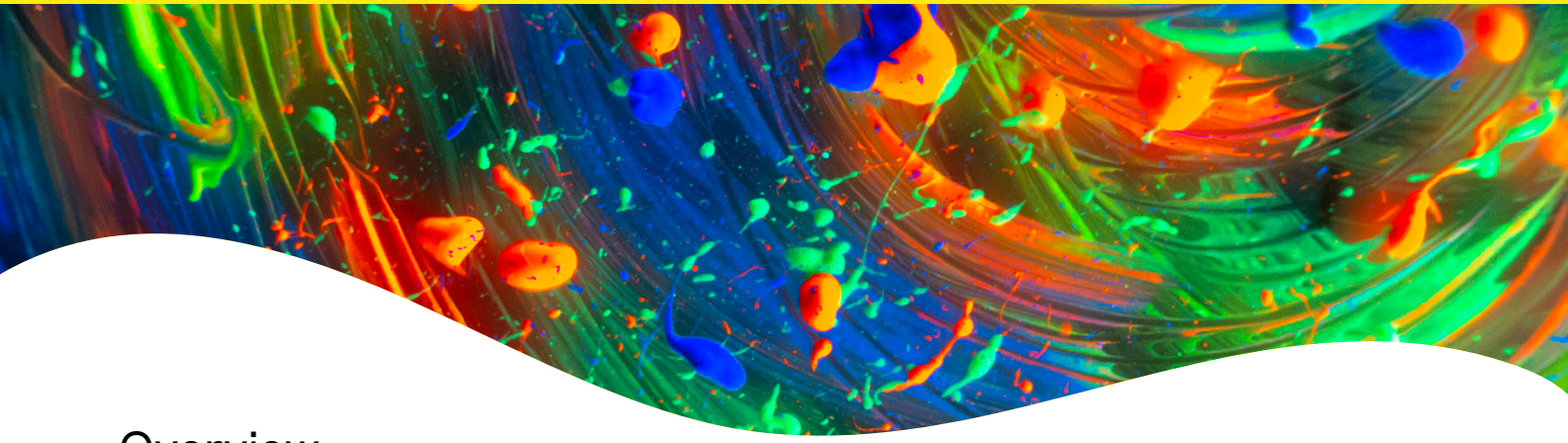
PhenoVue microglia differentiation staining kit 1x384.



Overview

In neuroscience research, the use of physiologically relevant cell models, such as primary cells, iPSC-derived neuronal cells, or co-cultured systems in 2D and 3D, is crucial to better replicate the pathophysiological conditions underlying disease onset. The reliable qualification of microglia cell models is especially important, given the lengthy and complex differentiation processes required, particularly for iPSC-derived cells.

Microglia, the immune cells of the brain, play a pivotal role in either neuroprotection or neuroinflammation depending on their environment. These highly sensitive cells adapt their functions in response to external conditions, adopting distinct morphologies that are characteristic of their activation state.

To address these needs, the PhenoVue™ Microglia Differentiation Staining Kit combines a specific antibody against Iba1, the major microglia marker, with general organelle staining probes. This combination allows for the simultaneous validation of Iba1 expression and the observation of cell morphology—two essential parameters for confirming successful differentiation and determining the activation state of microglia.

The PhenoVue™ Microglia Differentiation Staining Kit includes three fluorescent markers:

- **Iba1:** A protein predominantly expressed by macrophages and microglia. While its precise function remains unclear, Iba1 expression is upregulated during microglia activation and in response to nerve injury, CNS ischemia, or certain brain diseases. It is widely used as a key marker to distinguish microglia from other CNS cells such as neurons, astrocytes, and oligodendrocytes.
- **Actin:** A protein that polymerizes to form the actin cytoskeleton, commonly used to assess cell morphology.
- **PhenoVue Hoechst 33342:** for cell nuclei staining

Each component of the kit has been extensively validated and optimized, ensuring maximum spectral separation with no overlap, even when adding additional fluorescent stains, such as those compatible with the PhenoVue Fluor 488 channel, to measure other proteins or cellular events in parallel. Furthermore, the specificity of each reagent has been rigorously confirmed, making this kit an easy-to-use solution for qualifying microglia differentiation and assessing activation states based on cellular morphology.

