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Title: Whole Brain Single Cell Imaging and Analysis of Intact Neonatal Mouse Brains Using MRI, Tissue Clearing, and Light-Sheet Microscopy

Authors and Affiliations:

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Author Questionnaire

1. Microscopy: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No.**

If **Yes**, can you record movies/images using your own microscope camera?
N/A

If your protocol involves microscopy but you are not able to record movies/images with your microscope camera, JoVE will need to use our scope kit.

If your microscope does not have a camera port, the scope kit will be attached to one of the eyepieces and **you will have to perform the procedure using one eye**.

For the video, we will acquire images using the LaVision Ultramicroscope II. Everything will be recorded on the computer screen so there will be no need to attach a scope kit to the eyepiece.

2. Software: Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes**

If **Yes**, we will need you to record using screen recording software.

We recommend using the screen capture program OBS. JoVE's tutorial for using OBS Studio is provided at this link: https://www.jove.com/v/5848/screen-capture-instructions-for-authors?status=a7854k

As these files are necessary for finalizing your script, please upload all screen captured video files to your project page as soon as possible.

3. Filming location: Will the filming need to take place in multiple locations? **Yes**

If **Yes**, how far apart are the locations? **10 minute walking distance**

To ensure that your script can be filmed in one day, the Protocol section is restricted to **55 shots** (shots are the 3-digit numbers like 2.1.1, 2.1.2…etc)

Current Protocol Length

Number of Steps: 22

Number of Shots: 37

Introduction

1. **Introductory Interview Statements**

Videographer: Obtain headshots for all authors.

Your answers to these questions will become author interview statements, which authors will memorize and then deliver on camera.

- Enter the **full name** of the author who will deliver the statement.
- If possible, each author should deliver **no more than two statements**.
- Fill out **both** required statements, **one** optional statement may also be selected.
- Answer in full sentences, in a style suitable for being spoken aloud.
- Limit the length of each statement to **30 words or fewer**.
- Answers will be edited for length, clarity, and consistency with journal style guidelines.

REQUIRED: Why is your protocol significant? *OR* What key questions can this method help answer?

1.1. Jason Stein**:** With more than 100 million cells in the mouse brain and data sizes of whole-brain cellular resolution images approaching the terabyte scale, advanced image analysis tools are needed to accurately quantify cells and assess differences in anatomy. To address these issues, we implemented a group of image analysis tools called NuMorph to perform cell-type quantification within the mouse cortex after tissue clearing and imaging by a conventional light-sheet microscope.

REQUIRED: What is the main advantage of this technique?

1.2. Jason Stein**:** Our computational pipeline can preprocess images and quantify nuclei within the mouse cortex while maintaining a reasonable compromise between cell detection accuracy, imaging time, and computational resources. Furthermore, NuMorph can detect cell types by nuclear protein marker expression in other channels. This allows quantitative comparisons of brain structure at cellular resolution across genotype groups in a wild type/knockout design.

OPTIONAL: Do the implications of this technique extend toward the therapy (or diagnosis) of a particular disease, disability, or challenge? How so?

1.3. N/A

OPTIONAL: Are there any specific areas of research that this method could provide insight into? *OR* Can this method be applied to any other systems?

1.4. N/A

OPTIONAL: How would you expect an individual who has never performed this technique to struggle? Do you have any advice to offer to somebody who is trying this technique for the first time?

1.5. N/A

Introduction of Demonstrator on Camera

Complete this statement **ONLY** if any of the individuals who will be demonstrating the procedure on camera will not be delivering an Introductory Interview Statement.

1.6. Jason Stein**:** Demonstrating the procedure will be Felix Kyere and Ian Curtin, graduate students from my laboratory leading this project.

1.6.1. INTERVIEW: Author saying the above.

1.6.2. The named demonstrator(s) looks up from the workbench or desk or microscope and acknowledges the camera.

Ethics Title Card

1.7. Procedures involving animal subjects have been approved by the Institutional Animal Care and Use Committee (IACUC) at University of North Carolina at Chapel Hill.

Protocol

Please review this section to make sure that it accurately describes your protocol. Use **Track Changes** when making edits or revisions.

