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## Immunofluorescence Staining and High Content Imaging

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**We use this protocol and it's working**

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## Abstract

This protocol offers a description of immunofluorescence staining and high content imaging of iPSC induced neurons using the ImageXpress Micro Confocal High-Content Imaging System (Molecular Devices, San Jose, CA).

## Materials

**96 well optical bottom plate with a polymer base** (Fisher Scientific, 12-566-70).

**1x PBS, pH 7.4** (Fisher Scientific, 10-010-023)

**Sodium Azide 5%** (Avantor, BDH7465-2)

**Triton X-100** (Sigma, SLCD3244)

**Bovine Albumin Fraction V (7.5% solution)** (Fisher Scientific, 50-121-5315)

**DAPI (1mg/ml)** (Fisher Scientific, EN62248)

### Primary Antibodies

|  | Antibody              | Catalogue Number       | Concentration |
|--|-----------------------|------------------------|---------------|
|  | Mouse anti-Synapsin 1 | SySy, 106011           | 1:1,000       |
|  | Goat anti-tdTomato    | Thermofisher, TA150129 | 1ug/ml        |
|  | Chicken anti-MAP2     | Sigma, AB5543          | 1:5,000       |

### Secondary Antibodies

|  | Antibody                      | Catalogue Number           | Concentration |
|--|-------------------------------|----------------------------|---------------|
|  | Donkey anti mouse Alexa 488   | Fisher Scientific, A211202 | 1:1,000       |
|  | Donkey anti-chicken Alexa 647 | Fisher Scientific, EN62248 | 1:1,000       |
|  | Donkey anti-goat Alexa 568    | Fisher Scientific, A11057  | 1:1,000       |



## Immunofluorescence Staining

- 1 Wash iPSC derived neurons twice with 1X PBS and fix with 4% PFA for 00:30:00 in a 96 well optical bottom plate with a polymer base.
- 2 Store fixed neurons at 4C in 1x PBS with 0.02% sodium azide.
- 3 Aspirate sodium azide with pipette and permeabilize neurons in 150  $\mu$ L of 1x PBS with 0.5% Triton X-100 for 00:15:00 at Room temperature (RT) without shaking.
- 4 Aspirate PBST and block neurons with 300  $\mu$ L of 3% BSA and 0.1% Triton X-100 in 1x PBS for 01:00:00 at Room temperature .
- 5 Aspirate blocking buffer and stain neurons with primary antibodies mouse anti-Synapsin 1 (1:500), goat anti-tdTomato (1 $\mu$ g/ml), and chicken anti-MAP2 (1:5000), in blocking buffer by adding 100  $\mu$ L of blocking buffer for 01:30:00 at Room temperature or overnight at 4  $^{\circ}$ C .
- 6 Aspirate primary antibody solution and wash samples three times for 00:05:00 with 200  $\mu$ L 1x PBS with 0.1% Triton X-100 (0.1% PBST).
- 7 Aspirate 0.1% PBST and incubate samples with secondary antibodies in blocking buffer by adding 100  $\mu$ L for 01:00:00 at Room temperature in the dark.
- 8 Aspirate secondary antibody solution and wash neurons twice with 200  $\mu$ L of 0.1% PBST for 00:05:00 .
- 9 Aspirate 0.1% PBST and incubate neurons with DAPI (0.5 mg/ml) by adding 100  $\mu$ L at Room temperature for 00:10:00 .



10 Aspirate DAPI and add  250  $\mu\text{L}$  of 1x PBS containing 0.05% sodium azide.



11 Store plate at  4  $^{\circ}\text{C}$  until use.



## Image Acquisition

12 Allow plate to warm to RT before imaging.

13 Neurons are imaged using Molecular Devices (San Jose, CA) ImageXpress Micro Confocal High-Content Imaging System at both 20x and 40x.

14 The laser wavelengths used were DAPI, FITC, Texas Red, and Cy5.

15 Each well in the 96 well plate was imaged at 8 sites for 40x and 9 sites for 20x with 8-10 z stacks at 1  $\mu\text{m}$  step size. For the 40x objective the pixel size is 0.3438  $\mu\text{m}^2$  with a pinhole of 60  $\mu\text{m}$ , 20x objective pixel size is 0.6842  $\mu\text{m}^2$  also with a pinhole of 60  $\mu\text{m}$ .

## 20x Image Analysis

16 Acquired images are analyzed as 2D maximum projections. Analyze the first two morphometrics, mean number of branches per cell and mean length of neurite outgrowth per cell, with the built-in Neurite Outgrowth Application Module within the MetaXPress 6 software, version 6.7.2.290

17 Both mean number of neurite branches per cell and the mean length of neurite outgrowth per cell were calculated based on using the DAPI stain as a nuclear marker and the tdTomato stain, which labels excitatory neurons, as the neurite and cell body marker. The values used for subsequent analysis can be optimized depending on staining quality and cell density.

17.1 Define cell bodies to have an approximate maximum width of 30  $\mu\text{m}$ , a minimum area 300  $\mu\text{m}^2$ , and a pixel value of at least 1500 above local background level.

17.2 Define nuclei to have an approximate minimum width of 8  $\mu\text{m}$ , an approximate maximum width of 20  $\mu\text{m}$ , and a pixel value of at least 1500 above local background level.



- 17.3 Define neurite outgrowth to have a maximum width of 2  $\mu\text{m}$ , a minimum projection length of 15  $\mu\text{m}$  from the cell body, and a pixel value of at least 500 above local background level.

## 40x Image Analysis

- 18 Acquired images are analyzed as 2D maximum projections. Analyze the third morphometric, excitatory synapse density, using a custom synaptic assay module with MetaXpress 6 software. The values used for subsequent analysis can be optimized depending on staining quality and cell density.
- 18.1 Use Synapsin1 staining to identify puncta with an approximate minimum width of 0.5  $\mu\text{m}$ , an approximate maximum width of 2  $\mu\text{m}$ , and a minimum pixel value of 2500 above local background level.
- 19 The custom module will generate the number and area of Synapsin1 positive puncta within the colocalized MAP2+ and tdTomato+ signals.
- 19.1 Puncta density is generated by dividing the total area of puncta within the colocalized MAP2 and tdTomato staining by the area of MAP2+ and tdTomato+ signal within the neurites.