Product information

Product name	Part no.	Number of vials per unit	Shipping conditions
PhenoVue Microglia Differentiation Staining kit	PMIDIF11	7	Dry ice

Kit contents	Format	Packaging*	Storage
PhenoVue Hoechst 33342 nuclear stain	Liquid	1 vial of 70 µL (500x)	2-8 °C or below. Protect from light
PhenoVue Fluor 555 – Phalloidin (600x)	Desiccated	1 vial of 60 µL (600x)	-16 °C or below. Protect from light
PhenoVue mouse anti-Iba1 IgG2a antibody (100x)	Liquid	1 vial of 100 µL (100x)	-16 °C or below. Protect from light
PhenoVue Fluor 647 - Goat anti-Mouse antibody Highly Cross-Adsorbed (100x)	Liquid	1 vial of 100 µL (100x)	-16 °C or below. Protect from light
PhenoVue Paraformaldehyde, 4% solution	Liquid	1 vial of 25 mL (1x)	2-8 °C or below. Protect from light
PhenoVue Permeabilization, 0.5% Triton X-100 solution	Liquid	1 vial of 25 mL (5x)	2-8 °C or below. Protect from light
PhenoVue dye diluent A (5x)	Liquid	1 vial of 8 mL (5x)	16 °C or below. Protect from light

*Amount of reagents provided is sufficient for 1 x 384 well microplate using the recommended concentrations.

Storage and stability

- Upon receipt, store PhenoVue permeabilization 0.5% Triton X-100 solution and PhenoVue paraformaldehyde, 4% solution reagents at 2–8 °C, protected from light. Other reagents can be stored together at ≤ -16 °C or separately between ≤ -16 °C to 2-8 °C protected from light, as indicated in the table above. Avoid repeated freeze / thaw cycles.
- Allow the reagents to warm up to room temperature for 30 min before opening the vials and reconstitution. Aliquoted reagents must be stored at -16 °C or below and are stable for 6 months.
- After thawing, the PhenoVue dye diluent A (5x) may contain some aggregates which will not impair the product and image quality. If aggregate removal is preferred, the PhenoVue dye diluent A (5x) can be filtered (0.22 µm filter) prior to dilution. The resulting PhenoVue dye diluent A 1x must be stored at 2-8 °C for no more than 2 days.
- The stability of these products is guaranteed until the expiration date provided in the certificate of analysis, when stored at recommended and protected from light.

Spectral and photophysical properties

Product name	Maximum excitation wavelength (nm)	Maximum emission wavelength (nm)	Common filter set	Quantum yield (ϕ)	Epsilon* (ε in $M^{-1}.cm^{-1}$ at λ max)	Brightness (ϕ x ε)
PhenoVue Hoechst 33342	357**	455**	DAPI	dsDNA: 0.38 ssDNA: 0.22	43,000	Nd
PhenoVue Fluor 488	495	520	FITC	92%	73,000	65,320
PhenoVue Fluor 555	555	570	Cy3	10%	155,000	15,500
PhenoVue Fluor 647	650	670	Cy5	30%	240,000	72,000

* In PBS pH 7.4 ** In methanol with 0.2 M HCl
Nd: not determined.

Please note that the PhenoVue Fluor 488 green channel remains available for experimental flexibility, allowing users to incorporate additional markers tailored to their specific research needs.

