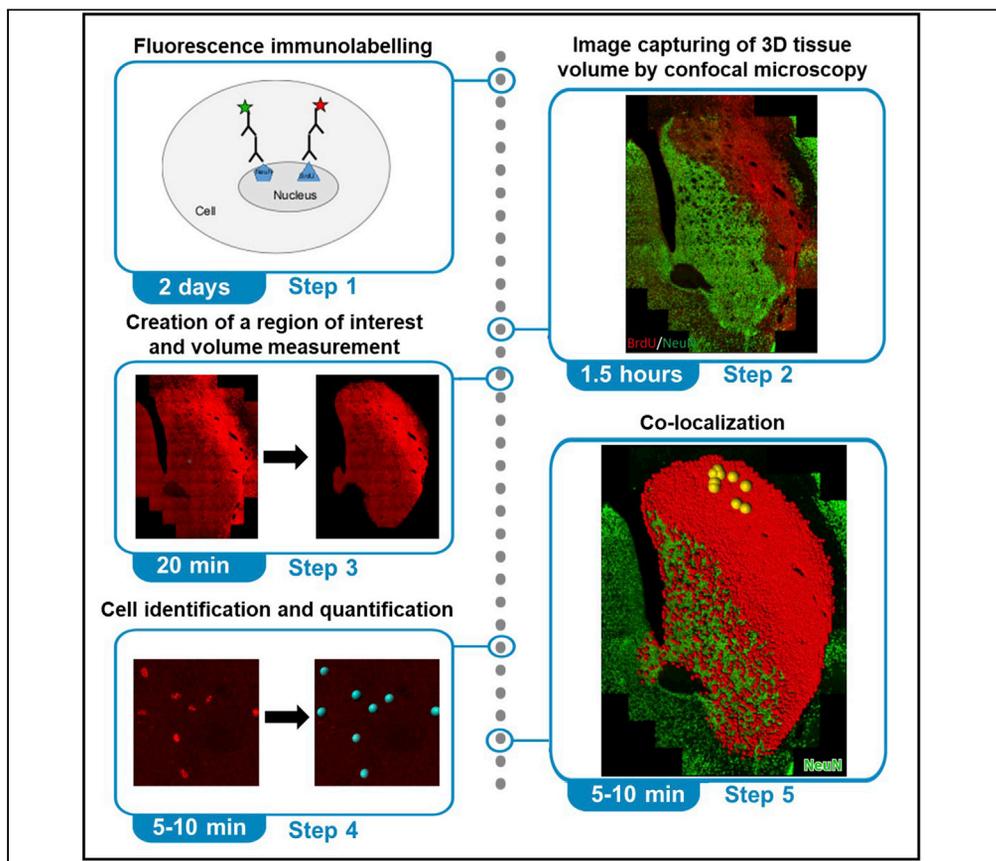


Protocol

Histological characterization and quantification of newborn cells in the adult rodent brain



This protocol describes the identification and characterization of newborn cells generated in the rodent brain after injury through birthdating with the thymidine analog 5-bromo-2'-deoxyuridine, followed by immunohistochemical labeling of fixed tissue sections. We also describe a software-assisted approach for automated detection and quantification of cells in large three-dimensional tissue volumes acquired using confocal microscopy. This approach facilitates the identification of low-frequency events that may be difficult to capture using manual counting methods, including stereology based on random sampling.

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Highlights

Newly generated cells can be immunolabeled using thymidine analogs such as BrdU

High-quality 3D image capture allows for accurate software-based cell detection

Automated cell detection quantifies immunolabeled cells in large tissue volumes

Workflow to assist identification of low-frequency events in large cell populations

Ermine et al., STAR Protocols
2, 100614
September 17, 2021 © 2021
The Authors.
<https://doi.org/10.1016/j.xpro.2021.100614>



Protocol

Histological characterization and quantification of newborn cells in the adult rodent brain

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SUMMARY

This protocol describes the identification and characterization of newborn cells generated in the rodent brain after injury through birthdating with the thymidine analog 5-bromo-2'-deoxyuridine, followed by immunohistochemical labeling of fixed tissue sections. We also describe a software-assisted approach for automated detection and quantification of cells in large three-dimensional tissue volumes acquired using confocal microscopy. This approach facilitates the identification of low-frequency events that may be difficult to capture using manual counting methods, including stereology based on random sampling. For complete details on the use and execution of this protocol, please refer to Ermine et al. (2020).

BEFORE YOU BEGIN

Birthdating in vivo using thymidine analogs

The use of detectable thymidine analogs that are incorporated into cells during S-phase of mitosis provides a powerful approach for birthdating the period of cell division. It is an approach that has contributed fundamentally to our understanding of cellular proliferation and differentiation during neurodevelopment as well as in the postnatal brain in both physiological and pathological settings. The technique has evolved from the use of tritiated thymidine followed by autoradiographic detection in the 1960-70s (Sidman, Miale and Feder, 1959; Sidman, 1970), to what are now more routinely used immunohistochemical procedures, where combinations of antibodies allow for the detailed phenotyping of birth-dated cells (for review see (Rowell and Ragsdale, 2012)). The most widely used thymidine analog has been 5-bromo-2'-deoxyuridine (BrdU) (Miller and Nowakowski, 1988), and there are a variety of commercially available antibodies for specific immunochemical detection of BrdU in cell culture or tissue preparations. A number of other halogenated uradines that can be detected with specific antibodies have also been described, including 5-iodo-2'-deoxy-uridine (IdU) and 5-chloro-2'-deoxy-uridine (CldU), as well as 5-ethynyl-2'-deoxyuridine (EdU), which can be detected using 'click chemistry' (Chehrehasa et al., 2009). Combinatorial use of different analogs has facilitated interesting applications, including studies on cell-cycle length or identification of distinct proliferative events in the same cell population (for review see (Llorens-martín and Trejo, 2011)).

