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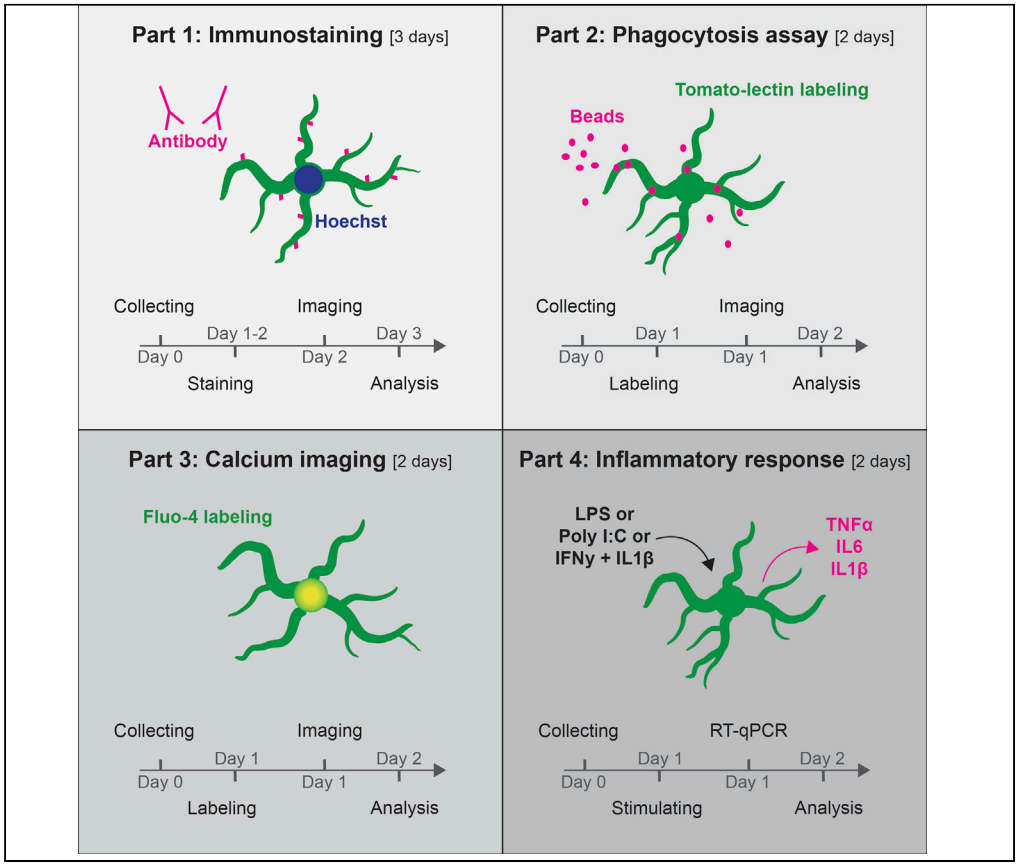
Assessing human iPSC-derived microglia identity and function by immunostaining, phagocytosis, calcium activity, and inflammation assay



To understand how potential gene manipulations affect *in vitro* microglia, we provide a set of short protocols to evaluate microglia identity and function. We detail steps for immunostaining to determine microglia identity. We describe three functional assays for microglia: phagocytosis, calcium response following ATP stimulation, and cytokine expression upon inflammatory stimuli. We apply these protocols to human-induced-pluripotent-stem-cell (hiPSC)-derived microglia, but they can be also applied to other *in vitro* microglial models including primary mouse microglia.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Functional characterization of human-iPSC-derived microglia

Immunostaining to assess microglia identity

Live imaging of microglial calcium and phagocytosis activity

Determine microglial transcriptional changes upon inflammatory stimulation

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Assessing human iPSC-derived microglia identity and function by immunostaining, phagocytosis, calcium activity, and inflammation assay

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SUMMARY

To understand how potential gene manipulations affect *in vitro* microglia, we provide a set of short protocols to evaluate microglia identity and function. We detail steps for immunostaining to determine microglia identity. We describe three functional assays for microglia: phagocytosis, calcium response following ATP stimulation, and cytokine expression upon inflammatory stimuli. We apply these protocols to human induced-pluripotent-stem-cell (hiPSC)-derived microglia, but they can be also applied to other *in vitro* microglial models including primary mouse microglia.

For complete details on the use and execution of this protocol, please refer to Bartalska et al. (2022).[1]

BEFORE YOU BEGIN

Biosafety standards and laboratory guidelines for handling and disposal of biological materials, including genetically modified materials have to be established and need to be followed.

Institutional permissions

For the use of hiPSC and human embryonic stem cell (hESC) lines, it has to be ensured to adhere to the national legislation and institutional requirements of the country. Legislation in some countries may require approvals via the Ethics Committee or the Institutional Review Board. Furthermore, the provider of the cell line has to ensure that informed consent has been obtained from the donor to derive and use hiPSCs or donated embryos for the derivation of hESC lines, and that the personal data and privacy of the donors are sufficiently protected. For several cell lines, the ethics information is already available in the European stem cell registry hPSCreg (https://hpscreg.eu) or in the NIH Human Embryonic Stem Cell Registry (https://grants.nih.gov/stem_cells/registry/current.htm).

Specifically for hESC, funding bodies of research projects might have additional requirements regarding approval procedures and reporting in place for projects.

Note: In case of primary human microglia, similar aspects have to be fulfilled.

Note: In case of primary murine microglia, the user has to have the approval of the animal procedure from the ethical committee of their institute and all relevant authorities.

Note: Legislation in some countries may require approval to use macrophage-like cell lines.

Table 1. Overview of protocols differentiating hiPSC into microglia-like cells

Protocol	Days (D) of differentiation to harvest microglia-like cells	Isolation strategy
Abud et al.[4]	>D35	FACS
Amos et al.[4]	>D40	FACS
Bartalska et al.[12]	>D35	Harvest cells from suspension
Brownjohn et al.[10]	>D23	Harvest cells from suspension
Douvaras et al.[5]	>D45	FACS
Guttikonda et al.[7]	>D17	Harvest cells from suspension
Haenseler et al.[8]	>D42	Harvest cells from suspension
Konttinen et al.[11]	>D24	Harvest cells from suspension
McQuade et al.[13,17]	>D28	Harvest cells from suspension
Muffat et al.[14]	>D60	Seed cells on Primaria plates
Pandya et al.[18]	>D29	MACS
Reich et al.[15]	>D30	Harvest cells from suspension
Takata et al.[9]	>D46	FACS

FACS, fluorescent activated cell sorting; MACS, magnetic activated cell sorting.

Source of microglia

For generating primary mouse microglia, we refer the reader to.[2]

For generating primary human microglia, we refer the reader to.[3]

For generating hiPSC-derived microglia-like cells from hESCs or hiPSCs, we refer to.[1,4–16] The users can decide to either choose one strategy of the published protocols in Table 1 or buy a commercially available kit (e.g., STEMdiff™ Microglia Differentiation Kit). For a detailed step-by-step protocol, we refer to.[16] Depending on the chosen protocol (Table 1), microglia-like cells will be generated within 3–5 weeks.

> ***Note:*** The community has not decided on a gold standard for generating microglia-like cells from hiPSC/hESC. In our experience, the microglial yield depends on the cell line used for differentiation than on the chosen protocol.

In this STAR Protocol, we performed all experiments on hiPSC-derived microglia-like cells.

General note

This protocol contains a set of four short protocols to evaluate microglia identity and function.

For immunostaining, see steps 1–12.

For beads-based phagocytosis assay, see steps 13–30.

For imaging calcium activity after ATP stimulation, see steps 31–55.

For inflammation assay, see steps 56–75.

Each protocol can be run independently. We typically perform these experiments in the above-described sequence. Based on the research question, individual or combination of assays can be selected.

