being plated for FLIP.

39. Culture the NSCs until they form neurospheres as shown in Figure 2. These neurospheres should be allowed to grow to a maximum diameter of approximately 100μm. At this stage, the NSCs will need to be passaged. Troubleshooting 1.
40. Transfer the entire media in the plate to a 15 mL tube.
41. Centrifuge the tube at 80g for 5 min at 20°C–25°C.
42. Aspirate the supernatant and resuspend the pellet in 100μL of warm trypsin prepared as outlined above.
43. Incubate the tube at 37°C in a water-bath for 5 min.
44. Add 200μL of trypsin inhibitor prepared as outlined above to the tube. Lightly tap the tube at this point.
45. Incubate the tube for 5 min at 20°C–25°C.
46. After the 5 min, use a 1mL pipette to pipette the mixture with the cells up and down approximately 20 times until the mixture turns cloudy.

47. Following this, count the cells. $4x10^6$ cells will be used for electroporation. Add the volume corresponding to the number of cells required into a 15mL tube.
48. Centrifuge tube at 200g for 4 min.
49. Aspirate the supernatant and resuspend the pellet in the Nucleofector Solution mixed with DNA earlier (Reagent preparation for electroporating NSCs with recombinant DNA).
50. Transfer the resuspended NSCs to the cuvettes supplied with the Nucleofector kit.
51. Insert the cuvette into the Nucleofector device and run the program.
52. Immediately afterward, take the cuvette out and add 1mL of prewarmed culturing media.
53. Plate $9x10^5$ cells per well in the coated Nunc Lab-Tek 4-well chambered coverglass. These chambered coverglass were previously coated with Poly-L-Ornithine and Laminin (Reagent preparation for culturing and plating NSCs for FLIP assays).
54. Plate the remaining cells in a 100x15mm dish with preheated culturing media.
55. After plating the NSCs, incubate the Nunc Lab-Tek 4-well chambered coverglass at 37°C for 16 h.
56. Following the incubation, wash the cells twice with 1mL of PBS to remove any dead cells and debris.
57. Add 1mL of prewarmed media 3 h before starting the FLIP assays.

⚠ CRITICAL: Once the cells are resuspended in the Nucleofector Solution, the following steps must be completed as quickly as possible until warm media is added to prevent high cell death.

FLIP assay

Timing: 30 mins per cell

After plating the NSCs and keeping them in 37°C incubator for 16 h, the FLIP assays can be performed on them. FLIP assays allow visualization of diffusion dynamics. An area is selectively bleached and the changes in the fluorescence intensities in the sample is measured, including in the area bleached. The rate of decay of fluorescence is an indirect measure of the diffusion dynamics of the specific protein. Using this, we have developed the following protocol to look for the presence of a diffusion barrier in a dividing NSC.

58. Place the Nunc Lab-Tek 4-well chambered coverglass with the NSCs on the stage.
    a. This protocol has been optimized for 63x oil immersive objective.
59. Using the eyepiece, scan the cuvette to search for a cell in mitosis.
    a. Ideally, cells in late metaphase should be found.
    b. Cells in mitosis would not be attached to the surface and therefore, scan approximately 5–10 μm above the surface as shown in Figure 3A.
60. Once the cell is found, wait for the cell to start anaphase. For a cell in metaphase, this waiting time is typically 5 min. Troubleshooting 2.
    a. To identify the start of anaphase, focus on the shape of the cell. Anaphase would result in the cell elongating along an axis.
61. As soon as the cell starts elongating, select a region adjacent to the future cleavage furrow as indicated in Figure 3B and Methods video S1.

62. Once the cell starts anaphase, select the best z-slice and immediately start the experiment. Troubleshooting 3.
    a. The range of laser power used for acquisition is between 1% and 4%. However, laser power used depends on the microscope and the lasers being used.
63. After the acquisition of the first image (the pre-bleach image), select a region with high GFP signal and use the region of interest drawn earlier to bleach it.
64. After the bleach, an image would be automatically acquired.