- The one-digit numbers represent **sections** of the video. The text will appear onscreen.
- The two-digit numbers (e.g. 2.1., 2.2.) represent **steps** of your protocol. The text will be recorded by a professional voiceover talent.
- The three-digit numbers (e.g. 2.1.1., 2.2.2.) represent the **shots** that our videographer will capture at your lab.
- To ensure that your protocol can be **filmed in one day**, the protocol is restricted to **25 steps** and/or **55 shots**.

Please use this draft script to help you prepare for filming day.

- Filming should take no more than 10 minutes per step. If a step will take more than 10 minutes, prepare the product from that step in advance.

2. Light-Sheet Imaging

2.1. To begin, carefully mount the sample in the correct sample size holder such that the sample is oriented with the z dimension no more than 5.2 millimeters in-depth due to the rated working distance of the Ultramicroscope II *(two)* microscope **[1].**

2.1.1. WIDE: Talent placing the sample in the holder.

2.2. Then insert the holder into the sample cradle such that the screw of the holder is at 45 degrees angle to the supports of the cradle **[1].** Next, place the cradle into a position such that the sample is oriented perpendicular to the light path **[2].**

2.2.1. Talent inserting the holder into the sample cradle.

2.2.2. Talent placing the cradle.

2.3. Afterward, set the zoom body on the microscope to 4x *(four-x)* magnification or higher yielding 0.75 micrometers per pixel **[1].**

2.3.1. Talent turning the zoom dial.

2.4. In the ImSpectorPro software, select a single light-sheet with an NA value of approximately 0.108 **[1]**. To ensure axial resolution is maintained along the width of the image, select horizontal dynamic focusing and apply the recommended

number of steps depending on the laser wavelength **[2].** Authors: How would you like the JoVE's voiceover to pronounce NA? **NA = Numerical Aperture**

2.4.1. SCREEN: Single light-sheet is being selected.

2.4.2. SCREEN: Horizontal dynamic focusing and the number of steps are being selected.

Authors: Please create screen capture videos of the shots labeled as SCREEN, create a screenshot summary, and upload the files to your project page as soon as possible: https://www.jove.com/account/file-uploader?src=19549328

2.5. Then adjust the fine focus for each channel with respect to the registration channel and laser power per channel with respect to the channel properties **[1].** Next, adjust the light-sheet width to about 50 percent to ensure sheet power is distributed optimally in the y dimension for this sample size **[2].**

2.5.1. SCREEN: Fine focus and laser power are being adjusted.

2.5.2. SCREEN: Light-sheet width is being adjusted.

2.6. Afterward, set the number of tiles with respect to the size of the sample with a recommended overlap of 15 percent between tiles **[1]** and capture images for each channel sequentially for each stack at a given tile position **[2].**

2.6.1. SCREEN: Number of tiles is being set.

2.6.2. SCREEN: Images are being captured.

3. Image Processing using NuMorph

3.1. First, download and install conda environment manager for Linux and NuMorph image processing tools **[1].** Authors: How would you like the JoVE's voiceover to pronounce NuMorph ? **NuMorph = New-Morph**

3.1.1. WIDE: Talent at the computer, downloading and installing conda environment manager and NuMorph.

3.2. On the command line, run Matlab and NM_setup.m from NuMorph to download and install image analysis software packages needed for analyses. Then specify sample names, input and output directories, channel information, and light-sheet imaging parameters by editing the file NM_samples.m. Authors: How would you like the JoVE's voiceover to pronounce NM_setup.m? **NM_setup.m = N-M-setup-dot-M**

3.2.1. SCREEN: Command is being given to run Matlab and NM_setup.m.

3.2.2. SCREEN: File is being edited.

3.3. For intensity adjustment, in NMp_template, set intensity adjustment to true and use processed images as false when working with a new set of images **[1].** Next,

set save images and save samples as true **[2].** Authors: How would you like the JoVE's voiceover to pronounce NMp_template? = N-M-p-template

3.3.1. SCREEN: Intensity adjustment and use processed images are being set.

3.3.2. SCREEN: Save images and save samples are being set.

3.4. Next, set tile shading to basic to apply shading correction using the BaSiC algorithm or manual to apply tile shading correction using measurements from Ultramicroscope II at specific light-sheet widths **[1].**