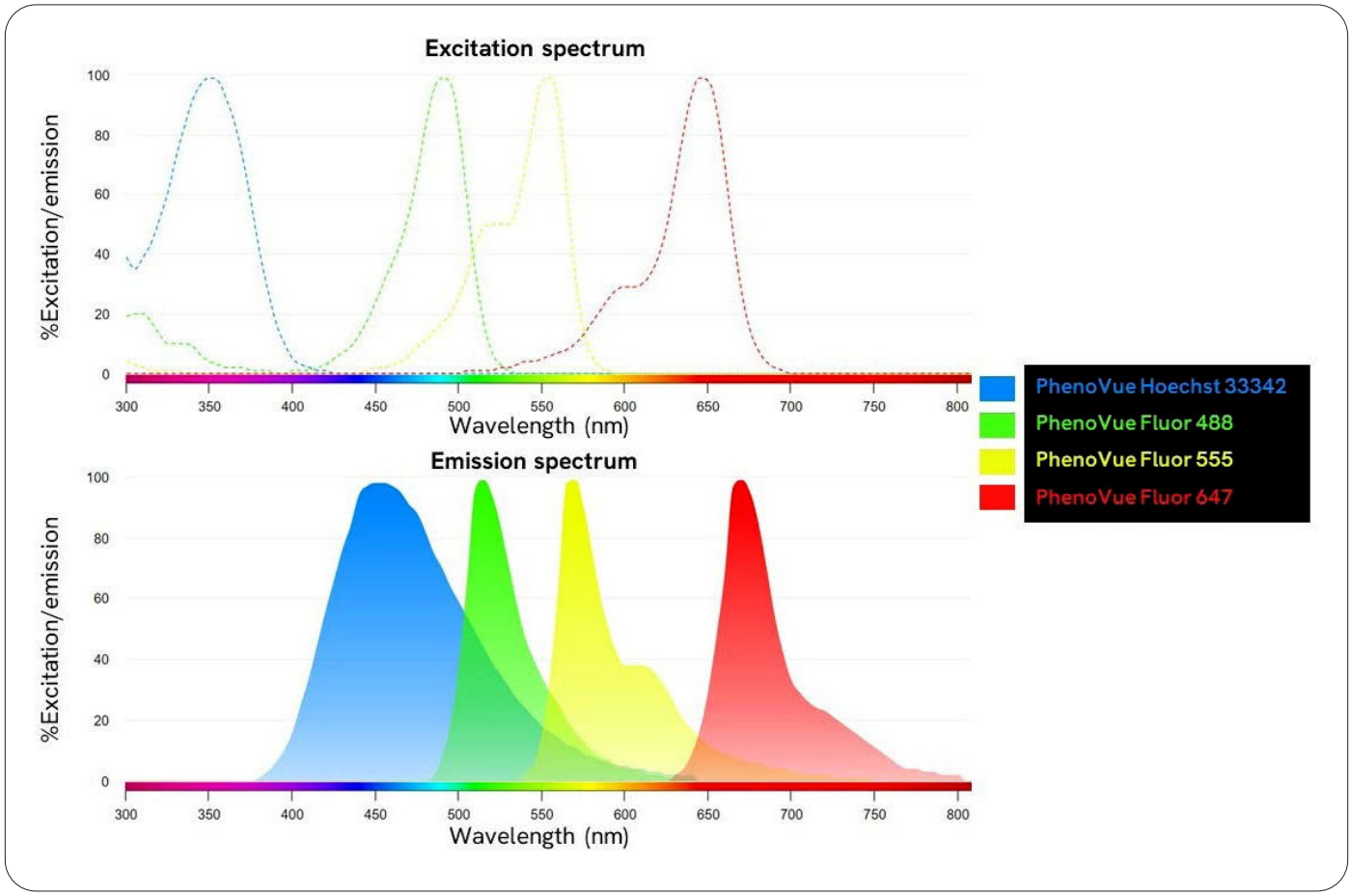


Figure 1: Excitation and emission spectra associated with the PhenoVue Microglia Differentiation Staining kit components.

Other materials and reagents not provided

Reagents or consumables	Usage
PBS	Washing buffer and diluent for PhenoVue permeabilization 0.5% Triton X-100 solution
Distilled H_2O	Dilution of PhenoVue dye diluent A (5x)
Anhydrous DMSO	Reconstitution of PhenoVue Fluor 555 – Phalloidin (600x)
PhenoPlate™ 384-well or 96-well	Cell plating, stimulation, staining and imaging
Aluminum single-tab foil	Plate sealing to protect fluorescent probes from light

Reagent reconstitution and preparation of staining solutions

	Reagents	Preparation of staining solutions
Buffers	PhenoVue Paraformaldehyde, 4% solution	Ready to use
	PhenoVue Permeabilization 0.5% Triton X-100 solution	Dilute 5 times in PBS
	PhenoVue Dye Diluent A (5x) *	Dilute 5 times in distilled H_20
Staining solution 1	PhenoVue anti-Iba1 antibody (100x)	Dilute 100 times in diluent A (1x)
Staining solution 2	PhenoVue Hoechst 33342	Dilute 500 times in diluent A (1x)
	PhenoVue Fluor 555 – Phalloidin (600x)	▪ Reconstitute with 60 µL DMSO ▪ Dilute 600 times in diluent A (1x)
	PhenoVue Fluor 647 - Goat anti-Mouse antibody Highly Cross-Adsorbed (100x)	Dilute 100 times in diluent A (1x)