One area where birthdating studies using BrdU have been fundamentally important is in our understanding of the proliferative response following brain injury, for example after modeling of stroke in rodents (Ermine et al., 2020). These studies are built around a 'pulse-chase' design, where BrdU is delivered systemically to animals to birthdate dividing cells during a defined 'pulse' period, followed by a 'chase' period that allows the labeled cells time to differentiate in order to facilitate



immunohistochemical phenotyping based on the expression of mature markers of cellular identity. The number of cells birthdated using the administered thymidine analog will be determined by a number of factors including cell cycle-length relative to the frequency and dose of BrdU delivery and also the survival of the cells relative to the chase period (for reviews see (Taupin, 2007; Llorens-martin and Trejo, 2011)). Previous studies in rodents have shown that intraperitoneal (i.p.) injection of BrdU at a dose of 50 mg/kg every 12 h is sufficient to label all dividing cells in the neurogenic subventricular zone (Arvidsson et al., 2002) although 100 mg/kg is also commonly used (Ermine et al 2020). The appropriate chase period should be determined based on the aim of the experiment. In the material described here, we have used a chase period of 3 weeks in order to allow for expression of mature markers of cellular identity in cells BrdU-labeled during a pulse period of 7 days (100 mg/kg i.p.; daily) directly following ischemic injury to the rat forebrain (Ermine et al 2020). We describe immunohistochemical procedures to identify newborn (BrdU+) cells generated in the first week after injury that survive, and differentiate as new neurons (NeuN+). We also describe a software-assisted approach for the identification and quantification of thousands of these newborn cells, characterized by expression of phenotypic proteins, in large 3-dimensional brain tissue volumes captured by confocal microscopy.

Tissue preparation

⌚ Timing: 2 days

Here we describe tissue preparation from rats that received a focal ischemic injury to the forebrain, followed by BrdU pulsing for 7 consecutive days (100 mg/kg in saline; i.p.) and then a chase period of an additional 3 weeks.

1. After 3 weeks the animals are euthanized by a lethal dose of pentobarbitone (100 mg/kg; Lethobarb® Virbac; Stock solution made of 29.7% pentobarbital sodium).
2. Perfuse transcardially with 4% paraformaldehyde (PFA, 4% in 0.2 M phosphate buffered saline (PBS) with 0.2% picric acid).
3. Collect the brains and further post-fix them for 2 h in 4% PFA.
4. Cryopreserve the brains in 20% sucrose-PBS solution for 1–2 days.
5. Freeze the brains on dry ice.
6. Using a freezing-microtome (Leica, Wetzlar, Germany) collect 40 µm-thick coronal sections in 1:12-series.

⚠ **CRITICAL:** The post-fix times should be limited to 2 h for BrdU labelling. The time between the last BrdU administration and euthanasia was set at 3 weeks in the present example (4 weeks from first injection). This allows time for the expression of mature markers of cell identity in the birth-dated cells, such as NeuN to identify neurons born during the BrdU labelling period.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Sheep anti-BrdU	Exalpha	A205P
Mouse anti-NeuN	Millipore	MAB377
Anti-sheep conjugated Dylight Fluorophores-488	Jackson ImmunoResearch	713545147
Anti-mouse biotin conjugated	Jackson ImmunoResearch	715065151
Streptavidin-550	Abcam	134348
DAPI	Sigma	D9542-1 mg

(Continued on next page)

<i>Continued</i>		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
BrdU	US Biologicals	CAS: 59-14-3
Lethabarb®	Virbac	1905213
PFA	Sigma	158127
Picric acid	Sigma	197378-100 g
H ₄ NaO ₅ P	Merck	1063461000
Na ₂ HPO ₄	Merck	1065805000
NaCl	ChemSupply	SA046-5KG
De-ionized formamide	Millipore	4610-OP
HCL 37%	Sigma	320331
Donkey serum	Merck	S30-100 mL
Triton X	Merck	1086031000
Sodium tetraborate	Ajax Finechem (Univar)	AJA460
Gelatin from porcine skin	Sigma	G25000-1 kg
Chromium potassium sulfate dodecahydrate	Merck	1010360250
Dako	Agilent	S302380-2
Experimental models: organisms/strains		
Sprague-Dawley (adult >10 weeks, female)	ARC	n/a
Software and algorithms		
Zen Black 2012 SP5	Zeiss	n/a
Imaris v9.0	Imaris	n/a
Other		
Microtome	Leica	n/a
Confocal microscope LMS 780	Zeiss	n/a

MATERIALS AND EQUIPMENT

Solutions used in this protocol

- **PBS**: stored at room temperature (~20°C) for 2 weeks maximum) in distilled water

Reagent (stored at room temperature (~20°C))	Final concentration	Amount for a total volume of 1 L of distilled water
H ₄ NaO ₅ P	20 mM	2.76 g
Na ₂ HPO ₄	80 mM	14.24 g
NaCl	154 mM	9 g

- **0.1 M Borax solution (store at ~20°C)**: 38 g of Na₂B₄O₇ (Sodium tetraborate) in 1 L of distilled water, buffered at pH 8.5.
- **2 M HCl (store at ~20°C)**: 200 mL HCl 37% and 800 mL of distilled water.
- **Blocking solution (store at 4°C, best used within few days)**: 5% serum (donkey used here, however ideally the serum is matched to the species the secondary antibodies are raised in and should never overlap with the primary antibody species), 0.5% Triton X and PBS.