3.4.1. SCREEN: Tile shading is being adjusted.

3.5. For image channel alignment, in NMp_template, set channel alignment to true and channel alignment method to either translation or elastix **[1].** Next, set stitch images, sift refinement, and load alignment parameters to true and overlap value of 0.15 to match tile overlaps during imaging **[2].**

3.5.1. SCREEN: Channel alignment and channel alignment method are being set.

3.5.2. SCREEN: Stitch images, sift refinement, load alignment parameters, and overlap value are being set.

3.6. To run preprocessing steps in Matlab, specify the sample name and set the configuration to NM_config(process,sample) **[1].** Then run any of the preprocessing steps by specifying NM_process(config,stage) while specifying the stage using intensity, align or stitch and check the output directory for output files for each of the stages **[2].** Authors: How would you like the JoVE's voiceover to pronounce NM_config(process,sample)? **NM_config(process,sample) = N-M-config-process-sample**

3.6.1. SCREEN: Sample name and configuration are being set.

3.6.2. SCREEN: NM_ process(config,stage) is being specified, and the output directory is being checked.

4. Image Analysis

4.1. Start with a 3D atlas image and an associated annotation image that assigns each voxel to a particular structure **[1].** Align both the atlas image and annotation file to ensure they match correctly in the right orientation **[2]**.

4.1.1. SCREEN: 3D atlas image software is being opened.

4.1.2. SCREEN: Atlas image and annotation file are being aligned.

4.2. After alignment, process the files in NuMorph to specify the inputs as described in the manuscript by executing the command **[1].**

4.2.1. SCREEN: Command is being given.

Video Editor: Please emphasize the following command on the screen:

"munge_atlas(atlas_file, annotation_file, resolution, orientation, hemisphere)".

4.3. In NMa_template, set resample images to true and resample resolution to match the atlas **[1].** Then specify the channel number to be resampled using resample channels **[2].**

4.3.1. SCREEN: Resample images and resolution are being set.

4.3.2. SCREEN: Channel number is being specified.

4.4. Afterward, set register images to true, specify the atlas file to match the file in the atlas directory, and set registration parameters as default. Then set save registered images to true.

4.4.1. SCREEN: Register images is being set, atlas file is being specified, and registration parameter is being set.

4.4.2. SCREEN: Registered image is being saved.

4.5. For nuclei detection, cell counting, and classification, set both count nuclei and classify cells as true **[1]**. Then set the count method to 3dunet and min_intensity to define a minimum intensity threshold for detected objects **[2]**. Authors: How would you like the JoVE's voiceover to pronounce 3dunet and min_intensity? **3dunet = three-D-you-net. Min_intensity = min-intensity**

4.5.1. SCREEN: Count nuclei and classify cells are being set.

4.5.2. SCREEN: Count method and min_intensity are being set.

4.6. Next, set classify_method to either threshold which is based on an unsupervised fluorescence intensity at centroid positions, or svm, which models a supervised linear Support Vector Machine classifier **[1].** Authors: How would you like the JoVE's voiceover to pronounce classify_method? **Classify_method = classify-method**

4.6.1. SCREEN: Classify_method is being set.

4.7. To perform analysis steps in Matlab, specify the sample name and set the configuration to NM_config(analyze,sample) **[1].** Next, run any of the analysis steps by specifying NM_analyze(config,stage) while specifying the stage using

resample, register, count, or classify and check the output directory for output files for each of the stages **[2].**

4.7.1. SCREEN: Sample name and config are being set.

4.7.2. SCREEN: NM_analyze(config,stage) is being specified, and the output directory is being checked.

4.8. In NMe_template, set update to true and compare_structures_by to either index **[1].** Then set the template_file and structure_table, which specifies all possible structure indexes and structures to evaluate while specifying cell counting and cell-type classification **[2].**

4.8.1. SCREEN: Update and compare_structures_by are being set.

4.8.2. SCREEN: template_file, structure_table, cell count, and cell type are being specified.

Protocol Script Questions

Authors: Please use the **step and shot numbers from the script above** (not step numbers from the manuscript) when answering the questions below. Do not include steps that will be screen-captured and do not list entire sections.

1) Which steps from the protocol are the most important for viewers to see? Please list 4 to 6 individual steps (steps are indicated with the 2-digit numbers, like 2.1, 2.2, etc.).

Click here to list 4 to 6 individual steps, using the step numbers from the protocol section of the video script.