Collecting hiPSC-derived microglia-like cells

Timing: 30 min (for step 1)

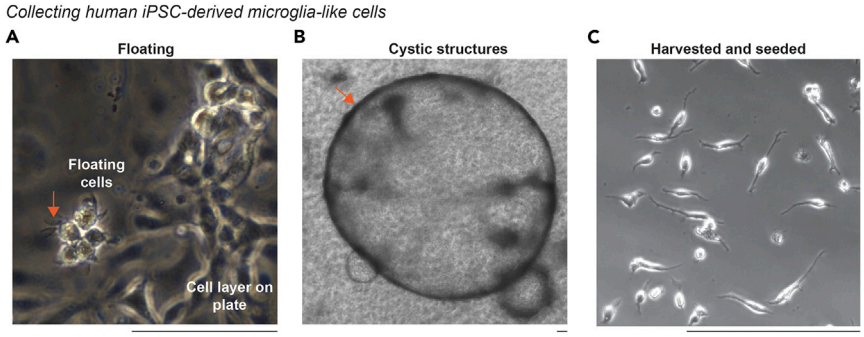

Figure 1. Collecting hiPSC-derived microglia-like cells

(A) Bright field images of (A), Branched cells floating in the supernatant. Arrow points to branches of floating cells. (B) Cystic structure that also appears in the supernatant (arrow). These structures should not be not collected. (C) Microglia-like cells attached to the dish after seeding. Cell bodies show a light halo and only a few branches. Scale bar: 100 μm. Panel (A)-(C) re-used with permission from.[1]

For this protocol, we used hiPSC-derived microglia-like cells that were collected from the supernatant from week 5 onwards twice per week as described in Bartalska et al.[1] At this time, the floating cells showed some small processes (Figure 1A).

Note: This procedure depends on the chosen microglial differentiation protocol (see Table 1). Besides harvesting the cells from the supernatant, several protocols require magnetic or fluorescent activated cell sorting (FACS) with selective markers.

1. (Day 0) Prepare *microglia medium* and *microglia medium supplemented with M-CSF* and pre-warm both media to 37°C in a bead bath.
2. Collect and pool the supernatant from 8 individual 6-cm microglia differentiation culture dishes into a 50 mL falcon tube.
   a. Place a 100 μm cell strainer on the falcon tube.
   b. Carefully collect the supernatant with a 1,000 μL pipette tip and rinse it through the filter.

Note: Depending on the protocol, the differentiation culture might contain cystic structures in suspension (Figure 1B). Using a 100 μm cell strainer prevents the collection of these structures.

3. Place the 50 mL falcon tube into a centrifuge and spin down the microglia-like cells at 200 × *g* for 4 min at room temperature (20°C–22°C).

Note: For continuous microglia collection over the next weeks, 3 mL/6-cm dish *microglia medium* can be added to the original microglia differentiation culture dishes and incubated at 37°C, 5% CO_2. The collection can then be repeated in 2–3 days.

4. After centrifugation, aspirate the supernatant e.g., with a vacuum pump.
5. Re-suspend the cell pellet in 1 mL *microglia medium supplemented with M-CSF* by pipetting up and down for five times.

⚠ CRITICAL: To minimize cell death, use a 1 mL pipette tip for re-suspension and do not pipette more than 10 times up and down.

Seeding cells

Timing: 15 min

Table 2. Overview seeding format, cell number and medium for functional assays

Functional assay	Plate format	Number of wells/replicate	Cell number/well	Medium
Immunostaining	8 well Ibidi-chamber	2	2.5×10^4–5×10^4	*Microglia medium supplemented with M-CSF*
Phagocytosis assay	8 well Ibidi-chamber	1	2.5×10^4–5×10^4	
Calcium imaging	8 well Ibidi-chamber	2	2.5×10^4–5×10^4	
Inflammation assay	24 well plate	4	5×10^4–1×10^5	*Microglia medium*

6. Count cells with a hemocytometer or an automated cell counter and determine the cell number.

   Note: The expected cell number of hiPSC-derived microglia-like cells is between 5×10^5–1×10^6 cells per total harvest from 8 individual pooled 6-cm dishes (troubleshooting 1).

7. For seeding the cells, adapt the required plate format and number, number of seeded wells and the medium usage for the respective downstream analysis to be performed (Table 2).

   Note: For imaging-based functional assays, we are using Ibidi cell culture chambers, which are suitable for most inverted microscopy techniques. Ibidi chambers meet all optical requirements for high resolution live imaging. Alternatively, cells can be seeded onto sterilized cover slips placed into 12- or 24-well plates.

8. Incubate collected cells at 37°C, 5% CO_2 overnight (~15–17 h).

   Note: Usually no coating of the plate is required as microglia attach to the plate.

   Note: HiPSC-derived microglia-like cells attach overnight to the surface of the plate (Figure 1C) (troubleshooting 2).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Alexa Fluor™ 488 – conjugated donkey anti-goat, 1:2000	Thermo Fisher Scientific	Cat#A11055 RRID: AB_2534102
Alexa Fluor™ 647 – conjugated donkey anti-rabbit, 1:2000	Thermo Fisher Scientific	Cat#A31573 RRID:AB_2536183
Rabbit monoclonal anti-CD45, 1:200	Cell Signaling Technology	Cat#13917P; RRID: AB_2750898
Mouse monoclonal anti-CX3CR1, 1:50	BioLegend	Cat#B355702; RRID: AB_2561726
Goat polyclonal anti-IBA1, 1:250	Abcam	Cat#ab5076; RRID: AB_2224402
Rabbit polyclonal anti-P2Y12, 1:100	Sigma-Aldrich	Cat#HPA014518; RRID: AB_2669027
Rabbit polyclonal anti-PU.1, 1:500	Cell Signaling Technology	Cat#2266S; RRID: AB_10692379
Mouse monoclonal anti-RUNX.1, 1:50	BioLegend	Cat#659302; RRID: AB_2563194
Rabbit polyclonal anti-TMEM119, 1:100	Abcam	Cat#ab185333, RRID: AB_2687894
Rabbit polyclonal anti-TMEM119, 1:100	Novus Biologicals	Cat# NBP2-30551; RRID: AB_2910564
Chemicals, peptides, and recombinant proteins		
Adenosine-5 Triphosphate (ATP)	New England Biolabs	Cat#P0756S
B27 without Vitamin A (50×)	Thermo Scientific	Cat#12587-010
Bovine Serum Albumin (BSA)	Thermo Scientific	Cat#A9418
DMEM, high glucose, GlutaMAX™ Supplement, pyruvate	Thermo Scientific	Cat#31966047
Donkey Serum	Thermo Scientific	#S30
DPBS, no calcium, no magnesium	Thermo Scientific	Cat#14190-250