STEP-BY-STEP METHOD DETAILS

Fluorescent immunolabeling protocol

⌚ Timing: 2 days

Free floating sections are transferred into glass scintillation vials and the vials are placed on an orbital rocker for the duration of the experiment in order to agitate the tissue sections. Generally, 1 mL of solution is sufficient for 1:12 series (used in the present example) up to a maximum of a

1:6 series for a rat brain (approximately spanning Bregma +4.7 mm to -8.0 mm—40 sections). For larger amounts of tissue, the incubation volume should be increased accordingly to ensure adequate diffusion of incubation solution throughout the tissue.

1. Denaturing pre-treatment

- a. Wash tissue for 5 min 3 times (3×) with PBS at room temperature (RT; ~20°C)
- b. Denaturing
 - i. Incubate free-floating sections in 1 mL of de-ionized formamide for 2 h at 65°C
 - ii. 3× 5 min washes with PBS
 - iii. Incubate in 1 mL of 2 M HCl for 30 min at 37°C
 - iv. 2× 5 min wash with 0.1 M Borax solution
 - v. Incubation in 1 mL of Borax solution for 20 min at RT
 - vi. 6× 5 min washes in PBS

Note: DNA denaturing using formamide as a pre-treatment allows greater access of the primary BrdU antibody to the DNA incorporated BrdU.

2. Labeling with primary antibodies

Primary antibodies:

- sheep anti-BrdU (Exalpha, A205P, 1:1000)
- mouse anti-NeuN (Millipore, MAB377, 1:1000)

- a. Incubate in 1 mL of primary antibodies diluted in blocking solution, overnight (~8–12 h) at RT
- b. 3× wash 5 min washes in PBS

Note: Tissue can be incubated for longer times (e.g. over a weekend) at 4°C.

3. Labeling with secondary antibodies

Here we describe direct conjugation of a fluorophore labeled secondary antibody (488) for direct detection of the BrdU labeled with primary sheep antibody and also indirect conjugation with a biotin labeled secondary antibody targeted to the mouse NeuN primary antibody. The biotin conjugation allows for amplification of the detectable signal via the addition of a streptavidin-fluorophore complex. Note that the capacity for biotin-streptavidin amplification to improve signal-to-noise ratio should be determined for each antibody by the user. We find that it is useful for certain antibodies where the specific signal can be low, including the mouse anti-NeuN described here.

Secondary antibodies:

- Direct: anti-sheep Dylight Fluorophore 488 (Jackson ImmunoResearch, 1:200)
- Indirect: anti-mouse biotin (Jackson ImmunoResearch, 1:400)

- a. Incubation in 1 mL blocking solution for 30–60 min
- b. Incubation in secondary antibodies in blocking solution for 2 h at RT
- c. 3× 5 min washes in PBS

Note: For all subsequent steps involving fluorophores, we reduce ambient light exposure by covering the scintillation vials (e.g. using Alfoil).

4. Amplification with streptavidin:

Detection of certain antibodies can benefit from streptavidin-biotin amplification. This step can also allow for detection using lower concentrations of primary antibody, which can improve the signal-to-noise ratio. Optimal results, including a range of primary antibody concentrations, should be determined by the user.

- a. Incubation with streptavidin-550 (1:400) in blocking solution for 1.5 h at RT
- b. 3× 5 min washes in PBS

5. Slide mounting:
 - a. Glass slides are previously double coated with gelatine (10 g/L, Sigma) and chromium potassium sulfate dodecahydrate (1 g/L, Merck) in distilled water. Slides are dried in an oven at 42°C for 48 h and left at room temperature (~20°C) for a further 48 h.
 - b. Tissue sections can be floated and arranged as individual sections in PBS in a glass petri dish and a fine paintbrush used to drag each section onto the coated glass slides. Slides are allowed to dry for ~10 min until the sections have adhered to the slide and then mounting medium is used (e.g., DAKO) to apply a coverslip, before final drying overnight (~8–12 h) in the dark. Slides should be stored at 4°C in the dark to reduce loss of signal from the fluorophore over time.

Notes: The immunohistochemistry was performed using free floating sections, that were agitated, which aids antibody penetration, but can also be performed on slide mounted sections, where the incubation solutions are applied to cover the tissue sections and washing is performed using e.g. coplin jars. Note that primary antibodies will vary in capacity to penetrate the tissue and thus tissue thickness will impact the final result. The concentration of detergents such as Triton X-100 can be increased to aid penetration in some cases.

6. **Optional: labeling with DAPI-** DAPI labeling can be included as a last step if the analysis requires identification of all nuclei. This will come after step 3c or 4b depending if the antibodies used require a streptavidin step or not.
 - a. After step 3c or 4b, incubate with DAPI (1:5000) in PBS for 5 min at RT
 - b. 3×5 min washes in PBS
 - c. Slide mounting as described

Image capturing of 3D tissue volumes by confocal microscopy

⌚ **Timing:** Will be dependent on the volume of the region of interest (ROI) and the performance of the microscope and associated software, including specific settings that determine the quality of the final image, such as laser scanning speed, resolution and averaging. In the example described here ([Figure 1](#)), the entire striatal volume in a single 40 μm section of rat brain was acquired in 1.5 h.