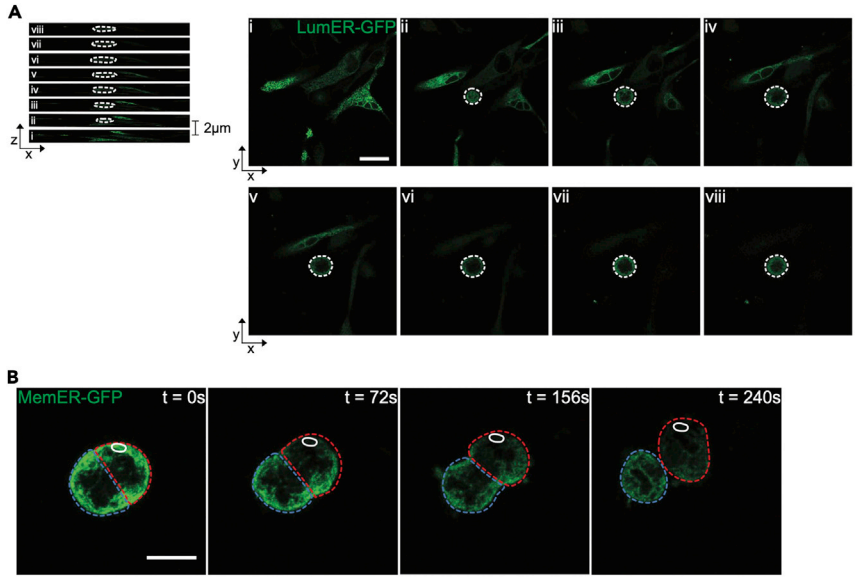

Figure 3. Fluorescence loss in photobleaching (FLIP) assay

(A) A z-stack along the z and x axis is shown where the gap between each image is 2um. The dividing cell expressing LumER-GFP is highlighted by the dotted circles (left). The same stack is shown along the x and y axis where the same cell is highlighted (right) starting up from the surface of the plate.

(B) A dividing cell expressing MemER-GFP (Sel1L-GFP) undergoing FLIP is shown. MemER-GFP labels the ER membrane. At the onset of anaphase, first frame, the region shown in white is bleached. Time-lapse images post-bleaching are shown where the dividing cell is divided into the bleached compartment (red) and unbleached compartment (blue).

Scale bar represents 20 μm (A) and 5 μm (B).

65. After the second image is acquired, select the region bleached earlier and bleach it again.
    a. Note, because the cell is undergoing anaphase, it is unlikely that the region bleached earlier will be in the exact same place again, hence the region of interest drawn earlier might need to be moved slightly to the corresponding area.
66. Repeat this process until there is no GFP signal visible in between the two dividing compartments of the cell. Troubleshooting 4.
    a. This process usually takes between 3 to 4 min approximately.
67. Once FLIP has been performed on the specific cell, save the image file as desired and then refer back to step 52 to include more cells.

⚠ CRITICAL: Only perform bleaching once the cell enters anaphase, i.e., starts elongating. Bleaching earlier would result in unreliable results.

Analyzing loss of fluorescence

Timing: 1h

68. To analyze the fluorescence loss in the bleached and the unbleached compartments, use ImageJ;
    a. Draw the regions of interest around the bleached and unbleached compartments.
    b. Measure the area and the intensity of the all the regions of interest for bleached and unbleached in the time-lapse. Divide the fluorescence signal by the area for each frame to calculate the fluorescence intensities for both the bleached and the unbleached.
    c. Draw a region of interest around a dark region with no fluorescence. Divide the fluorescence signal by the area to calculate the background fluorescence intensity.

d. Subtract the background fluorescence intensities for each frame from both the bleached and the unbleached fluorescence intensities.

69. Use the following equation to normalize the fluorescence intensities for the bleached and unbleached compartments. Note, the first frame was taken pre-bleach, hence we here normalize the fluorescence for both compartments to the pre-bleach intensity. This analysis is performed in Excel. Troubleshooting 5.

$$Normalized\ Intensity = \frac{Fluorescence\ intensity\ n^{th}\ Frame}{Fluorescence\ intensity\ 1st\ Frame} \times 100$$

70. After performing the above calculation for both compartments for all cells, compile the data from all cells into a table in a separate sheet.
    a. The time, in seconds, is calculated as follows where 12 is the interval between frames.

$$Time = (Frame\ N - 1) \times 12$$

    b. The normalized values for the bleached and the unbleached compartments calculated before.

Time	Bleached	Unbleached
0	100%	100%
12	80%	95%

71. Export the data from Excel into an XY table in Prism.
72. In Prism, fit the data to one-phase decay, which can be found under Analyze> Non-linear Regression. This fits the bleached and the unbleached data to the following equation.

$$Y = (Y_0 - Plateau) \times e^{-Kx} + Plateau$$

Where Y_0 is the y-intercept.

Under constraints, set this to be equal to 100.

Plateau is the value of Y at X infinity.

Under constraints, set this to be equal to 0.

K is the rate constant.

Under constrains, set this unconstrained.

