Standard Operating Procedure
Imaging RosetteArray™ Well Plates

Overview: This document summarizes the microscope settings needed to:

1) Acquire Z-stack images of immunostained tissues within RosetteArray™ plates/kits;
2) How to Use RosetteDetect™

Equipment Required:

- This SOP is written for a Nikon A1R Confocal Microscope, and it should be amended for other imaging platforms.
- Computer(s) with Image Capture Software, Internet Access, and spreadsheet processing software

Materials Required:

- Immunostained 96-well RosetteArray™ plate (DAPI (405 nm), N-Cadherin (488 nm), Pax6 (561 nm)) mounted with Glass Antifade Mountant Solution (Catalog #: P36984, ThermoFisher Scientific) and cultured using Neurosetta's Standard Operating Procedure

Protocol Overview:

1. Image Capture Settings/Parameters
2. How to Use RosetteDetect™

Protocol:

1. Image Capture Settings/Parameters

High-quality inputs generate high-quality outputs. Clients should use a confocal microscope to capture Z-stack images of their RosetteArray™ samples. Appropriate image capture settings/parameters are crucial for generating images that are amenable to RosetteDetect™ processing and analysis.

1. Using a negative control well, adjust the Z position of a 20x objective to get a resolved, digital view of a single rosette structure within a tissue of interest. Center the tissue within your frame. A violet corrected 20x objective is recommended but a normal 20x objective is sufficient to magnify your view of the tissue. You may use Perfect Focus to center your defined Z on the lumen within the tissue.
2. Set the digital frame zoom size to 2 (for forebrain tissues) or 3 (for spinal tissues), These values may be different for non-Nikon confocal microscope systems.
3. Set the digital frame resolution to 512x512 pixels.

4. Set the pinhole size to 1.5 – 2. This pinhole size may differ depending on the type of confocal microscope system you work with.
5. If you are using a resonance-like mode of acquisition, you may need to apply line averaging to your capture settings to get a resolved view of cell nuclei and N-Cadherin expression. For clients working with Nikon Confocal Microscope systems, I recommend 8x Line Averaging.
6. Apply laser power and gain (photomultiplier tube sensitivity) values to your 405 nm, 488 nm, and 561 nm – or comparable PMT/channels-to produce a multi-signal fluorescent image without excessive pixel oversaturation.

NOTE: Users need to centralize tissues within the X and Y bounds of the capture frame (see Figure 1).

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7. Generate a Z-stack image. The Z-stack image should feature 5 separate slices that span a Z range of 8-10 µms. For Forebrain tissues, the step height of the slices should be 2.5 µms. For spinal tissues, the step height of the slices may be 2-2.5 µms. Users should program their Z-stack acquisition tool (e.g., symmetric mode on Nikon A1R confocal microscopes) so that the third/center slice features the lumen of the rosette structure. The second and fourth adjacent slices may also feature portions of the rosette structure. RosetteDetect™ supported file formats include: TIFF, ND2 (Nikon), LIF (Leica), OIB (Olympus), and CZI (Zeiss).

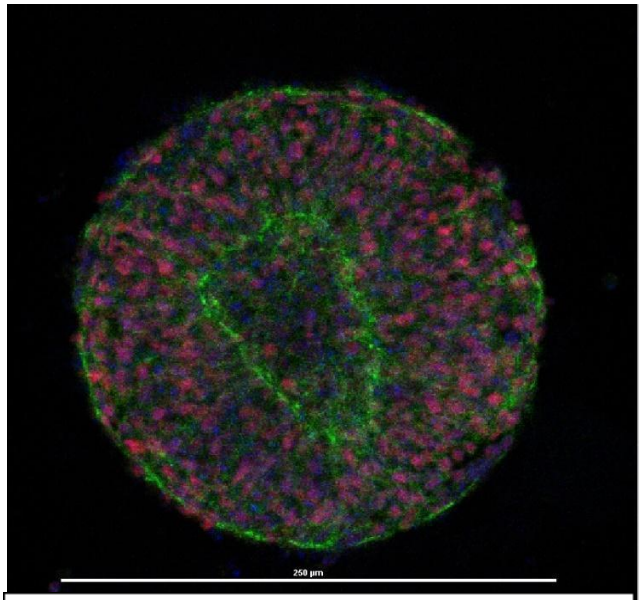

Figure 1: A 250-µm diameter forebrain tissue featuring a single neural rosette structure was captured with a violet-corrected, 20X objective (third slice). The tissue is centered in the capture frame and does not touch the boundaries of the image frame. N-Cadherin (green), Pax6 (red), Cell Nuclei (blue). Scale bar = 250 µms.

2. How to Use RosetteDetect™

Neurosetta's RosetteDetect™ is a web-based image analysis software designed to provide valuable insights into your RosetteArray™ images. After imaging RosetteArray™ plates using recommended procedures, simply upload your images into the RosetteDetect™ portal and our AI-powered software will provide a tabulated readout of the relative cell count (nucleic acid stain), neural induction (Pax6 stain), and rosette formation and morphology (N-cadherin stain) for each micropatterned tissue. For more detailed instructions see Neurosetta's Standard Operating Procedure.