High quality images are required to facilitate accurate identification of specific, antibody-labeled, fluorescent signal using automated image analysis packages, such as the Imaris software described below. We used a Zeiss confocal microscope (LMS 780, Carl Zeiss AG, Oberkochen, Germany) running Zen black software (2012 SP5). Images were acquired using a 20× air objective (NA of 0.8), at a resolution of 1024×1024, acquisition resolution of 2.4 pixels/μm and using a laser speed of 0.64 μs per pixel. Anatomical landmarks were used to define the striatum as the region of interest (ROI) and the tiling function was used to capture consecutive fields of view spanning the entire ROI. Optical sections were captured in series throughout the Z-axis (tissue depth) for each field of view. Post-processing using the 'stitch' function was used to produce a composite 3-dimensional volume of the entire striatum.

7. Adjust channel-specific settings such as laser power and gain to optimize signal strength and specificity for each fluorophore.
8. Define the ROI on the X and Y planes using microscope-specific software, typically defined as a 'tiling' feature.
9. Define the Z range and the optical section interval. Note that the absolute Z position defining the upper and lower limits of the tissue may vary across large X-Y areas. This may be due to the microscope stage not being completely flat or slight variations in tissue properties. This can be addressed by defining a Z range that oversamples to compensate. This can be achieved by defining upper and lower Z limits at diagonally opposite points of the ROI and selecting final Z values that will include the entire tissue volume within the ROI.

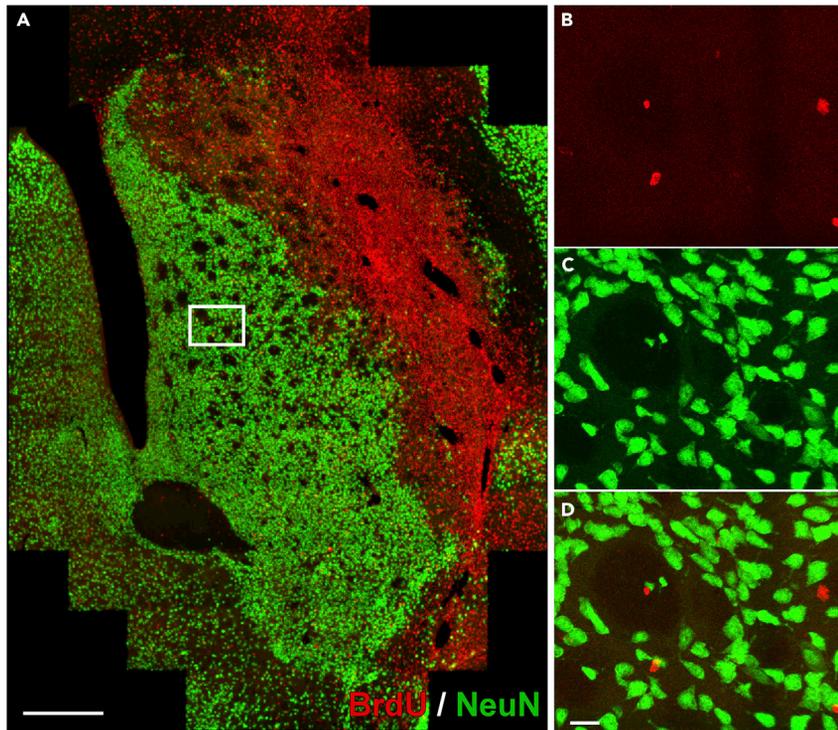


Figure 1. Image capturing of 3D tissue volumes by confocal microscopy

(A) Immunohistochemistry for BrdU (red) and NeuN (green) in the rat striatum 3 weeks after ischemic injury. The image was acquired as single fields of view using a 20× objective and the tiling function used to assemble the final montaged image of the entire striatum.

(B–D) The boxed area is shown at greater magnification and illustrates well-defined cell structures suitable for analysis using automated imaging software.

Scale bars: (A) 500 μm and (B–D) 50 μm .

The section interval should be set using the Nyquist principle, for the present example we set the interval as 2*pixel size (with a pixel size of 0.42 μm , the section interval was set at <0.84 μm).

- Following image acquisition, a post-processing ‘stitch’ function should be performed in order to generate a composite 3-dimensional ROI that can be handled as a single tissue volume in the image analysis software.

Software-based approaches for automated quantitation

Software packages for automated image analysis are increasingly being used for a variety of applications in biology, ranging from generation of images for robust illustration of cellular and sub-cellular morphology to efficient, automated quantitation of large data sets, including cell counting. Commonly available platforms include popular open-source software such as Image J/FIJI and Cell Profiler as well as commercial platforms such as Imaris and Amira. These software packages employ morphometric analysis tools, whereby fluorescent signals in tissue volumes are rendered in binary as either ‘spots’ or ‘surfaces’. Depending on the parameters used, these compartments can define and quantify a wide variety of morphological features ranging from whole cell bodies to dendrites and subcellular structures. Here we describe a workflow using Imaris 9.0 (Bitplane) for the automated identification and quantitation of new, BrdU+ cells generated in the striatum following ischemic injury as well as neuronal identity based on co-labeling for NeuN. This application thus demonstrates automated counting for large numbers of cells as well as high-throughput screening for rare events