73. Once the data is fitted, plot the data and the fitted curves to assess how well the data fits plotted curve.
74. Transfer the results from the fits under the results tab to an Excel sheet for both bleached and unbleached data.
75. Rearrange the equation above as follows.

$$X = \frac{\ln\left(\frac{Y - Plateau}{Y_0 - Plateau}\right)}{-K}$$

Note, X denotes the time it takes to reach certain fluorescence.

76. From the data transferred from Prism, substitute the value of K. The value of Y should be set at 80. The values for Plateau and Y_0 have already been preassigned to 100 and 0, respectively. Perform this calculation for both the bleached and the unbleached compartments.
    a. The calculate value of X would be the time it takes for the compartment to reach 80% of the original fluorescent signal, i.e., lose 20% of fluorescence.
77. To calculate the strength of the barrier, use the following equation.

$$Barrier\ Strength = \frac{X_{Unbleached}}{X_{Bleached}}$$

78. To calculate the total error in the barrier strength calculations, use the following equation

$$Total\ Error\ for\ each\ compartment = -seK \times \frac{\ln\left(\frac{Y - Plateau}{YO - Plateau}\right)}{K^2}$$

Where *seK* (standard error in K) is already calculated in Prism and should be in the data copied over from fits.

79. Calculate the standard error for each compartment using the following equations. Note, the equations for the bleached and the unbleached standard error are not the same.

$$Barrier\ Index\ Standard\ Error_{Bleached} = X_{Unbleached} \times \frac{total\ error\ for\ Bleached}{X^2_{Bleached}}$$

$$Barrier\ Index\ Standard\ Error_{Unbleached} = \frac{total\ error\ for\ Unbleached}{X_{Bleached}}$$

Where X for each compartment was calculated earlier.

80. Then, following this, sum the barrier index standard error for both the bleached and unbleached to calculate the standard error in the barrier strength calculation

$$Standard\ Error\ in\ Barrier\ Strength = Barrier\ Index\ Standard\ Error_{Unbleached} + Barrier\ Index\ Standard\ Error_{Bleached}$$

EXPECTED OUTCOMES

As has been already established, mouse NSCs establish a diffusion barrier in the ER membrane (bin Imtiaz et al., 2021; Moore et al., 2015). Using the setup described in this protocol, the loss of fluorescence in the bleached compartment should be significantly higher than the loss in fluorescence for the unbleached compartment when ER membrane is tagged with a fluorescent marker. Any

difference in the loss of fluorescence will be reflected in the final calculations of the diffusion barrier strength. Barrier strength of 1 reflects no barrier to the diffusion between the two compartments. A value higher than 1 indicates that there is a barrier to free diffusion between the two compartments analyzed.

LIMITATIONS

This protocol is based on mouse NSCs and the FLIP assays were performed using a Zeiss LSM800 microscope. The timings and the bleaching settings may need to be adjusted for other microscopes and software. Moreover, the density at which cells are plated may need to be adjusted for best results depending on the experimental setup.

TROUBLESHOOTING

Problem 1

Cultured NSCs take longer than 2 weeks to form neurospheres. This may happen when culturing NSCs from old animals as they have a low proliferation rate (step 39).

Potential solution

Be gentle when resuspending the cell pellet during when the media is changed. This is to avoid breaking neurospheres that may just be forming. Gentle resuspension is critical for NSCs derived from old animals.

Problem 2

The dividing cell takes longer than 5 min to complete anaphase. This can be typically assessed by looking at the morphology of the cell. If it doesn't change drastically over 5 min, the cell is not showing typical behavior (step 58).

Potential solution

Confirm the incubation temperature of the stage is set at 37°C.

Problem 3

The cell never enters anaphase (step 60).

Potential solution

It is likely that the cell was wrongly identified as a dividing cell. Cells programmed for apoptosis also round up and may appear very bright.

Problem 4

The dividing cell takes longer than 5 min to complete anaphase (step 64).

Potential solution

This may be because the incubation temperature of the stage is below 37°C. Confirm that the stage is set to the right temperature.

Problem 5

The normalized fluorescence intensities are above 100% for an individual cell (step 67).

Potential solution

This is typical as the change in cell size (area measured) can affect the normalized fluorescence intensities. This problem will be solved once cell intensities are averaged.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Sebastian Jessberger (jessberger@hifo.uzh.ch).

Materials availability

Genetic markers of the endoplasmic reticulum will be provided upon request and execution of suitable Mutual Transfer Agreement.

Data and code availability

This study did not generate any new datasets or codes.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.100695.

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AUTHOR CONTRIBUTIONS

M.K.b.I. wrote the manuscript. S.J. revised the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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