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
DyLight® 594 labeled Lycopersicon Esculentum (Tomato) Lectin	Szabo-Scandic	Cat#VECDL-1177
16% Formaldehyde (w/v)	Thermo Scientific	Cat#PI-28908
Ham's F-12 Nutrient Mix, GlutaMAX™ Supplement	Thermo Scientific	Cat#31765035
Hoechst 33342 (1:5000)	Thermo Scientific	Cat#H3570
Leibovitz's L-15 Medium	Thermo Scientific	Cat#21083027
Human IFNy	Sigma-Aldrich	Cat#SRP3058-100UG
Human IL1β	Thermo Scientific	Cat#RIL1BI
Lipopolysaccharid (LPS)	Sigma-Aldrich	Cat#L5886-10MG
Human M-CSF	PeproTech	Cat#300-25
MEM Non-essential Amino Acid Solution (100×)	Merck	Cat#M7145-100ML
Penicillin-Streptomycin (10,000 U/mL)	Thermo Scientific	Cat#15140122
Poly I:C	Tocris Bioscience	Cat#4287
Sodium azide	VWR	Cat#786-299
Triton X-100	Sigma	Cat#T8787
Critical commercial assays		
Fluo-4 Direct™ Calcium Assay Kit	Thermo Scientific	Cat#F10471
innuPREP RNA Mini Kit 2.0	Analytik-Jena	Cat#845-KS-2040050
LunaScript RT Super-Mix Kit	New England Biolabs	Cat#E3010L
Luna Universal qPCR Master Mix	New England BioLabs	Cat#M3003L
pHrodo™ Red E. coli BioParticles™ Conjugate for Phagocytosis	Thermo Scientific	Cat#P35361
Experimental models: Cell lines		
Human: iPSC cell line SC 102A-1	BioCat	hPSCreg.eu: SBLi006-A
Human: iPSC cell line NCRM-5 (CR0000005)	RUCDR Infinite Biologics	hPSCreg.eu: CRMi001-A
Oligonucleotides		
Oligonucleotides for RT-qPCR	Bartalska et al.[1]	See Table 8
Software and algorithms		
Imaris (version 9.3)	Bitplane Imaris	http://www.bitplane.com/imaris/imaris; RRID: SCR_007370
ImageJ	Schindelin et al.[19]	https://imagej.net/; RRID: SCR_003070
R (version 4.1.0)	R Core Team[20]	https://www.rstudio.com/; RRID: SCR_001905
Package: Xlsx (version 0.6.1)	Dragulescu[21]	
Package: ggplot2 (version 3.0.0)	Wickham[22]	RRID: SCR_014601
PeakCaller	Artimovich et al.[23]	
Other		
µ-Slide 8 Well chamber	IBIDI	Cat#80826
24 well plate	Corning	Cat#3527
6-cm dish	VWR	Cat#734-0007
100 µm cell strainer	Corning	Cat#352360
Automated cell counter	Bio-Rad	Cat#1450102
Flex Cycler 2 (Thermocycler)	Analytika Jena	Cat#844-00069
Hemocytometer	Merck	Cat#59629-1EA
LightCycler 480	Roche	Cat#05015243001
LSM 880 confocal/Airyscanner microscope	Carl Zeiss GmbH	N/A
NanoDrop 2000 Spectrophotometer	Thermo Scientific	Cat#ND2000USCAN
Plan-Apochromat 20× Air objective NA 0.8	Carl Zeiss GmbH	Cat#420650-9901-000
Plan-Apochromat 40× oil immersion objective NA 1.4	Carl Zeiss GmbH	Cat#420762-9900-000

MATERIALS AND EQUIPMENT

Microglia medium		
Reagent	Final concentration	Amount
DMEM (Dulbecco's Modified Eagle Medium) 1× with GlutaMAX	N/A	75 mL
F12 (F12 Nutrient Mixture) 1× with GlutaMAX	N/A	25 mL
B27 without Vitamin A (50×)	2% (v/v)	2 mL
NEAA (Non-essential amino acids) 100×	1% (v/v)	1 mL
Penicillin/Streptomycin 100×	1% (v/v)	1 mL

Note: Store the *microglia media* at 4°C for 4 weeks.

Microglia medium supplemented with M-CSF		
Reagent		
Microglia medium		1 mL
M-CSF Recombinant Human Protein (50 μg/mL)	50 ng/mL	1 μL

⚠ CRITICAL: Add the supplement only shortly before applying the medium to the cells. M-CSF stock solution can be aliquoted and stored at −20°C for 12 months.

Note: The cytokine M-CSF is commonly used in hiPSC-derived microglia differentiation protocols and is known to impact microglia survival. For us, the application of M-CSF resulted in an improved cell attachment and a more ramified morphology.

4% (v/v) Formaldehyde solution		
Reagent	Final concentration	Amount
16% (v/v) Formaldehyde solution	4% (v/v)	1 mL
1× PBS (phosphate-buffered saline)	N/A	3 mL

Note: Prepare freshly before use.

Blocking solution (10 mL)		
Reagent	Final concentration	Amount
BSA (bovine serum albumin) in dH_2O	1% (w/v)	100 mg
Sodium azide	0.1% (w/v)	10 μL
Donkey serum	10% (v/v)	1 mL
Triton X-100	2% (v/v)	200 μL
10× PBS	1×	1 mL
dH_2O	N/A	Up to 10 mL

Note: The *blocking solution* can be prepared in advance and stored in aliquots at −20°C for 6 months.

Antibody solution (10 mL)		
Reagent	Final concentration	Amount
BSA (bovine serum albumin) in dH_2O	1% (w/v)	100 mg
Sodium azide	0.1% (w/v)	10 μL

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
Donkey serum	3% (v/v)	300 µL
Triton X-100	2% (v/v)	200 µL
10× PBS	1×	1 mL
dH_2O	N/A	Up to 10 mL

Note: The *antibody solution* can be prepared in advance and stored in aliquots at −20°C for 6 months.

STEP-BY-STEP METHOD DETAILS

Part 1: Immunostaining of microglia marker

Timing: 3 days

Microglia identity can be defined with the following selective markers (Table 3):

Note: A unique microglia-selective marker has not yet been found. All listed markers also label other cell types such as blood-derived macrophages, perivascular macrophages, and similar . Thus, a combination of validated markers should be used to investigate microglia cell identity.

Note: TMEM119 (transmembrane protein 119) has been described as a microglia-specific marker,[24] and has been used in various studies to confirm microglia identity.[7,8,25] However, when we analyzed non-isolated microglia embedded in mesenchymal tissue, we could not observe TMEM119 expression in microglia-like cells.[1] Due to this ambiguity, we decided against including TMEM119 as a selective marker.

Note: Ideally, the antibodies used for hiPSC-derived microglia-like cells are cross-validated for their specificity in human tissue because several papers only refer to the specificity in mouse tissue. In Bartalska et al.,[1] we have validated the above set of markers in human brain tissue.

Here, we describe an example immunostaining for IBA1, P2Y12, and PU.1 (Figure 2A).

1. (Day 0) Seed collected microglia cells as described in "before you begin – seeding cells". Adjust the culture format and the cell number as outlined in Table 2.
   a. Incubate cells at 37°C, 5% CO2, overnight (~15–17 h).
   b. Prepare the solutions *formaldehyde solution* , *blocking solution*, and *antibody solution*.
2. (Day 1) Fixing microglia-like cells.
   a. Aspirate the medium.
   b. Add 200 µL PBS (phosphate buffered saline) per well to wash the cells.
   c. Aspirate the PBS.

Table 3. Overview microglia marker

Gene name/alternative name	Abbreviation	Function
Cluster of differentiation 45/protein tyrosine phosphatase receptor type C[17]	CD45	Membrane tyrosine phosphatase
Chemokine (C-X3-C) receptor 1[26]	CX3CR1	Chemokine receptor
Ionized calcium binding adaptor molecule 1/allograft inflammatory factor 1[27]	IBA1/AIF1	Calcium-binding protein
Purinergic receptor P2Y G-protein-coupled 12[28]	P2Y12	Purinergic receptor
Hematopoietic transcription factor PU.1[29]	PU.1	Transcription factor
Runt-related transcription factor 1[30]	RUNX1	Transcription factor

d. Add 200 μL of 4% (w/v) *formaldehyde solution at room temperature* (20°C–22°C) per well (troubleshooting 3).

⚠ CRITICAL: Handle formaldehyde always in a fume hood.

e. Incubate cells for 15 min in the fixative at room temperature (20°C–22°C).
f. Collect the *formaldehyde solution* with a pipette and dispose it in the aldehyde waste disposal.

⚠ CRITICAL: Formaldehyde is toxic and requires specific aldehyde waste disposal.

g. Add 200 μL PBS per well.
h. Incubate cells for 5 min in PBS.
i. Aspirate PBS.
j. Repeat **steps 2g–i** in total of three times.

Note: The cells can also wash for longer time.

Pause point: Fixed cells can be stored in 200 μL PBS at 4°C for up to 3 months. To prevent evaporation of the PBS, the dish should be wrapped with parafilm.

3. (Day 1–2) Staining of fixed cells.
   a. Incubate cells in 150 μL *blocking solution* for two hours at room temperature (20°C–22°C).