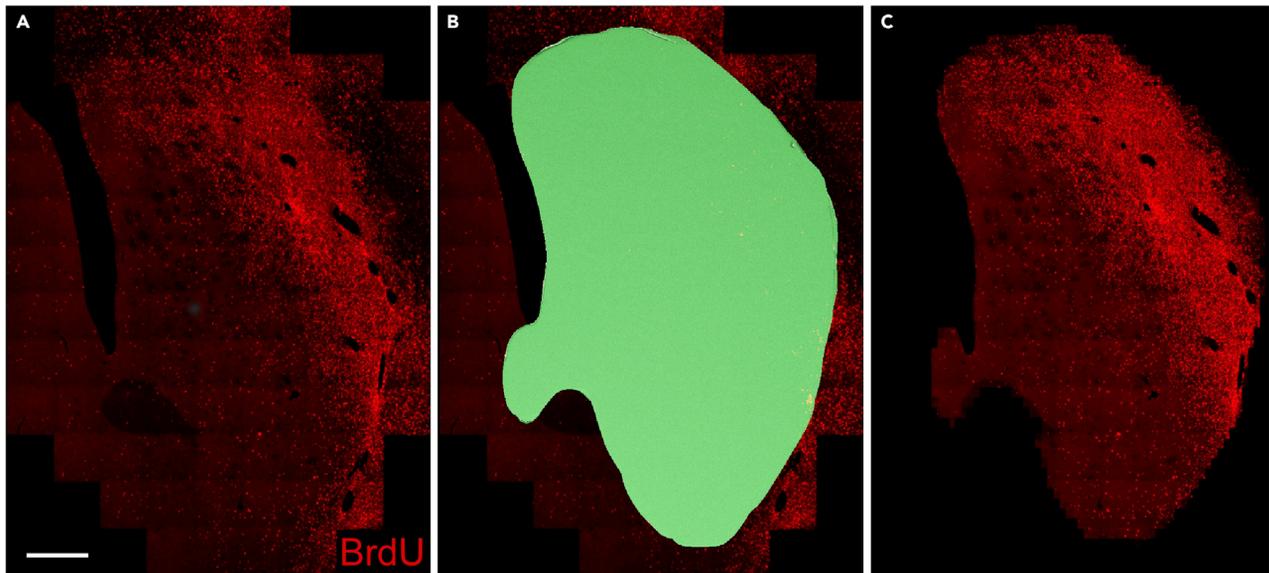


Figure 2. Surface creation for a region of interest

(A) The original BrdU signal in a 3D tissue volume before ROI selection.

(B) The surface created from the ROI selection is shown in green.

(C) A new surface channel is generated containing only information within the ROI. Scale bar: 500 μm .

Creation of a region of interest and volume measurement using the surface function

⌚ Timing: 20 min

The ROI creation is a completely flexible and user-defined function and can be performed as a manual delineation, for example based on anatomical structure, such as the striatum illustrated here (Figures 2A–2C). Immunohistochemical labeling can also be used for more refined anatomical delineation at the molecular level. Only a single channel needs to be selected – we used the BrdU channel in this example.

11. Select 'Surface' function in the software.
12. Select 'Skip automatic detection, edit manually' for more accurate delineation of the ROI.
13. Select pencil tool and draw the contour of your ROI. We used every second Z level to define the ROI on the X-Y plane in order to accommodate changes in the ROI throughout the depth of the image volume. A 3D surface volume was then created by the software (Figure 2B).
14. Use the 'Mask Selection' function to apply the surface to your desired channel – BrdU is shown in the example here (Figure 2). A duplicated channel is then created excluding any signal outside the surface defining the ROI (Figure 2C).
15. Specifications including the ROI volume can be found in the Statistic selection, under the detailed tab for this surface.

Cell counting using the spot tracer tool (spot creation Wizard)

⌚ Timing: 5–10 min

The spot creation tool can be used to identify and count cells within the ROI. Here we describe examples for BrdU (Figures 3A–3D) and NeuN (Figures 3E–3H).

16. Select the add new Spot to begin to define the parameters for automated cell detection. Part of the workflow allows for the selection of a small sampling square within the ROI, 'Segment only a