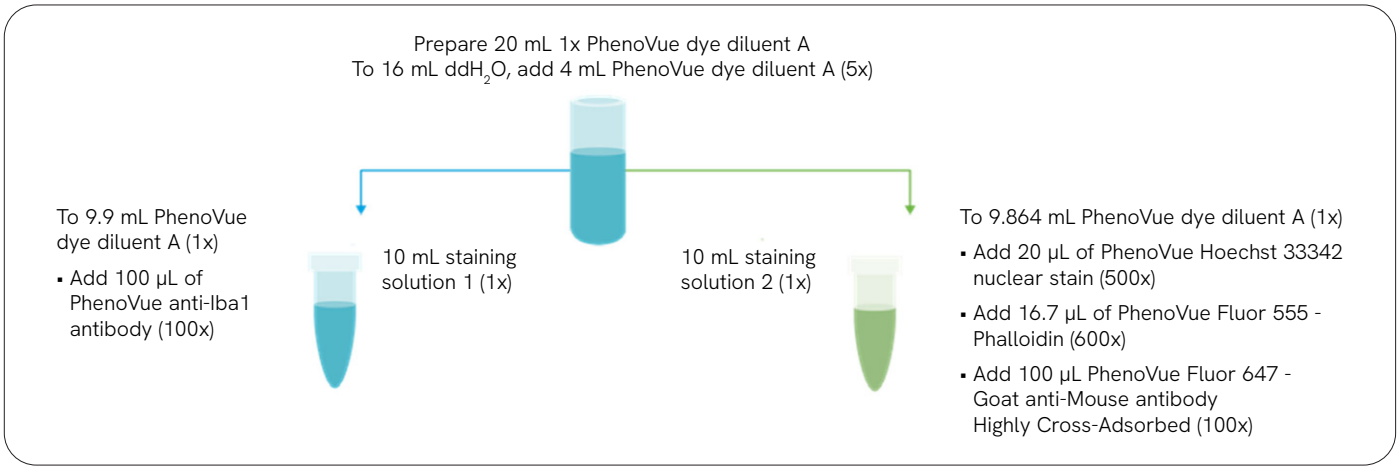
* PhenoVue dye diluent A 1x can be utilized for the saturation step.

- **Prepare stock solution of buffers and diluent:**
  - Fixative solution: PhenoVue paraformaldehyde, 4% solution is ready-to-use.
  - Permeabilization solution (0.1% Triton): Dilute stock solution of PhenoVue permeabilization 0.5% Triton X-100 solution 5 times in PBS.
  - Saturation and staining diluent solution: Dilute stock solution of PhenoVue dye diluent A (5x) 5 times in distilled H_2O to give a 1x solution.
- **Prepare the staining solutions in PhenoVue dye diluent A (1x)**

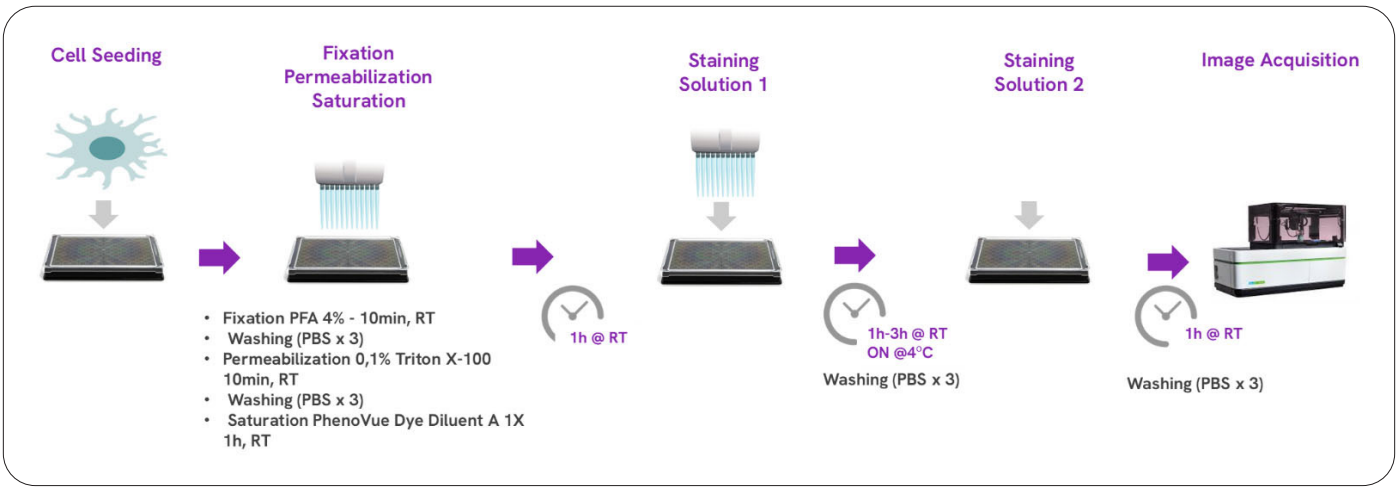
  (Protect stock and staining solutions from light)
  - **Staining solution 1:**
    - PhenoVue anti-Iba1 antibody: Dilute 100 times in diluent A (1x)
  - **Staining solution 2:**
    - PhenoVue Hoechst 33342 nuclear stain: Dilute 500 times in diluent A (1x)
    - PhenoVue Fluor 555 – Phalloidin: Reconstitute with 60 µLDMSO, then dilute 600 times in diluent A (1x)
    - PhenoVue Fluor 647 - Goat anti-Mouse antibody Highly Cross-Adsorbed: Dilute 100 times in diluent A (1x)

Example preparation of staining solutions

The following example describes the preparation of 10 mL staining solution 1 and 10 mL of staining solution 2, sufficient for 1 x 384-well plate (or 2 x 96-well plates).