   ⚠ CRITICAL: Do not incubate the Ibidi-chamber on a shaker to avoid cell detachment.

   b. Prepare the primary antibodies in diluting the desired antibody concentration in 150 μL *antibody solution* per well. For an estimate of the dilution factor, see the list of primary antibodies in the key resources table.
      i. Well 1: Mix 0.6 μL goat-anti-IBA1 and 1.5 μL rabbit-anti-P2Y12 to get a 1:250 and 1:100 dilution, respectively.
      ii. Well 2: Mix 0.6 μL goat-anti-IBA1 and 0.3 μL rabbit-anti-PU.1 to get a 1:250 and 1:500 dilution, respectively.

   Note: Due to antibody batch effects, each primary antibody has to be validated for their optimized concentration ideally with testing different dilution factors.

   c. After the 2 h, aspirate the *blocking solution*.
   d. Add the *antibody solution* containing the desired primary antibodies.
   e. Incubate the plate overnight (~15–17 h) at room temperature (20°C–22°C).

   Note: It might be beneficial to transfer the plate to 4°C to prevent the denaturation of reagents and evaporation of the solution.

   f. Aspirate the antibody solution.
   g. Perform the washing step described in **steps 2g–j.**
   h. To perform the secondary antibody staining, prepare in total 300 μL antibody solution and add 0.15 μL goat-anti-Alexa Fluor 488 and 0.15 μL rabbit-anti-Alexa Fluor 647 correlating to a 1:2000 dilution.

   Note: Due to antibody batch effects, each secondary antibody has to be validated for their optimized concentration with varying dilution factors.

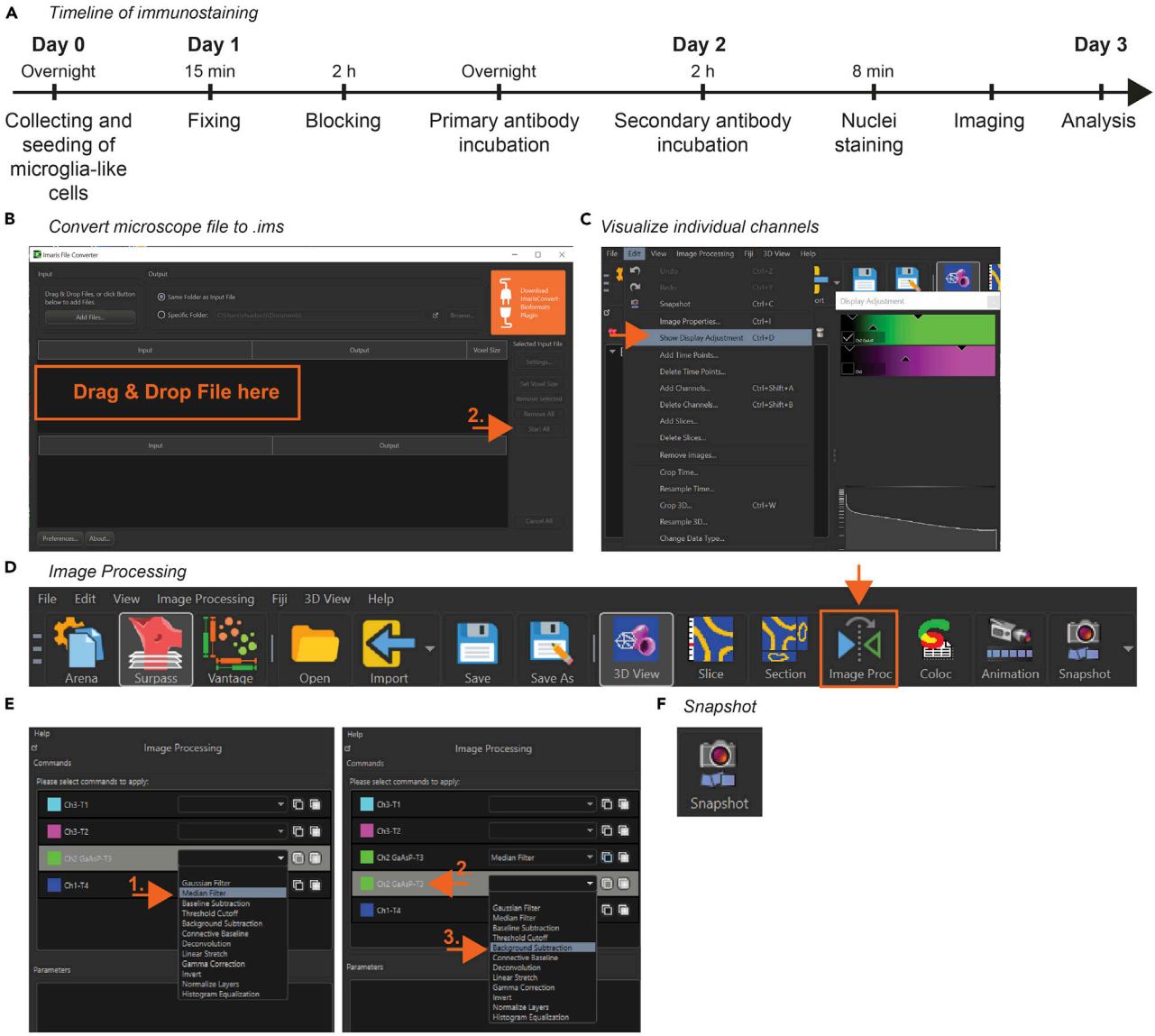

Figure 2. Immunostaining

(A) Schematic overview of immunostaining timeline. H: hours. Min: minutes.

(B–F) Screenshots of Imaris software version 9.9.1. (B) Converting microscope file to imaris file. First, drag and drop files into orange frame and second, click "*Start all*" (arrow). (C) "*Display Adjustment*" for image channel visualization (arrow). (D) Imaris icon for image processing (orange frame and arrow). (E) Arrows indicate: first "*Median Filter*", second "*Source Channel*" and third "*Background Subtraction*". (F) "*Snapshot*" icon.

Note: The fluorophore of the secondary antibodies should be chosen based on the excitation/emission spectra available at the microscope.

i. Add 150 µL of the secondary antibodies containing *antibody solution* into each well.

j. Incubate the cells light-protected for 2 h at room temperature (20°C–22°C).

⚠ CRITICAL: Light exposure causes fading of the fluorophore signal. Therefore, the Ibidi-chamber should be either covered with aluminum foil or kept in a box.

k. Aspirate antibody solution.

l. Perform the washing step described in **steps 2g–j.**
m. To stain the cellular nuclei, dilute 0.1 μL Hoechst 33342 (1:5000) in 500 μL PBS to obtain a *Hoechst-PBS solution.*
n. Add 200 μL of the diluted *Hoechst-PBS solution* per well.
o. Incubated the cells light-protected for 8 min.
p. Perform the washing step described in **steps 2g–j.**

Pause point: The samples can be stored in 200 μL PBS at 4°C in the dark until imaging.

Note: If the samples are stored longer than 1 week, the Ibidi-chamber should be wrapped with parafilm to prevent dry-out of the samples.

4. (Day 2) Imaging of stained cells.
   a. Place the Ibidi-chamber into the specimen holder of the microscope.

Note: Ibidi-chambers can only be imaged with an inverted microscope. We used a LSM880 inverted confocal microscope. Alternatively, an epifluorescence microscope or a spinning disk confocal microscope can be used.

   b. Set up acquisition parameters on the microscope optimized for the excitation/emission spectra of the secondary antibody.
   c. Use a 20× objective to find the region-of-interest and to determine whether the cells expressed the investigated marker.
   d. Use a 40× oil immersion objective to focus on small subset of microglia to investigate expression details.

Note: To distinguish signal from background, a control sample should be imaged at the same time that has been only stained with the secondary antibody.

Analysis

Timing: up to 15 min

Here, we describe how to process images to take snapshots of the immunostaining of interest.