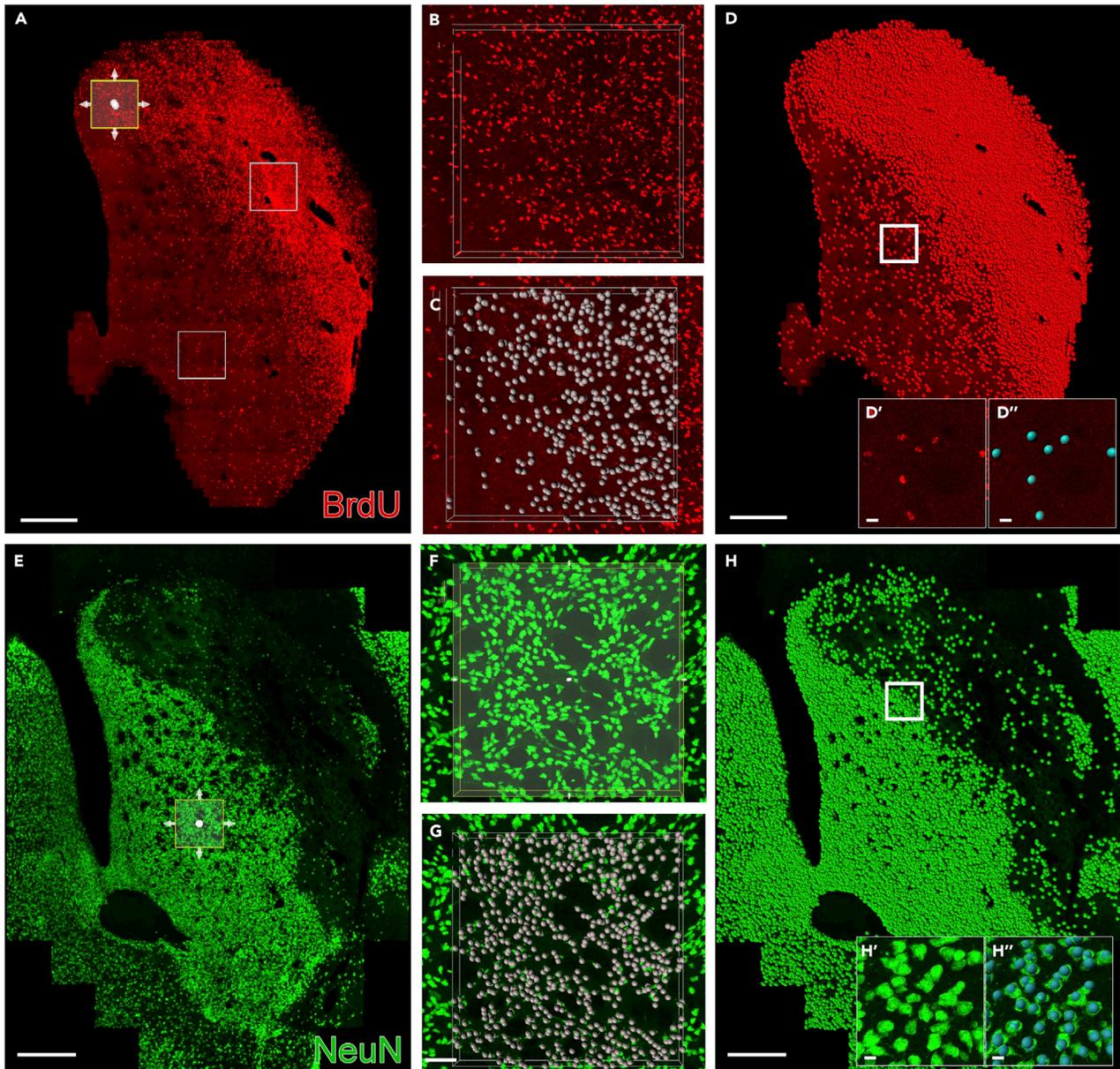


Figure 3. Cell counting – Automatic spot creation

(A and E) represents the striatum with the 3 sampling squares for BrdU and the single sampling square for the NeuN channel respectively. (B and F) show a closer view of the region of interests.

(C and G) are showing the cells selected as positive labeling based on their intensity and marked by the gray spots.

(D and H) represent the BrdU and NeuN cells selected by the spot tracer in the overall striatum respectively. The boxed area, (D', D'', H', and H''), respectively represent a higher magnification.

Scale bars: (A, D, E, and H) 500 μm ; (B, C, F, and G) 50 μm ; (D', D'', H', and H'') 10 μm .

region of interest', in order to preview the results of automated selection against actual cells defined by the histological label.

17. Position the sampling square in a representative portion of the image. In cases where the signal is not uniform, for example for detection of BrdU in the example presented here, we recommend the creation of 3 sampling squares in representative areas of the ROI (Figure 3A). If the marker is uniformly represented in your ROI. e.g., as shown for NeuN, a single sampling square may be sufficient (Figure 3E).

18. Select the source channel for automated cell detection.
19. Define the minimum diameter of the cell population of interest. This will avoid automated identification of artefacts and noise corresponding to objects below this size. In the example described here, NeuN is a relatively homogeneous labeling of nuclei with a minimum diameter defined as 6 μm . BrdU labeling is nuclear but punctate in distribution and a smaller size of 4.5 μm was appropriate to ensure capturing of all cells. Background subtraction option is recommended to remove the background.
20. Spot detection thresholding step using the Quality filter. The threshold can be allocated automatically according to default setting or set manually. A live preview feature within the sampling square allows the user to verify automated cell detection annotated against the histological signal in order to select the appropriate threshold (Figures 3B, 3C, 3F, and 3G). This can be useful when the signal quality varies across samples. In the example shown here, we found that for NeuN, the signal intensity was consistent between samples and a fixed threshold could be used across all brain sections. However, for the BrdU signal, signal quality varied across brain sections and the threshold had to be adjusted accordingly (while blinded to sample identity).
21. The final step applies the parameters selected for the sampling square (if used) to the entire ROI and the number of spots/cells can be found under the Overall tab. A new channel is generated with the annotated representation of cells.
22. Repeat all steps for additional channels where required.

Note: Settings should be defined by the user according to the material and will vary widely according to the distribution of the labelled proteins and the quality of the acquired image. As a general rule we found that investing more time in quality of the image acquisition translated to more efficient and accurate automated cell detection using morphometric software.

Co-localization using the spot co-localization tool

⌚ Timing: 5–10 min

Example of BrdU/NeuN co-localization (double labeling; Figure 4). We found an automated approach to be particularly useful for detecting low frequency co-localization events in large tissue volumes containing large numbers of cells where it would be prohibitive to manually inspect all cells for co-localization. Co-localization is a post-processing function on data acquired from the spot creation sequence on >1 channel.