Experimental workflow



Protocols

Cell culture

Seed cells in PhenoPlate 384-well imaging microplates* (or any other convenient cell culture vessels). Incubate in the appropriate cell culture conditions, usually 37 °C, 5% CO_2 until 50-70% confluency.

Fixed-cell imaging

Rinse briefly in phosphate-buffered saline (PBS) then proceed with cell fixation.

1. **Fixation:** Add 25 µL ready-to-use PhenoVue paraformaldehyde 4% solution for 10 min at room temperature
2. **Washing:** Wash three times with PBS.
3. **Permeabilization:** Add 25 µL PhenoVue permeabilization solution diluted at 0.1% Triton X-100 in PBS for 10 min at room temperature.
4. **Washing:** Wash three times with PBS for 5 min.
5. **Saturation:** Incubate with 25 µL PhenoVue dye diluent A 1x for 1h at room temperature.
6. **Staining solution 1:** Remove saturation buffer and add 25 µL per well of solution 1. Incubate for at least 1h at room temperature.
7. **Washing:** Wash three with PBS for 5 min.

* PhenoPlate 96-well microplates may also be used. Adjust the cell density accordingly and increase the corresponding volumes by 2-fold.

8. **Staining solution 2:** Add 25 µL per well of staining solution 2 and incubate for 1h at room temperature protected from light.
9. **Washing:** Wash three times with PBS for 5 min.
10. **Acquire images on an imaging device.**

Tips

- Reagent concentrations have been carefully optimized to minimize fluorescence crosstalk when using Revvity's high-content imaging instruments. Increasing these concentrations may lead to higher crosstalk.
- Optimal fluorescence intensities are typically achieved after 1 to 3 hours of incubation with primary antibodies at room temperature. While overnight incubation at 4 °C is possible, it is less recommended.
- When using 384-well microplates, we recommend centrifuging the plate for 1 minute at 500 g and room temperature between each step to ensure reagents settle at the bottom of the wells.
- For reconstituting PhenoVue Fluor 555 – Phalloidin, avoid using methanol or other alcohol-based or aqueous solvents. Anhydrous DMSO is preferable, as it preserves the integrity of actin filaments, resulting in brighter staining.
- The PhenoVue anti-Iba1 antibody has been validated exclusively on human models.
- For added flexibility, the PhenoVue Fluor 488 channel is available for detecting additional proteins or events using other compatible fluorescent stains. In such cases, the following considerations should be taken into account:
  - The PhenoVue anti-Iba1 antibody is a Mouse IgG2a.
  - The secondary PhenoVue Fluor 647 Goat anti-mouse antibody is highly cross-adsorbed. While it is highly specific to mouse antibodies, it is not isotype-specific and can therefore recognize IgG1, IgG2a, IgG2b, IgG2c, and IgG3.
  - You can easily combine a primary antibody with PhenoVue Fluor 488 secondary antibodies, as long as the primary antibody is not of mouse origin. For instance, PhenoVue Fluor 488 Goat anti-Rat (part number: 2GXRT488H1) or anti-Rabbit (part number: 2GXRB488H1), both highly cross-adsorbed, are compatible for such applications.
  - You can also use a Mouse primary antibody of a different isotype than IgG2a. However, in this case, you must replace the secondary Goat anti-Mouse antibody included in the kit with a highly specific anti-Mouse IgG2a antibody. The secondary antibody used to detect the additional Mouse primary antibody must be highly isotype-specific and should not detect Mouse IgG2
  - For example, in a microglia/neuron co-culture, the PhenoVue Fluor 647 Goat anti-Mouse antibody provided in this kit can be substituted with our PhenoVue Fluor 647 Rat Anti-Mouse IgG2a Highly Cross-Adsorbed Antibody (part number: 2RTXM647G2AH1). Additionally, you can incorporate Mouse IgG1 primary antibodies, such as PhenoVue anti-Nestin (part number: PANEST1) or PhenoVue anti-MAP2 (part number: PABMAP2), alongside our PhenoVue Fluor 488 Rat Anti-Mouse IgG1 Highly Cross-Adsorbed Antibody (part number: 2RTXM488G1H1). See how it works in Figure 4.