5. Software requirements:
   a. Get access to Imaris and obtain a license: https://imaris.oxinst.com/packages.
6. Convert microscope images to .ims files using the Imaris File Converter (Figure 2B).
   a. Drag and drop the microscope images into the Imaris File Converter.
   b. Press "*Start all*".
7. Open the converted files in Imaris.
8. Open "*Display Adjustment*" to visualize individual image channels (Figure 2C).
9. If necessary, process the images by clicking the "*Image Processing*" icon on the toolbar (Figure 2D).
10. Process images (Figure 2E).
    a. In the dropdown box of each channel select "*Median filter*".
    b. Select the newly generated channel.
    c. Apply "*Background Subtraction*" for each channel and press "*OK*".
11. Visualize the channel or channels of interest.
12. Select the icon "*Snapshot*" (Figure 2F).

Note: As an alternative, the open-source software Fiji/ImageJ can be used.

Part 2: Beads-based phagocytosis assay

Timing: 2 days

Microglia have been shown to phagocytose e.g., apoptotic cells, invading microbes, or synapses.[31–33] This phagocytic behavior can be recapitulated by quantifying the uptake of beads over time.[34] To visualize microglia in a non-fixed environment, tomato lectin (Lycopersicon *esculentum*) can be used, which selectively labels human microglia and blood vessels.[35]

13. (Day 0) Seed collected microglia cells as described in "before you begin – seeding cells". Adjust the culture format and the cell number as outlined in Table 2. Incubate cells at 37°C, 5% CO2 overnight (~15–17 h) (Figure 3A).
14. (Day 1) Preheat the microscope chamber to 37°C.

Note: Depending on the microscope heating chamber, this process will take approximately 30 min. Meanwhile you can proceed with steps 15 and 16.

Note: No CO_2 and humidity control is necessary.

15. Set up the microscope.
    a. Use a 20× air objective.
    b. Activate the 488 nm and 568 nm laser channels.
    c. Set up the microscope so that the two channels 488 nm and 568 nm can be simultaneously imaged and bleed-through is limited.
    d. Set up a time series with 1 cycle/minute and 80 cycles in total.
    e. Activate z-stack option.
    f. Activate an autofocus for each cycle to compensate drifting on the z-axis.

Note: We used a LSM880 inverted confocal microscope. Alternatively, an epifluorescence microscope or a spinning disk confocal microscope can be used.

16. Staining of attached microglia cells for live cell imaging.
    a. Prepare *lectin staining solution* by adding 0.2 µL tomato-lectin to 200 µL DBPS.
    b. Pre-warm *DPBS*, *lectin staining solution*, and *Leibovitz's L-15 medium* to 37°C in a bead bath.
    c. After pre-warming the solutions, aspirate the medium from the Ibidi-chamber.
    d. Wash the cells with 200 µL pre-warmed DPBS.
    e. Aspirate DPBS.
    f. Add 200 µL of the *lectin staining solution*.
    g. Transfer the cells for 20 min at 37°C, 5% CO_2 incubator.

Note: We use a tomato-lectin, which is conjugated to green fluorophore, and the beads are conjugated to a red dye. Alternatively, also other lectins and bead color combinations can be used.

Optional: If the cell line endogenously expressed a fluorophore, the tomato-lectin labeling step can be omitted.

17. Live cell imaging.
    a. Before imaging, aspirate *the lectin staining solution*.
    b. Wash the cells with 200 µL DPBS.
    c. Add 390 µL pre-warmed *Leibovitz's L-15 medium*.
    d. Protect the stained cells from light and transfer the cells to the microscope.
    e. Place the Ibidi-chamber into the specimen holder.

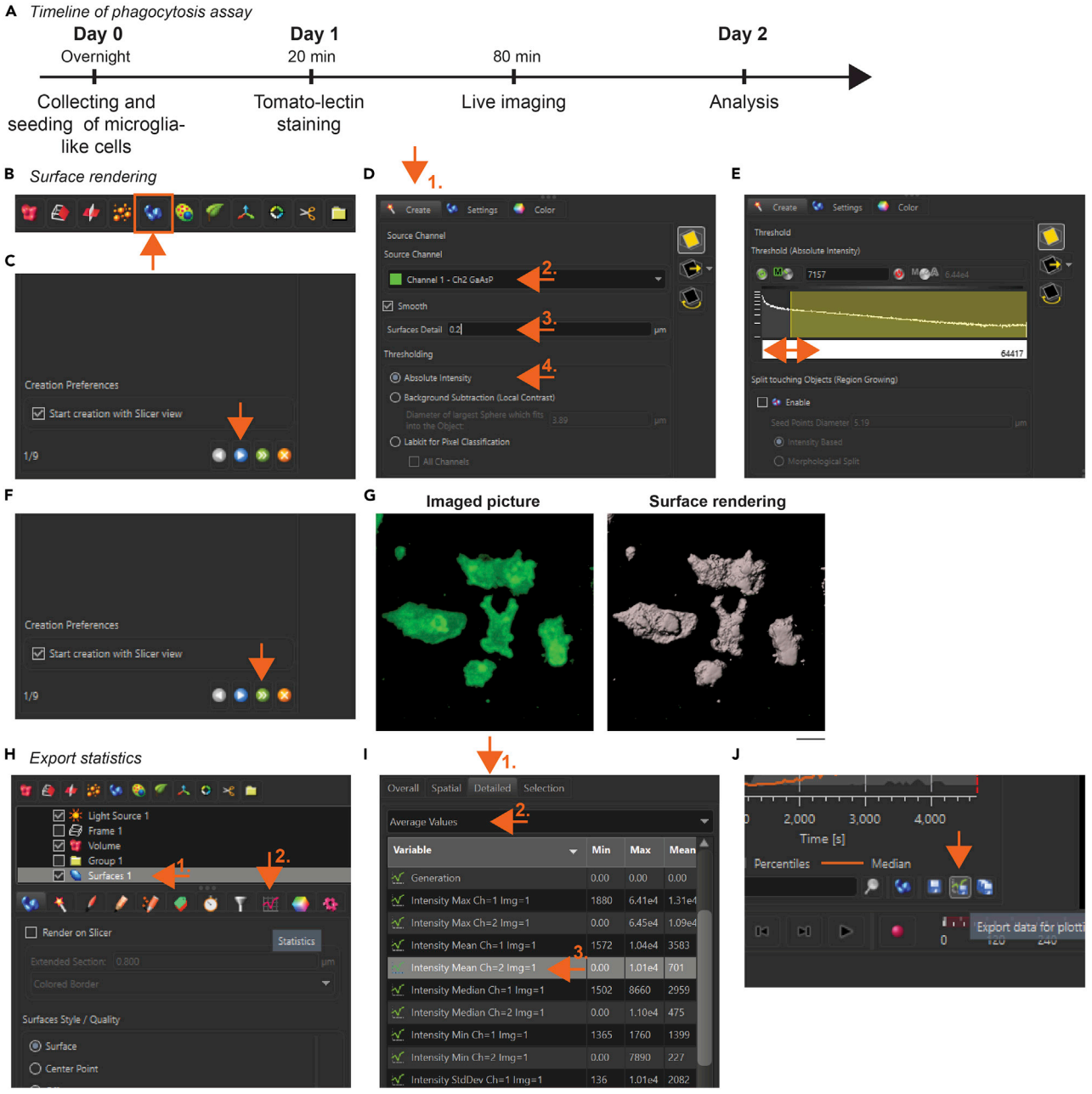

Figure 3. Phagocytosis assay

(A) Schematic overview of phagocytosis assay timeline. Min: minutes.

(B–J) Screenshots of Imaris software version 9.9.1. (B) Icon for *"Surface rendering"* (arrow). (C) Blue icon for *"Next"* (arrow). (D) Arrows point to: first *"Create"*, second *"Source channel"*, third *"Surface detail"* and fourth *"Threshold (Absolute Intensity)"*. (E) Adjustment of *"Threshold (Absolute Intensity)" (arrow)*. (F) *"Finish"* button (arrow). (G) Example of imaged microglia-like cells (left) and the corresponding surface rendered cells (right). (H–J) Export statistics. (H) Arrows indicate: first *"Surface"* tab and second the *"Statistics"* icon. (I) Arrows indicate: first *"Detailed" tab, second "Average Values"* and third the *"Intensity mean (Ch=2)* within the microglia surface *(Img=1)"*. (J) Icon for *"Export data for plotting"* (arrow).

f. Select a representative region-of-interest for imaging.

g. Set a z-stack to acquire the entire volume of the microglia-like cells.

