Sep 15, 2022

Optical Fractionator protocol

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dx.doi.org/10.17504/protocols.io.yxmvm2719g3p/v1

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ABSTRACT

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DOI

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PROTOCOL CITATION

Andrew Hunter 2022. Optical Fractionator protocol. **protocols.io** https://protocols.io/view/optical-fractionator-protocol-cgp8tvrw

CREATED

Sep 15, 2022

LAST MODIFIED

Sep 15, 2022

PROTOCOL INTEGER ID

70112

MATERIALS TEXT

Information sources
http://www.stereology.info/number/
http://www.stereology.info/the-optical-fractionator/
http://www.stereology.info/the-fractionator-principle/
The Stereo Investigator user guide is within software help menu.

Rules of thumb:
● You should aim to count roughly 200 cells to get a CE of <0.1 (even better if <0.05; but much less than 0.05 means that you're probably doing more work than you need to).
● You should use about 10 sections covering the entire structure of interest (therefore, you are looking to count about 20 cells per section). For each brain, the choice of the first section should be random with respect to the structure of interest; thereafter the section interval should be constant (ie. if taking every third section from serial sections, the choice of whether the first section is section 1, 2, or 3 should be random, but then you would take sections 1, 4, 7…, or 2, 5, 8…, or 3, 6, 9… respectively).
● Counting frame size should give an average of 3–4 cells counted per frame. This may appear to suggest that a grid size that gives 5–7 counting frames per section would be ideal; but in practice more counting frames per section are needed (~10), this is because many counting frames will fall at the edge of the structure and will have few or no cells to count. The CE calculation is complex, but more smaller counting frames tends to give a lower CE than fewer larger counting frames.
● The image acquisition workflow will only image one field of view per counting frame. This means that counting frame size is limited by the field of view. At 100×, 50 × 50 µm is about as large as the counting frame can be. At 40×, sizes up to 100 × 100 µm are OK.
If the counting frame is too large imaging will proceed as normal, but you may get odd behaviour in the optical fractionator workflow.
● The guard zone should take you beyond fluctuations in the slice surface. Ideally it would be at least one cell diameter (if counting cells), but for immunostained tissue a guard zone this thick is unlikely to ever be practical.
● The probe thickness would ideally be much greater than one cell diameter (if counting cells), but again for immunostained tissue this is unlikely to ever be practical.
● For immunostained tissue, top guard zone + probe thickness should be less than antibody penetration depth. Obviously this has to take precedence over the previous two rules (you can't count cells that you can't see).

Image acquisition workflow

1 Note, these instructions are for fluorescently labelled tissue, imaging one channel only. They will
be much the same if imaging in two or three channels, but will have to set up multi-channel imaging first.

2 To minimize bleaching turn the LED off as often as possible during the workflow.

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3 It is possible to place two slides on the stage, set up all the structure outlines first and then do the imaging in a single step.

4 This doesn't save much time and increases the chance of an error (which, almost always, means starting over), so these instructions are for drawing and imaging
the sections one at a time.

5 Turn on stage and LED before the computer.

6 Start 'Stereo Investigator' (select monochrome mode) and click a reference point (location irrelevant).

7 Click 'Live image' (for some reason it won't let you start the workflow until you have done this) then go to 'Acquisition>Acquire SRS Image Stack Workflow'

8 If you want to fill out subject info wait until you have entered the thickness of the first section, there appears to be a bug which blanks this information the first time you enter a section thickness

9 Enter sampling parameters, serial section information. If loading existing parameters, do this first.

10 Move to 10× lens

11 Select Low Mag Lens' -> 10×/0.25NA

12 Move stage to first section. Click 'Next Step'. Draw structure outline. Right-click and select 'close contour' when done.

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13 When you finish counting, Stereo Investigator can give you a volume estimate based on your outlines. If
you are interested in this estimate it is worth spending some time on your outlines.

14 However, the math for the optical fractionator does not use this volume estimate , so if you are not interested in this volume estimate then the outline is just used to delineate the area of interest for counting: if your stain marks cells that you are not going to be counting ideally all cells to be counted should be within the outline and all cells not to be counted should be outwith the outline.

15 Click ‘Next Step’. Draw structure outline. Right-click and select ‘close contour’ when done. When you finish
counting, Stereo Investigator can give you a volume estimate based on your outlines. If you are interested in this estimate it is worth spending some time on your outlines. However, the math for the optical fractionator does not use this volume estimate , so if you are not interested in this volume estimate then the outline is just used to delineate the area of interest for counting: if your stain marks cells that you are not going to be counting ideally all cells to be counted should be within the outline and all cells not to be counted should be outwith the outline.

16 In macro view zoom onto outline that you just drew (useful later)

17 It’s often useful to check the accuracy of your structure outline at 20×. Move to 20× lens (and change to 20× in Stereo Investigator) and focus to the top of the slice, then focus 10 to 15 um into the slice. Use ‘Go to’ in macro view or ‘joy track’ mode to move around your outline checking it. Can use ‘Edit>Select Objects’ to select outline and adjust. RIght-click and end object selection when done.

18 Click ‘Next Step’. Click within the outline just drawn

19 Click ‘Next Step’. Select Acquire Lens’ -> 100× Oil/1.3NA (or high power lens of choice)

20 Move to 100× lens (add oil). Will likely have to focus down (par-focus calibration on the 100× needs checked?)

21 This is a good chance to check exposure. Move around the STN by clicking within the

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macro view (‘Go to’ is automatically on at this point), and adjust exposure as necessary. Ensure that ‘Camera Histogram’ is adjusted to full width

22 Click ‘Next Step’. Set the counting frame size (see ‘note on counting parameters’ at the end of this
protocol)

23 Click ‘Next Step’. Set the grid size (see ‘note on counting parameters’ at the end of this protocol)

24 Click ‘Next Step’. Set the guard zone and probe thickness (see ‘note on counting parameters’ at the end of
this protocol). If using immunostained tissue, the guard zone + probe thickness should not go deeper into the section than your antibody penetration.