Safety information

Chemical reagents are potentially harmful, please refer to the Safety Data Sheet (SDS) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Applications

- High-content analysis / high-content screening
- Imaging microscopy

Recommendations for acquisition settings

The PhenoVue Microglia Differentiation Staining Kit allows for multiplexing with 3 to 4 colors simultaneously. For optimal fluorescent signal and image quality on a Revvity high-content screening system, we recommend the following acquisition settings, depending on the instrument used:

HCS instruments		PhenoVue Hoechst 33342	PhenoVue Fluor488	PhenoVue Fluor 555	PhenoVue Fluor 647
Opera Phenix® Plus 5 lasers	Excitation laser (nm)	375	488	561	640
	Emission filters (nm)	435-480	500-550	570-630	650-760
Opera Phenix Plus 4 lasers	Excitation laser (nm)	405	488	561	640
	Emission filters (nm)	435-480	500-550	570-630	650-760
Operetta CLS 4 or 8 LED - 1600 & 1601	Excitation LED (filters) (nm)	370 (355-385)	475 (460-490)	550 (530-560)	630 (615-645)
	Emission filters (nm)	430-500	500-550	570-650	655-760

Assay Validation

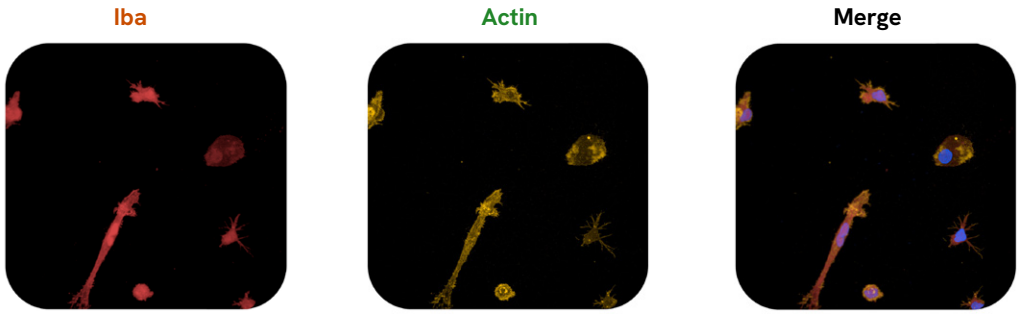
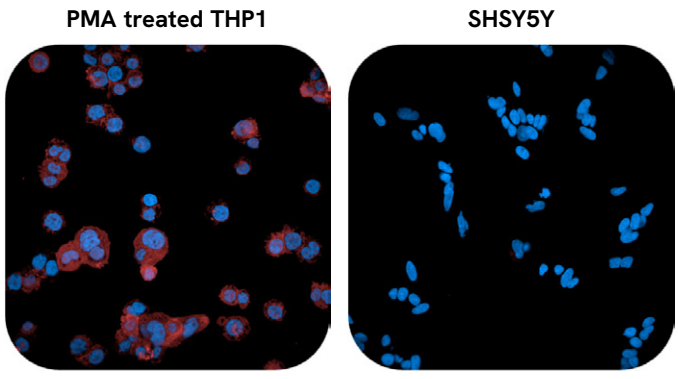


Figure 1: Human iPSC-derived microglia, differentiated and matured for 50 days, were provided by INM collaborators from Montpellier (Coralie Clua Provost and Carole Crozet, INSERM U1298/Université de Montpellier). The cells were seeded in PhenoPlate 96-well microplates at a density of 15,000 cells per well. After fixation, permeabilization, and saturation, the cells were stained using the PhenoVue Microglia Differentiation Staining Kit (part number: PMIDIF11). Images were captured on the Opera Phenix Plus high-content imaging system with 5 lasers, using a 63x water immersion objective in confocal mode.



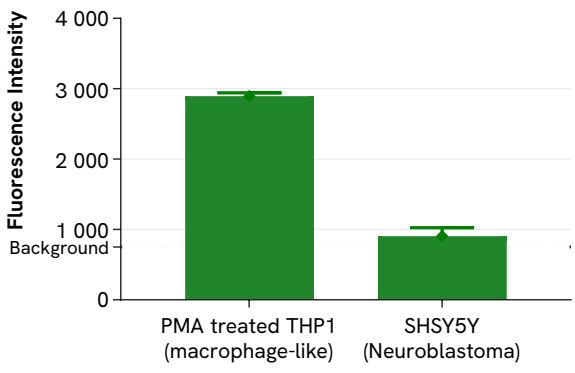


Figure 2: PMA-treated THP1 cells (macrophage-like, positive control) and SHSY5Y cells (neuroblastoma, negative control) were stained with the PhenoVue anti-Iba1 antibody and PhenoVue Fluor 647 Goat anti-Mouse IgG (H+L) highly cross-adsorbed antibody. Images were captured using the Operetta CLS high-content analysis system with an 8-LED light source, a 40x water immersion objective, and confocal mode. As expected, no fluorescent signal was detected in SHSY5Y cells, while the PMA-treated THP1 cells displayed whole-cell labeling, confirming the specificity of the PhenoVue anti-Iba1 antibody.