⚠ CRITICAL: The autofocus and the acquisition of one z-stack needs to be finished within 1 min. Therefore, you need to adjust the acquisition speed and number of planes.

h. Before starting the time series, remove the lid of the Ibidi-chamber.
i. Start recording.
j. Acquire 20 min of baseline.
k. Meanwhile, re-suspend beads at 1 mg/mL in pre-warmed *Leibovitz's L-15 medium* according to manufacturer's instructions.
l. Shortly before use, sonicate beads for 30 s at room temperature (20°C–22°C) to homogenously disperse particles.
m. After the acquisition of cycle 20, immediately add 10 μL of freshly sonicated beads in *Leibovitz's L-15 medium* to the Ibidi-chamber without stopping the recording.

⚠ CRITICAL: Avoid touching the Ibidi-chamber while adding the beads to prevent the loss of the region-of-interest. Therefore, the lid should be removed before starting the acquisition.

Note: It is not necessary to re-suspend the solution since we perform a qualitative description and thus the beads do not need to be equally distributed.

n. Keep recording for the remaining 60 min to image the bead uptake.

18. In total, repeat the assay three times from three independent differentiations.

Analysis

Timing: up to 30 min

In the following example, we describe how to quantify the microglia uptake of beads as a parameter of phagocytic activity.

19. Software requirements:
    a. Get access to Imaris and obtain a license: https://imaris.oxinst.com/packages.
    b. To perform the described analysis, the surface rendering function is required.
    c. Get access to R here: https://www.rstudio.com/.
20. For opening and processing the images see **steps 6–10.** Then apply "*Median Filter*" and "*Background Subtraction (local contrast)*" to both channels. Visualize the microglia channel.
21. Click the icon "*Surface rendering*" to create a new surface (Figure 3B).
22. In the newly opened window press the blue icon for "*Next*" (Figure 3C).
23. Create the surface of microglia (Figure 3D).
    a. Select the "*Create*" tab.
    b. In "*Source channel*", select the channel that labels microglia.
    c. Enter the surface detail resolution and keep it consistent across the experiment. In our analysis, we used a surface detail of 0.2 μm.

Note: Imaris is automatically adjusting the value in function of the resolution.

    d. Select ''Absolut intensity'' as the thresholding method.
24. Press the blue icon for "*Next*" (Figure 3C).
25. Adjust the threshold with the slider to visualize microglia surface and minimize background. Use the microglia channel as a reference (Figure 3E).
26. Press the green button for "Finish" (Figure 3F).

Note: This step creates the surface of the microglia-like cells (Figure 3G).

27. Next, determine the intensity mean of the 568 nm channel within the microglia-like cell created surfaces. For this, open the statistics of the created surfaces (Figure 3H).

a. Select the newly generated "*Surface 1*" tab.
b. Select the "*Statistics*" icon.

28. Four new tabs will appear (Figure 3I).
    a. Select "*Detailed*".
    b. Select "*Average Values*".
    c. Select "Intensity Mean Ch=2 Img=1".
29. Select the icon "Export data for plotting" and export data as .xlsx (Figure 3J).
30. To visualize the exported data use e.g., R.

Note: We used the xlsx package[21] to load the excel file into R, the *ggplot2* package[22] to generate plots, and geom_abline() to visualized the 95% confidence interval.

Part 3: Imaging calcium activity after ATP stimulation

Timing: 2 days

Intracellular calcium (Ca^{2+}) has been shown to modulate various microglia properties such as proliferation, migration, and phagocytosis.[36] ATP (adenosin-tri-phosphat) is an important modulator of microglial Ca^{2+}-signaling by activating specific ionotropic and metabotropic purinergic receptors.[37,38] Here, we determine the activity of microglia-like cells based on fluctuations in intracellular Ca^{2+}-events upon stimulation with ATP.

Note: Fluo-4 is an easy-to-use synthetic Ca^{2+}-indicator[39] and suitable for live cell imaging of microglia.[40] Spontaneous and ligand stimulated changes in intracellular Ca^{2+}-levels can be assessed with high sensitivity.

31. (Day 0) Seed collected microglia cells as described in "before you begin – seeding cells". Adjust the culture format and the cell number as outlined in Table 2.
    a. Incubate cells at 37°C, 5% CO_2 overnight (~15–17 h) (Figure 4A).
32. (Day 1) Prepare *1×* Fluo-4 *Ca^{2+}-assay solution* according to manufacturer's instructions and pre-warm 550 µL to 37°C in a bead bath to stain 2 wells of the Ibidi-chamber.

Note: Reconstituted *Fluo-4 Ca^{2+}-assay solution* can be aliquoted and stored for 2 weeks at −20°C.

33. Prepare 200 µL of 10 mM ATP in Leibovitz's L-15 medium.
34. Label attached microglia-like cells with the *1× Fluo-4 Ca^{2+}-assay solution.*
    a. Aspirate the medium.
    b. Add 250 µL of *1× Fluo-4 Ca^{2+}-assay solution* per well.
    c. Incubate cells at 37°C, 5% CO_2 for 30 min.
    d. Remove cells from the incubator, protect the cells from light, and incubate them at room temperature (20°C–22°C) for another 30 min as suggested by the manufacturer's recommendation for other cell types.
    e. Aspirate *1× Fluo-4 Ca^{2+}-assay solution.*
    f. Add 270 µL of pre-warmed *Leibovitz's L-15 medium.*

Note: Label both wells of the Ibidi-chamber simultaneously.

35. Set up the microscope and image calcium activity.
    a. Place the Ibidi-chamber into the specimen holder.
    b. Activate the 488 nm laser channel.
    c. Use a 20× air objective.
    d. Select an area with around 50 cells per field-of-view.

e. Set a time series with 1 cycle/500 ms and a total recording time of 360 s.
f. Before starting the acquisition, remove the lid of the Ibidi-chamber.
g. Record 180 s/360 frames of baseline activity.
h. Immediately afterwards, add 30 µL of the *ATP in Leibovitz's L-15 medium* into the well for a final ATP concentration of 1 mM.
i. Continue the acquisition for the following 180 s.

Note: To minimize photo bleaching, reduce laser power and gain. We used 0.5% laser power of a 25 mW Argon-Multiline laser and a gain of 500 (troubleshooting 4).

Note: Do not stop recording while applying ATP.

⚠ CRITICAL: To prevent the loss of the region-of-interest, avoid touching the Ibidi-chamber with the tip while adding ATP. Therefore, the lid should be removed before starting the acquisition. Pipette the ATP solution carefully onto the surface of medium. Do not re-suspend the medium.

36. To obtain a control, repeat **steps 35d–i** for the second well. Instead of ATP, add *Leibovitz's L-15 medium* at **step 35h.**
37. In total, repeat the assay three times from three independent differentiations.

Analysis

Timing: 1 h

Here we describe a method to visualize Ca^{2+} events occurring in single microglia-like cells with and without stimulation.

38. Software requirements:
    a. Get access to Fiji/ImageJ here: https://imagej.net/software/fiji/.
    b. Get access to R studio here: https://www.rstudio.com/.
39. Open Fiji to batch convert the confocal image .lsm files to .tif files (Figure 4B).
    a. Select "Process".
    b. Select "Batch".
    c. Select "Convert".
40. A "*Batch Convert*" window opens (Figure 4C).
    a. Select "*Input*" directory to define the folder with the files to be run in the batch.
    b. Select "*Output*" directory to define where the files will be saved.
    c. Press the "*Convert*" icon.
41. Record and create the macro code (Figure 4D).
    a. Press "*File*" and "Open" to load an image.
    b. Select "*Plugins*".
    c. Select "*Macros*".
    d. Select "*Record*".
42. The "*Recorder*" window appears (Figure 4E).
43. Apply a Gaussian Blur to the image (Figure 4F).
    a. Select "Process".
    b. Select "Filters".
    c. Choose "Gaussian Blur".
44. Adjust the parameters (Figure 4G).
    a. Set the "Sigma (radius)".
    b. Select "Preview" to check the image before starting the processing.
    c. Select "OK".