23. Run spot detection as described above on the channels of interest.
24. From the 'Tools' tab select the 'Colocalize Spot Xtension'.
25. Select the 2 spot datasets of interest.
26. Select a threshold that corresponds to the distance above which the 2 spots would not be considered co-localized. This should be user defined based on the cellular distribution of the markers used - e.g., nuclear or cytoplasmic. For the 2 nuclear markers used in the present example we set the threshold as 2 μm . This should be applied across all samples.
27. A new spot dataset is created and annotated according to the co-localized cell population.
28. The number of co-localized cells can be found under the Statistic tab of the newly created 'coloc-cell' tab.
29. The degree of verification of co-localization will be user-specific and depend on the application. For our own work (Ermine et al 2020) we used this automated co-localization approach as a screening step to identify low-frequency events in large tissue volumes (striatum) but manually verified co-localization of each event by confocal microscopy. Alternatively, where there are a large number of events identified by automated software, it is recommended to manually verify a percentage of the identified cells in order to attribute a margin of error to the software generated value. In our study we found that this co-localization tool was ~95% correct. An example of

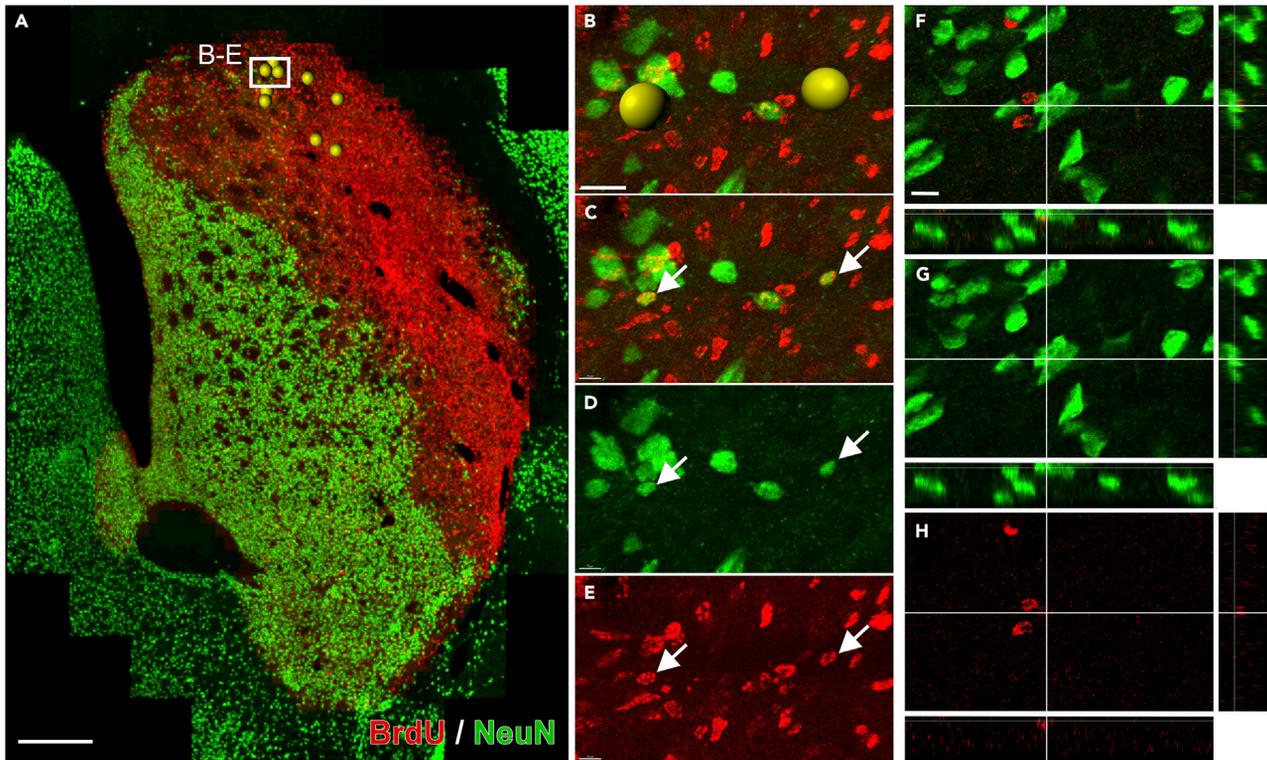


Figure 4. Co-localization using the spot co-localization tool

(A) Cells identified by the co-localization function are annotated as yellow spheres in the ROI (striatum).

(B–E) Enlarged view of the boxed areas show the identified cells as well as the immunohistochemical signal for the two channels used to detect both cell types using the 'Colocalize Spot Xtension'.

(F–H) Enlarged view of a false positive co-localization detected by this tool.

Scale bars: (A) 500 μ m and (B–H) 10 μ m.

correct double positive cells for BrdU/NeuN is presented in [Figures 4B–4E](#), as well as an example of false positive double labeling in [Figures 4F–4H](#).

Note: The ROI creation is a user defined step based on the area required for quantification. The co-localisation analysis can be done on multiple regions, for example the striatum can be divided into dorsal and ventral, however, unique ROIs need to be pre-defined prior to the cell counting step, as each ROI needs to be treated separately for spot creation and cell counting. The colocalization step for each region can then be processed as described.