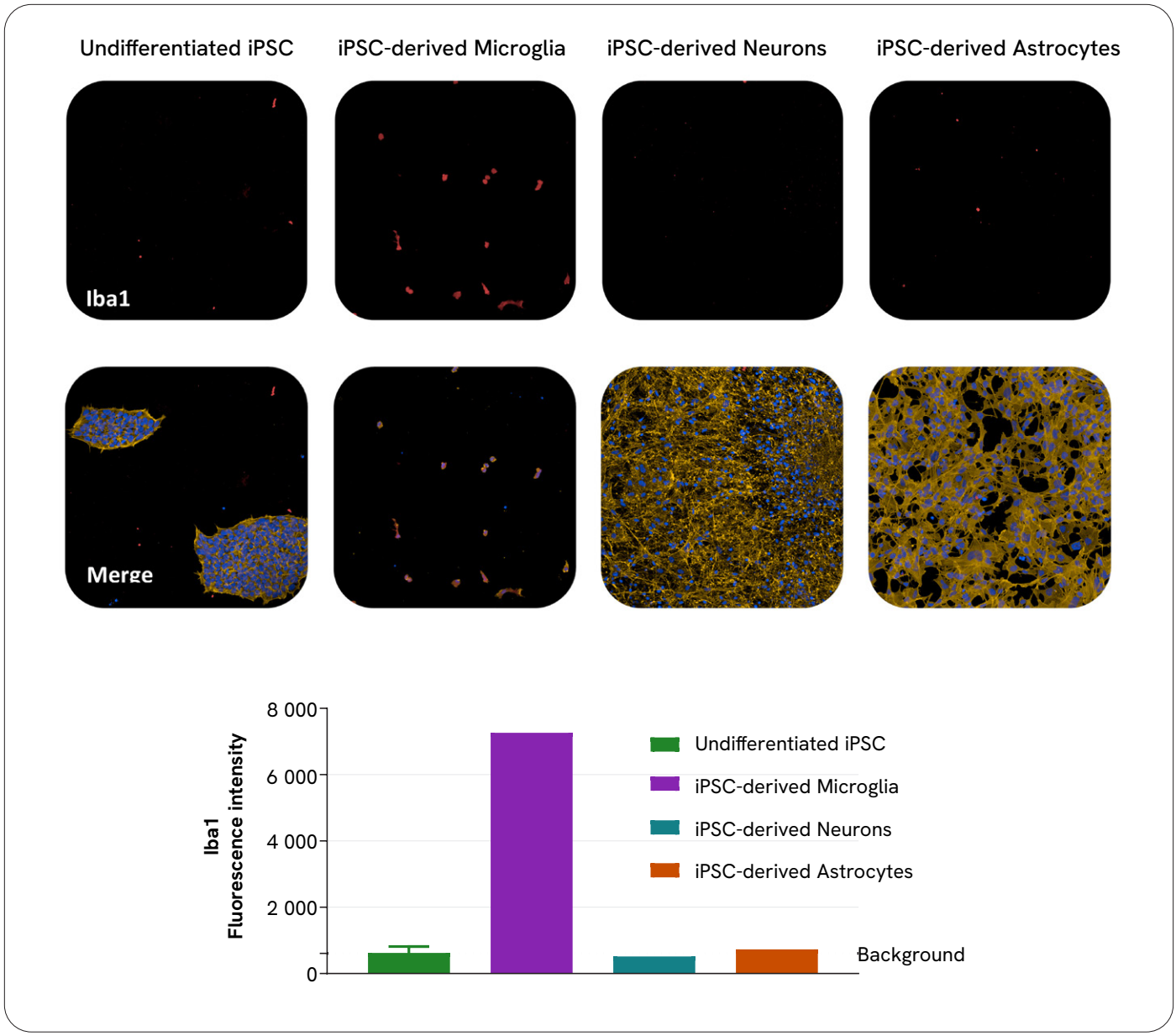


Figure 3: Human undifferentiated iPSC cells (15,000 cells/well), 50-day differentiated and matured human iPSC-derived microglia (15,000 cells/well), and 30-day differentiated human iPSC-derived neurons (45,000 cells/well) were provided by INM collaborators from Montpellier (Coralie Clua Provost and Carole Crozet, INSERM U1298/Université de Montpellier). Additionally, human iPSC-derived astrocytes (15,000 cells/well), provided by IXCells, were seeded in PhenoPlate 96-well microplates following the supplier's recommended protocol. All cells were fixed, permeabilized, saturated, and stained using the PhenoVue Microglia Differentiation Staining Kit. Images were acquired using the Opera Phenix Plus high-content imaging system, equipped with 5 lasers, a 20x water immersion objective, and confocal mode.

