Standard Operating Procedure: Imaging RosetteArray® Well Plates and Analyzing Images Using RosetteDetect™

Overview: This document summarizes the microscope settings needed to:

1. Acquire Z-stack images of immunostained tissues within RosetteArray plates/kits;
2. Analyze Z-stack images using 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. A confocal microscope should be used to capture Z-stack images of 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. If you are working with Nikon Confocal Microscope systems, 8x Line Averaging is recommended.
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. Adjust the laser power and gain settings for the N-Cadherin channel so that a line scan tool (Figure 1) generates a pixel intensity profile with peaks (Figure 2, asterisks) that have amplitudes greater than 85% of your y-axis scale range. The pixel intensity peaks should be distinguishable from the valleys on the intensity plot.

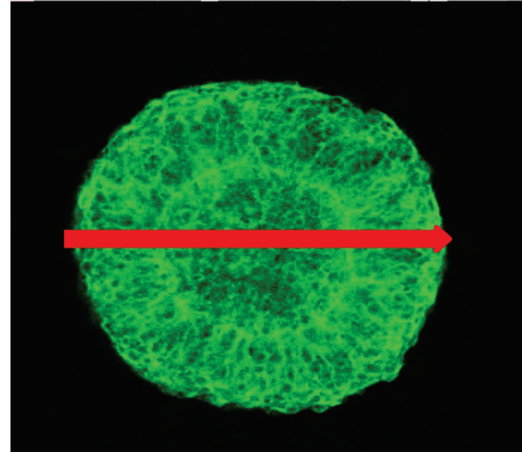

Figure 1: Line Scan tool applied across third plane of a control tissue (N-Cadherin channel).

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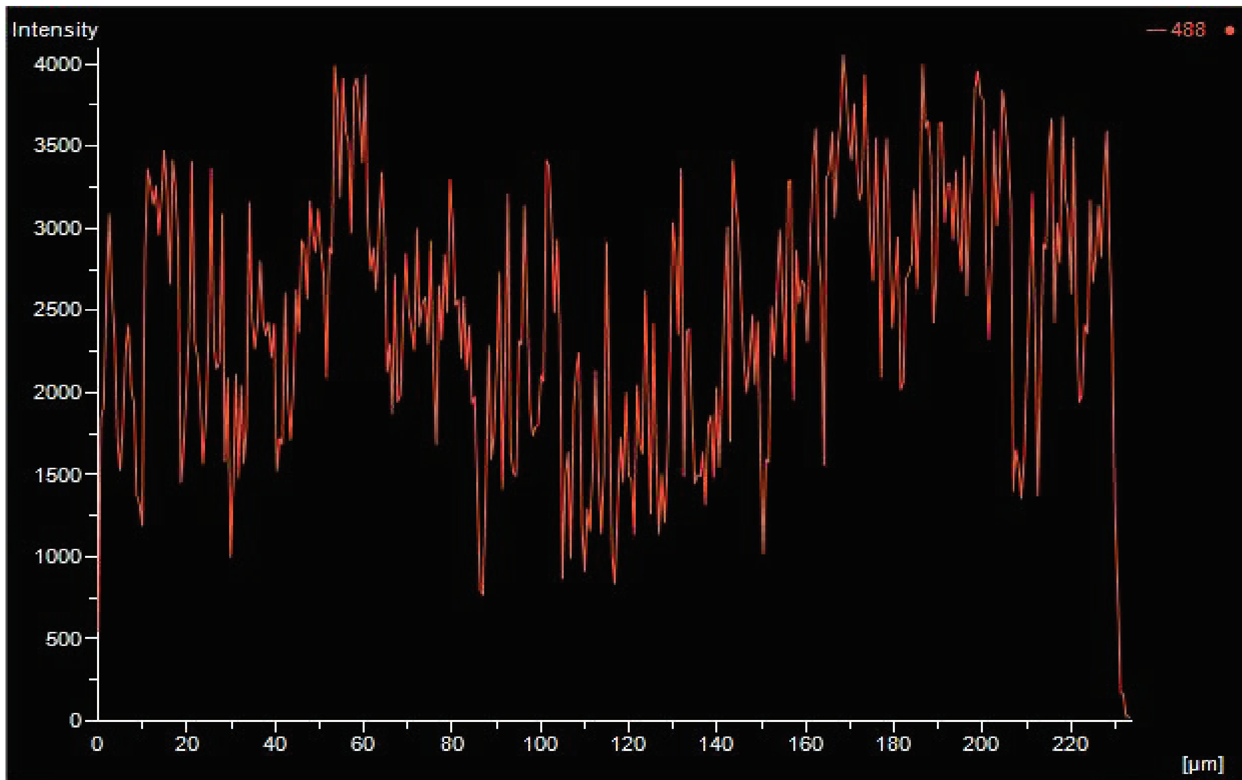

Figure 2: Pixel Intensity profile plot derived from use of line scan tool. The x-axis represents the length of the applied line scan tool (micrometer units) and the y-axis represents the pixel intensity range (0-4096 arbitrary units). The plotted line indicates the pixel intensity value along the length of the line scan tool. Asterisks denote peaks of high pixel intensity (indicative of rosette ring structure).

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

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. Z-stack acquisition tools (e.g., symmetric mode on Nikon A1R confocal microscopes) should be programmed 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).

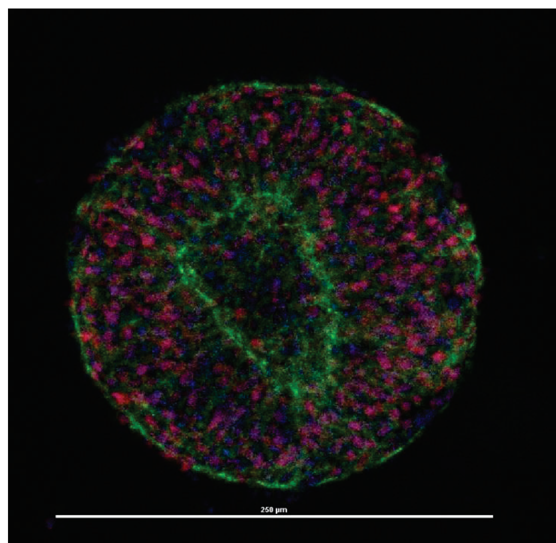

Figure 3: A 250-µm diameter forebrain tissue featuring a single neural rosette structure was captured with a violet-corrected, 20X objective (third slice). The tissue is centered in the capture frame and does not touch the boundaries of the image frame. N-Cadherin (green), Pax6 (red), Cell Nuclei (blue). 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.