25 Adjust ‘mounted section thickness’ (this is probably only necessary if you intend to image the whole section thickness)

26 This is a good opportunity to measure section thickness. Section thickness is critical to the calculations for the optical fractionator, so it is worth getting this measurement as accurate as possible. Click ‘Set’ and use the section thickness tool to measure thickness at one or (preferably) more locations within your structure. The section thickness tool will keep a running average of your measurements. Note down the average thickness and also double click on the section in the ‘Serial Section Manager’ to record the mounted thickness there.

27 Select ‘Collect images for’ -> Probe & Top Guard Zone

28 Click both check boxes to collect additional images above and below (see ‘note on counting parameters’ at the end of this protocol)

29 Distance between images = 1 µm. Click ‘Next Step’

30 Fill out file names, storage folder, etc. Click ‘Next Step’

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31 Method for identifying top of section = At every 1 acquisition site. Click ‘Set Top of Section’ and follow instructions. When close to the edge of your outline it can be useful to use the macro view to guide you as to what is within and outwith the outline: set the top of the section as the first area within the outline that comes into focus
when focusing down onto the section.

32 Click ‘Next Step’. The configuration can be saved at this step if desired

33 Click ‘Next Step’. Click the start acquiring button (blue arrowhead)

34 When acquisition is complete, repeat the following steps for all sections:
Click ‘Next Slide’ (even if next section is on same slide)
- ‘Select Low Mag Lens’ -> 10×/0.25NA
- Move to 10× lens
- Move stage to next section (the first time you do this you may need to click ‘Live Image’ and then go into ‘Joy Track’ mode; on subsequent repeats this will happen automatically).
- Click ‘Next Step’
- Draw structure outline
- In macro view zoom onto outline
- Edit outline at 20× if you want to.
- Click ‘Next Step’
- Click within the outline just drawn
- Click ‘Next Step’
- ‘Select Acquire Lens’ -> 100× Oil/1.3NA
- Move to 100× lens (add oil).
- Move around the structure by clicking within the macro view and adjust exposure as necessary.
- Click ‘Next Step’
- Click ‘Next Step’
- Click ‘Next Step’
- Set ‘mounted section thickness’ as before
- Click ‘Next Step’
- Click ‘Next Step’
- Click ‘Set Top of Section’ and follow instructions.
- Click ‘Next Step’
- Click ‘Next Step’
- Click the start acquiring button (blue arrowhead)

Counting workflow

35 If you have just completed imaging, click the ‘new file’ button; I don’t think that this should be

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necessary but I've had some problems if a data file is already loaded before I start the optical fractionator workflow. These instructions are for a single marker, but you can count multiple markers simultaneously with this workflow.

36 Select 'Probes>Optical Fractionator Workflow' and click 'Load subject data from existing file' to open the .dat file that you made in the imaging workflow.

37 Click the '+' next to 'Section 1' in the Regions of Interest list (left of the screen)

38 Click the '+' next to 'User Line 1' (or whatever line you used to draw your outlines)

39 Click on 'Acquired Stacks'. Click the 'Start Counting' button (blue arrowhead).

40 Focus with 'page up' and 'page down' keys to top of section & click 'OK'. Click 'Do Not Measure'

41 At left of screen choose a marker (I like marker 10)

42 Click zoom out in macro view and then zoom in on to your outline (this helps when a counting frame falls on the edge of an outline to determine what is inside and outside of the outline)

43 Focus down through tissue clicking on cells as they come into focus, but only if they do so within the probe thickness (the counting frame will be yellow above and below counting depth), and only if they are within the outline and within the counting frame. The counting frame includes all cells that touch the green lines, but does not include any that touch the red lines (see http://www.stereology.info/counting-rules/).

44 You can improve contrast if needed using the 'Image Adjustment' histogram at the top right of the screen (click inside of it to see the histogram)

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45 Continue until all sites on the section have been visited (A 'Run Completed' message box appears)

46 Select 'Acquired Stacks' from next section, and click the blue run button

47 Repeat until all sections are counted, then click 'I'm finished counting'.

Getting the data

48 Click 'Display Probe Run List' button (in the toolbar). Select sections (click first one, hold 'shift' key and click the last one). Click 'view results'. Enter section interval. Click 'Edit Mounted Thickness' and enter your mean section thickness.

49 It is important to check the coefficient of error (CE). Stereo Investigator gives a number of different CE estimates, check that they are all less than 0.1. For reporting in papers the Gunderson, m=1 estimate is probably a good one to use (there are special cases where the m=0 estimate is preferred including if the section sampling interval is greater than 8).

50 For NeuN labelled mouse STN cell nuclei (coronal sections at 70 µm) try:
- Section interval: 1 (at 70 µm there will only be ~8 sections in total)
- Grid size: 150 × 150 µm
- Counting frame size: 50 × 50 µm
- Upper guard zone thickness: 2 µm
- Probe thickness: 8 µm
- Image 3 µm above and 8 µm below probe and guard zone

For FVB mouse STN these parameters gave: count = ~11000 cells (CE = ~0.08), volume = ~0.09 mm 3 , density = ~121000 cells per mm 3 .

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