EXPECTED OUTCOMES

This protocol will generate immunohistological data for the identification and characterization of proliferative cells in the rodent brain generated during a defined period. This type of data remains a frontline approach for research that seeks to understand the proliferative response to injury and particularly the generation of new neurons as part of a 'self-repair' process. The protocol will also generate quantitative data using an automated, software-based approach in order to determine the number and types of cells generated in large 3D tissue volumes. A particularly useful outcome for this type of work is the high through-put interrogation of very large numbers of birth-dated cells as a screening tool to identify rare events such as neurogenesis. In addition to the quantitative data, this protocol will generate annotated representations showing the spatial distribution of defined cell types across large, 3-dimensional tissue volumes. These images are useful for illustrative purposes as part of various data presentation formats including publication.

LIMITATIONS

The accuracy of automated cell counting will ultimately be determined by the quality of the image obtained from the immunohistochemical labeling of the cells of interest. The suitability of a morphometric approach to delineating individual cells will become limited under certain conditions including:

- High degree of background signal leading to poor signal-to-noise ratio.
- High density of cells in images acquired at low (<20×) magnification.
- Non-uniform cellular distribution of the target protein. E.g. organelle-specific or punctuate patterns.

Under circumstances where these limitations cannot be refined, the advantages of automated cell counting may be offset by a low degree of accuracy and thus conventional stereological or manual counting approaches will be more appropriate.

TROUBLESHOOTING

Describe any problems that might arise from running the protocol and provide possible solutions. Make sure to flag them in the corresponding steps of the protocol and refer to this section. If applicable, this section can also be the space to include information about alternates, i.e., what reagents and/or equipment has some flexibility and what cannot be changed.

Problem 1

Image doesn't open in Imaris software (Software-based approaches for automated quantitation).

Potential solution 1

2-dimensional images, e.g., acquired only on X and Y axes, cannot be processed for cell identification by most morphometric software platforms, including Imaris. The image needs to be generated using a microscope that can acquire information on as a 3-dimensional tissue volume on X, Y and Z axes. Other platforms such as Image J can perform basic cell counting functions on 2-dimensional images using optical density rather than morphometry. In addition, if the tissue volume is a montage composed of individual tiles, the image needs to be pre-processed before opening in Imaris, using a Stitch function.

Problem 2

Marker intensity is different throughout the sample and some cells are not included in the spot counting (Cell counting using the spot tracer tool, steps 16–22).

Potential solution 2

In some cases this can be addressed by using the surface tool function in place of the spot tool. Where the surface creation can't be validated during the live preview as a more accurate method of cell detection, the number of surfaces can be used to determine cell number. However, care should be taken where there is a high cell density in the sample to assess overlapping surfaces where 2 cells might be detected as 1 surface. In this case the total surface volume can be used to compare the evolution of cell number between 2 groups.

Problem 3

Cells have different shapes and the spot function is allocating 2 spots for 1 cell or not counting all the cells if the minimum diameter for spot detection is increased (steps 19–21 in Cell counting using the spot tracer tool)

Potential solution 3

There are 2 options to address this issue:

Option 1: Increase the minimum diameter for spot detection (for example we defined the spot diameter for BrdU as 4.5 μm to accommodate the punctuate labeling, but as 6 μm for NeuN).

Option 2: The spot function in Imaris can be directed to account for difference in shape of the marker of interest, by using the 'Region Growing' option. This function will increase the "region" of spot detection, by defining a threshold based either on the absolute intensity of the signal or on local contrast. The threshold can be set automatically or adjusted manually so each cell can be represented by a single spot.

Problem 4

The signal to noise ratio is too high and cannot be improved during the imaging (step 7 in Image capturing of 3D tissue volumes by confocal microscopy)

Potential solution 4

In this case, it is possible to post-process the image to clean up the background as much as possible before opening the image in the analysis software. This step is called deconvolution. Deconvolution restores the image and generally improves an image through mathematical operations. Different software are available including DeconvolutionLab and PSF Generator, which are open source software.

Problem 5

The spot threshold cannot be set accurately to match the marker being investigated (step 20 in Cell counting using the spot tracer tool).

Potential solution 5

Different filters can be used for the spot thresholding. In our example, applying the Quality filter, Imaris chooses a default threshold setting based on the data from the image. However, if the threshold is not accurate and the spots created are not the best match, a new filter type can be chosen for the threshold setting to best outline the marker of interest. This needs to be decided based on the image analyzed by the experimenter.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Charlotte Ermine (charlotte.ermine@florey.edu.au)

Materials availability

This study did not generate new unique reagents.

Data and code availability

Original data for BrdU and NeuN positive cells and co-localization following focal ischemic stroke in rat is available in the original paper [Ermine et al. 2020 \(https://doi.org/10.1016/j.isci.2020.101175\)](https://doi.org/10.1016/j.isci.2020.101175).

ACKNOWLEDGMENTS

The authors thank Mong Tien for expert technical assistance in the tissue preparation and immunohistochemical procedures. This work was supported by NHMRC project grant 1130734. We thank the Microscopy Facility at the Florey Institute of Neurosciences and Mental Health for their support. The Florey Institute of Neuroscience and Mental Health acknowledges the strong support from the Victorian government and in particular the funding from the Operational Infrastructure Support Grant.

AUTHOR CONTRIBUTIONS

C.M.E. contributed to the experimental procedures and analysis. C.M.E. and L.H.T. contributed to the conception and design of the study. C.M.E. wrote the manuscript; L.H.T. and C.A.C. edited approved the final version. L.H.T. sourced funding for the work.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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