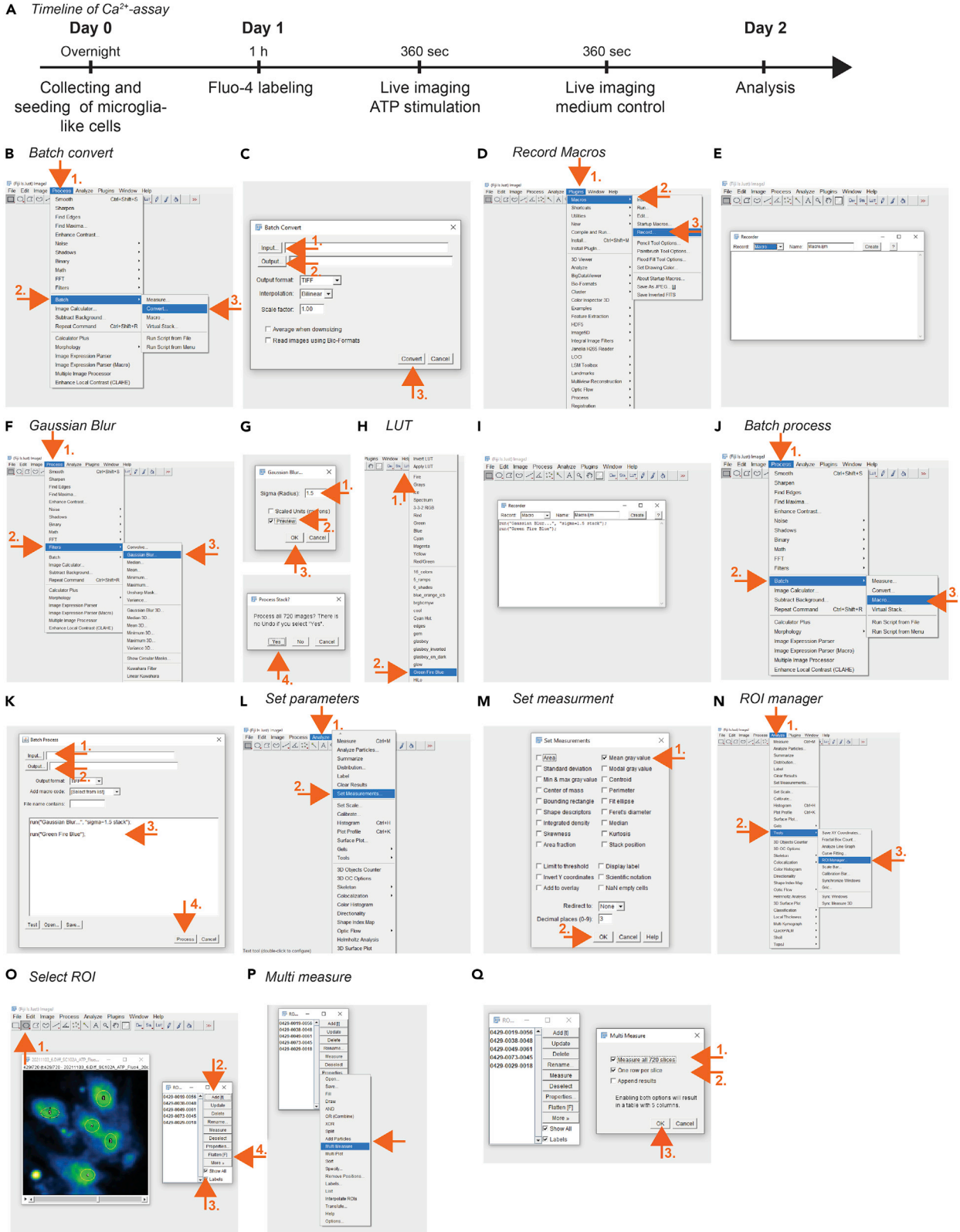
A Timeline of Ca2+-assay
Day 0
Overnight
Collecting and seeding of microglia-like cells
Day 1
1 h
Fluo-4 labeling
360 sec
Live imaging ATP stimulation
360 sec
Live imaging medium control
Day 2
Analysis
B Batch convert
C
D Record Macros
E
F Gaussian Blur
G
H LUT
I
J Batch process
K
L Set parameters
M Set measurment
N ROI manager
O Select ROI
P Multi measure
Q

Figure 4. Ca^{2+}-activity of baseline and ATP-stimulated microglia activity

(A) Schematic overview of Ca^{2+}-imaging timeline. ATP: Adenosin-tri-phosphat. Ca^{2+}: calcium. H: hour. Sec: seconds.

(B–P) Screenshots of ImageJ software version 1.8.0. (B) Batch convert. Arrows indicate: first *"Process"*, second *"Batch"* and third the *"Convert"* tab. (C) *"Batch Convert"* window. Arrows point to: first *"Input"* directory, second *"Output"* directory and third to the *"Convert"* tab. (D) Macro recording. Arrows point to: first *"Plugins"*, second *"Macro"* and third to the *"Record"* tab. (E) Recorder window. (F) Gaussian Blur. Arrows point to: first *"Process"*, second *"Filter"*, and third *"Gaussian Blur"*. (G) Adjusting Gaussian blur. Arrows point to: first "Sigma (radius)", second "Preview", third "OK" and fourth to the "Yes" tab. (H) Look-up-table (LUT). Arrows point to: first *"LUT"* and second *"Green Fire Blue"*. (I) Macro code displayed in "Recorder" window. (J) Batch process. Arrows point to: first *"Process"*, second *"Batch"* and third *"Macro"*. (K) Batch Process window. Arrows indicate: first *"Input"* directory, second *"Output"* directory, third the macro code and fourth the *"Process"* tab. (L) Set parameters. Arrows indicate: first *"Analyze"* and second *"Set Measurements"*. (M) Set measurement window. Arrows indicate: first *"Mean gray value"* and press *"OK"*. (N) Regions of interest (ROI) Manager. Arrows indicate: first *"Analyze"*, second *"Tools"* and third *"ROI manager"*. (O) Selecting ROI. Arrows point to: first *"Elliptic shape"*, second *"Add"* and third to *"Shows all"* and *"Labels"* and forth *"More"*. (P) Multi Measure windows. *"Multi measure"* (arrow).

(Q) Arrows indicate: first *"Measure all xxx slices" second "One row per slice"* and third the *"Ok"* tab.

d. Select "Yes" to process all images.

45. Apply a look-up-table (LUT) to the images (Figure 4H).
    a. Select "*LUT*".
    b. Choose one of the listed options.

Note: We apply a Gaussian Blur with a sigma (radius) of 1.5 to smoothen the pixels and "Green Fire Blue" as LUT to visualize the cells.

