APR 05, 2024

Protocol Citation: David Sirkin, Gregory Tracy, Hanwen Zhang, Lilia Peyton, Ada McCarroll, Jubao Duan 2024. Immunofluorescence Staining and High Content Imaging. **protocols.io** https://protocols.io/view/immunofluorescence-staining-and-high-content-imagi-dbn22mge

Protocol status: Working
We use this protocol and it's working

Created: Apr 03, 2024

Last Modified: Apr 05, 2024

Immunofluorescence Staining and High Content Imaging

David Sirkin[1], Gregory Tracy[1], Hanwen Zhang[1], Lilia Peyton[1], Ada McCarroll[1], Jubao Duan[2]

[1]Endeavor Health/NorthShore University HealthSystem;
[2]NorthShore University HealthSystem/University of Chicago

David Sirkin
NorthShore

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).

PROTOCOL integer ID: 97722

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. 30m

2 Store fixed neurons at 4C in 1x PBS with 0.02% sodium azide.

3 15m
Aspirate sodium azide with pipette and permeabilize neurons in 150 µL of 1x PBS with 0.5% Triton X-100 for 00:15:00 at Room temperature (RT) without shaking.

4 1h
Aspirate PBST and block neurons with 300 µL of 3% BSA and 0.1% Triton X-100 in 1x PBS for 01:00:00 at Room temperature .

5 1h 30m
Aspirate blocking buffer and stain neurons with primary antibodies mouse anti-Synapsin 1 (1:500), goat tdTomato (1ug/ml), and chicken anti-MAP2 (1:5000), in blocking buffer by adding 100 µL of blocking buffer for 01:30:00 at Room temperature or overnight at 4 °C .

6 5m
Aspirate primary antibody solution and wash samples three times for 00:05:00 with 200 µL 1x PBS with 0.1% Triton X-100 (0.1% PBST).

7 1h
Aspirate 0.1% PBST and incubate samples with secondary antibodies in blocking buffer by adding 100 µL for 01:00:00 at Room temperature in the dark.

8 5m
Aspirate secondary antibody solution and wash neurons twice with 200 µL 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 µL at Room temperature for 00:10:00 . 10m

10 Aspirate DAPI and add 250 µL of 1x PBS containing 0.05% sodium azide.

11 Store plate at 4 °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 µm step size. For the 40x objective the pixel size is 0.3438 μm^2 with a pinhole of 60 µm, 20x objective pixel size is 0.6842 μm^2 also with a pinhole of 60 µm.

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 µm, a minimum area 300 μm^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 µm, an approximate maximum width of 20 µm, and a pixel value of at least 1500 above local background level.

17.3 Define neurite outgrowth to have a maximum width of 2 µm, a minimum projection length of 15 µm 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 µm, an approximate maximum width of 2 µm, 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.