The PhenoVue Microglia Differentiation Kit offers a free 488 channel, allowing the addition of a marker of interest such as MAP2 or Nestin as illustrated here, which is particularly beneficial for co-culture experiments

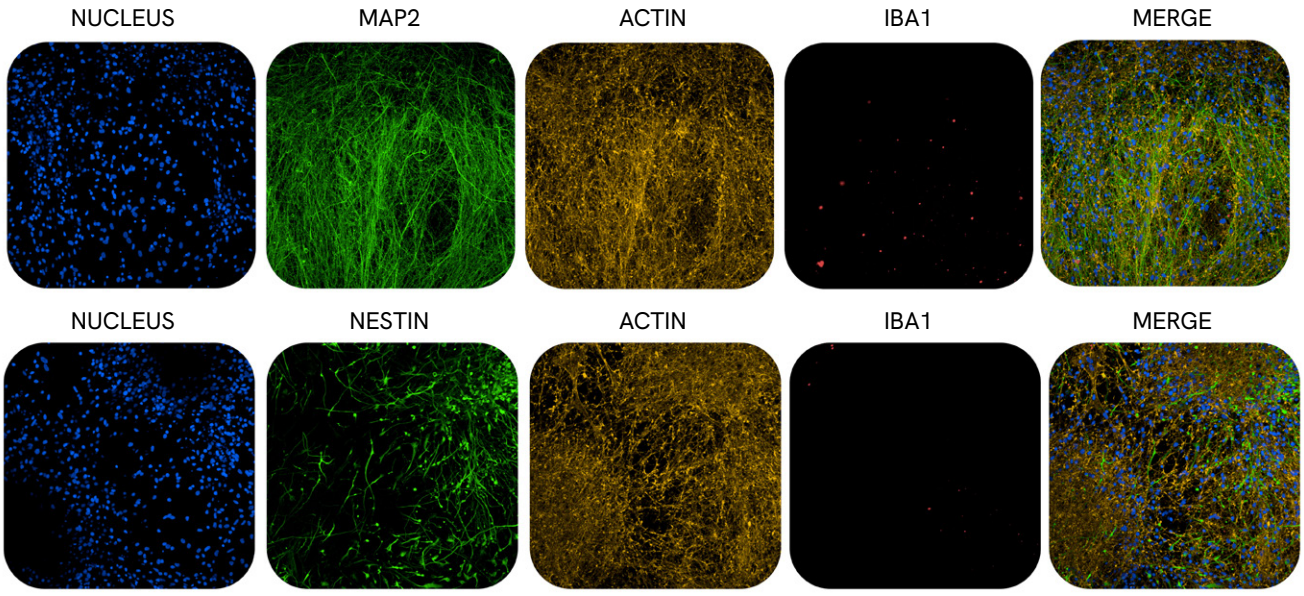


Figure 4: Human iPSC-derived neurons, differentiated for 30 days from undifferentiated iPSCs (45,000 cells/well), were provided by INM collaborators from Montpellier (Coralie Clua Provost and Carole Crozet, INSERM U1298/Université de Montpellier). The cells were seeded in PhenoPlate 96-well microplates, then fixed, permeabilized, saturated, and stained using the PhenoVue Microglia Differentiation Staining Kit.

As detailed in the "Tips" section, the PhenoVue Fluor 647 Goat anti-Mouse antibody from the kit was replaced with PhenoVue Fluor 647 Rat Anti-Mouse IgG2a Highly Cross-Adsorbed Antibody (part number: 2RTXM647G2AH1). In addition, Mouse IgG1 PhenoVue anti-Nestin (part number: PANEST1) or PhenoVue anti-MAP2 (part number: PABMAP2) antibodies were added in combination with PhenoVue Fluor 488 Rat Anti-Mouse IgG1 Highly Cross-Adsorbed Antibody (part number: 2RTXM488G1H1).

As expected, no Iba1 expression was detected in these iPSC-derived neurons. However, some cells expressed nestin (neuronal progenitor cells), while others expressed MAP2 (indicating more mature and well-differentiated neurons). No crosstalk was observed between fluorescence channels. Images were acquired using the Opera Phenix Plus high-content imaging system with 5 lasers, a 20x water immersion objective, and confocal mode.