Steps 2.4.2; 3.2.1; 4.2.1; 4.7.2

2) If a dissection or stereo microscope is required for your protocol, please list all shots that will be visualized using the microscope (shots are indicated with the 3-digit numbers, like 2.1.1, 2.1.2, etc.).

N/A

Results

Please review this section to make sure that it accurately reflects your findings.

- Use **Track Changes** when making edits or revisions.
- If you would like the video to include different results, please revise this section.
- When revising, please keep the length of the voiceover below 200 words. Current word count: 219. (Voiceover is the text that follows the two-digit numbers)
- Please note that the video cannot include voiceover without an accompanying visual.

5. Results: Quantification of Nuclear Markers Within Mouse Brain After Imaging on a Light-Sheet Microscope Using NuMorph

5.1. Tissue clearing using the iDISCO+ protocol and neuronal layer specific nuclei markers resulted in clearly defined cell groups of upper and lower layer neurons in the isocortex **[1].** Authors: How would you like JoVE's voiceover to pronounce iDISCO+? **iDISCO+ = eye-disco-plus**

5.2.1. LAB MEDIA: FIGURE 3.

5.2. Cell counting using NuMorph was dependent on successful preprocessing steps involving intensity adjustment, channel alignment and stitching **[1].**

5.3.1. LAB MEDIA: FIGURE 4A.

5.3. However, errors in preprocessing steps could result in improper stitching leading to improper alignment and stitching **[1]** and thus, result in images with in focus and out of focus pattern **[2].**

5.4.1. LAB MEDIA: FIGURE 4B.

5.4.2. LAB MEDIA: FIGURE 4C.

5.4. In order to count nuclei from specific brain regions, the stitched images will be annotated using atlas, allowing annotations to be overlaid on brain regions **[1].**

5.5.1. LAB MEDIA: FIGURE 5A.

5.6. The centroids of nuclei were detected with a trained 3D-Unet model in NuMorph with around 12 million total nuclei that were To-Pro-3$^+$ in the isocortex with about 2.6 million Brn2$^+$ and 1.6 million Ctip2$^+$ nuclei **[1].** Authors: How would you like JoVE's voiceover to pronounce To-Pro-3$^+$, Brn2$^+$, and Ctip2$^+$? **To-Pro-3 = tow-pro-three. Brn2 = brain-two. Ctip2 = see-tip-two**

5.6.1. LAB MEDIA: FIGURE 5B,C.

5.7. About 3.7 and 2.9 million To-Pro-3$^+$ total nuclei in the basal ganglia and hippocampal allocortex were detected, respectively. However, the Brn2$^+$ cells detected in these two brain regions were negligible and only about 1.5 and less than 1 million Ctip2$^+$ cells were detected each in the basal ganglia and hippocampal allocortex **[1]**.

1.1.1. LAB MEDIA: FIGURE 5D.

Conclusion

6. Conclusion Interview Statements

Below are prompts for interview statements that can be used to further emphasize the significance of your protocol.

- Answer **one** or **two** of the prompts below.
- Limit the statements to **30 words**.
- Answer the questions in full sentences; you will need to memorize and deliver the interview statements during filming.
- Indicate the **full name** of the author who will deliver each statement.

What is the most important thing to remember when attempting this procedure? Please indicate the steps (*e.g.*, 2.4., 2.5.) in the Protocol section of the script that this advice applies to.

6.1. Felix Kyere: Good segmentation is achieved when nuclei are visually distinct in the images, so make sure to do visual quality checks during acquisition. Also ensure that the conda environment is properly set up in step 3.1 to ensure no errors are encountered in the downstream analysis.

Following this procedure, what other methods can be performed? What questions would these additional methods answer?

6.2. Felix Kyere: In addition to cell counting, this pipeline allows for integration with other segmentation tools that measure cell size and shape, which can then be compared across genotype groups.

After its development, did this technique pave the way for researchers to explore new questions within a specific scientific field? If so, how?

6.3. Felix Kyere: With our pipeline, we can identify how brain anatomy changes at cellular resolution, leading to identification of cell types and brain regions important for disease risk.

Thank you for addressing our questions. We will incorporate your answers and suggestions, and send you the final script before your filming day. You will also receive detailed preparation instructions in the email accompanying the final script.