46. Save the macro code in the "*Recorder*" window (Figure 4I).
47. Batch process the images (Figure 4J).
    a. Select the "*Process*" tab.
    b. Choose "*Batch*".
    c. Select "*Macro*".
48. The "*Batch Process*" window opens (Figure 4K).
    a. Select the "*Input*" directory.
    d. Select the "*Output*" directory.
    e. Insert the macro code into the empty field.
    f. Select "*Process*" to start the batch processing.
49. Set analysis parameters (Figure 4L).
    a. Select the "*Analyze*" tab.
    b. Choose "*Set measurements*".
50. The "*Set measurements*" window opens (Figure 4M).
    a. Select "Mean grey values".
    b. Press "OK".
51. Open processed file and select your regions of interest (ROIs) (Figure 4N).
    a. Open file and select the "Analyze" tab.
    b. Go to "*Tools*".
    c. Choose "*ROI Manager*".
52. Manually select ROIs (Figure 4O).
    a. Select ROIs on the center of individual cells using an elliptic shape.
    b. Select "*Add*" to have the ROI listed.
    c. Select "*Show all*" and "*Labels*" to visualize and annotate all selected ROIs.
    d. Repeat **step 52b** for all cells of interest.
53. Measure the "Mean intensity levels" for each frame (Figures 4O–4Q).
    a. Select "*More*" and "*Multi Measure*" in the ROI manager to measure the mean grey value for each ROI.
    b. Activate the two options: "*Measure all slices*" and "*One row per slice*" in the *"Multi measure"* window.
    c. Press "*OK*".
54. Export the "*Mean intensity values*".
55. To visualize the exported data use e.g., R.

Note: We used the xlsx package[21] to load the excel file into R, the *ggplot2 package*[22] to generate plots. We normalized the intensity of each cell to its average intensity throughout the entire 360 s of recording and performed a one-sample t-test to compare different conditions.

Optional: An alternative approach is PeakCaller, a MATLAB script that can be used for the quantification of intracellular Ca^{2+}-transients.[23] The output provides various parameters describing the peaks such as amplitude, frequency, rise and decay time and the half maximum width. We used this script to quantify and visualize the sum of Ca^{2+}-events occurring in microglia-like cells within experimental conditions over the whole duration of recording. We used following parameters: required rise = 20% absolute; max. lookback = 700 pts; required fall = 30% absolute; max. lookahead = 700 pts; trend control = exponential moving average (2-sided); trend smoothness = 100; interpolate across closed shutters = true.

⚠ CRITICAL: PeakCaller detects peaks which are not reflecting Ca^{2+}-events. For this reason, we additionally filtered the output in R by including only peaks with a height >0.15 and a FWHM >5.

Part 4: Inflammation assay

Timing: 2 days

Pathological conditions caused by injuries or infections increase the expression of pro-inflammatory cytokines such as IL1β, IL-6 and TNFα in microglia.[33] In this protocol, we identify changes in the mRNA transcript levels in response to inflammatory stimuli. To treat microglia, a combination of IFNy and IL1β,[33] bacterial lipopolysaccharide (LPS)[41] or polyinosinic:polycytidylic acid (poly I:C)[42] can be used.

56. Before starting the experiment, order primers. Alternatively, primers can be also designed for the gene-of-interest.
    a. Download the RNA sequence of your gene-of-interest from e.g., NCBI.
    b. Copy the sequence into Primer-Blast.
        i. Set the product length to 70–200 base pairs (bp).
        ii. Keep the default settings.
    c. Choose a primer pair based on the following parameters:
        i. Primer length: 18–24 bp.
        ii. G/C content: 40%–60%.
        iii. Tm (melting temperature): 50°C–60°C.
        iv. Avoid self-complimentary structures (<8).
        v. Avoid cross-dimer formation (<5).
    d. Order Primers.
    e. Validate primers.
        i. Run a RT-qPCR of a test sample (e.g., untreated harvested microglia-like cells) and check the PCR product size by running an agarose gel.
        ii. Run a RT-qPCR on serial dilutions of your test sample to check the efficiency.
57. (Day 0) Seed collected microglia cells as described in "before you begin – seeding cells". Adjust the culture format and the cell number as outlined in Table 2 and incubate cells at 37°C, 5% CO_2 overnight (~15–17 h) (Figure 5).
58. (Day 1) Pre-warm 2,000 µL *microglia medium* for 4 wells.
59. Dilute stock solutions of inflammatory stimuli IFNy, IL1β, LPS and poly I:C in *microglia medium* to the desired final concentrations:
    a. Prepare 500 µL of IFNy + IL-1β with a concentration of 10 ng/mL each.[43]
    b. Prepare 500 µL of LPS with a concentration of 100 ng/mL.[4]

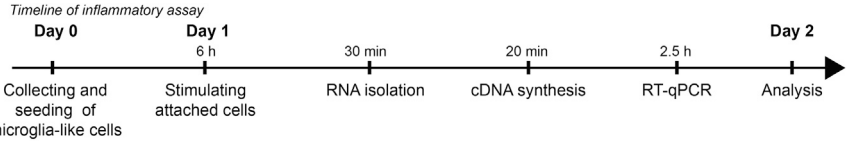

Figure 5. Inflammation assay

Schematic overview of inflammation assay timeline. cDNA: complementary DNA. H: hour. Min: minutes. RT-qPCR: Real-time quantitative PCR (polymerase chain reaction).

c. Prepare 500 µL of poly I:C with a concentration of 50 µg/mL.[44]

60. Stimulate attached microglia-like cells.
    a. Aspirate the medium.
    b. Add 500 µL of diluted IFNγ + IL1β into well 1, 500 µL of diluted LPS into well 2, 500 µL of diluted poly I:C into well 3 and 500 µL *microglia medium* only (control condition) into well 4.
    c. Incubate cells for 6 h at 37°C, 5% CO_2.
61. Isolate RNA with *innuPREP RNA Mini Kit 2.0* as described in the manufacturer's instructions.

⚠ CRITICAL: Work on RNAse-free bench and keep samples on ice. Carefully clean working area before starting RNA isolation with 20% bleach to inhibit RNAse activity.

    a. Take out plate from incubator and place on RNAse-free bench.
    b. Aspirate supernatant.
    c. Add lysis buffer and proceed as described in the manufacturer's instructions.

Note: Sometimes microglia-like cells detach from the surface upon stimulation (troubleshooting 5).

    d. Measure the RNA concentration with Nanodrop Spectrometer.
        i. Perform a reference measurement "blank" of the nuclease-free water in which you diluted your RNA samples.

Note: The expected RNA yield per condition should be between 100-800 ng for each condition (troubleshooting 6).

⚠ CRITICAL: To assess purity of RNA, check that the $A_{260/280}$ ratio is between 1.9–2.1 (troubleshooting 7).

Pause point: Isolated RNA can be stored at −80°C. However, it is recommended to proceed immediately to cDNA synthesis to reduce the risk of RNA degradation.

62. Perform cDNA synthesis using the LunaScript RT SuperMix Kit according the manufacturer's instructions.
    a. Dilute all samples to the same concentration of input RNA for each condition within experimental repetitions.
    b. For each sample, prepare one reaction for cDNA synthesis according to Table 4 and mix by up-and-down pipetting.
    c. Prepare one no RT-control of one sample per biological replicate (Table 5) and mix by up-and-down pipetting.
    d. Prepare a no-template control according to Table 6 and mix by up-and-down pipetting. Set up thermocycler accordingly to Table 7.

Note: Alternative synthesis protocols can be applied.

Table 4. Reaction for cDNA synthesis

Reagent	20 µL reaction	Final concentration
LunaScript RT SuperMix (5×)	4 µL	1×
RNA Sample	15 µL	Up to 1 µg
Nuclease free water	1 µL	N/A

Pause point: Synthesized cDNA can be stored at −20°C.

63. Prepare samples for gene expression analysis with Luna Universal qPCR Master Mix in 384 well plates.
    a. Clean the working area with 20% bleach solution and handle all procedures on ice.
    b. Dilute primers (Table 8) in nuclease-free dH_2O to a concentration of 1 µM.
    c. Determine the total volume for the appropriate number of reactions, plus 10% overage.
        i. 4 housekeeping genes and 3 genes-of-interest (Table 8).

    Note: We are using 4 housekeeping genes, which results in a more accurate and reliable normalization compared to the use of only one single housekeeping gene.

        ii. 4 conditions and no-template control.
        iii. Calculate in technical triplicates.
        iv. Add 10% bonus.

$$number\ of\ reactions = (5\ samples * 3\ replicates) + 10\%$$

    d. Dilute cDNA 1:10 in nuclease-free dH_2O.
    e. Thaw ready-to-use master mix on ice.
    f. Prepare master mix for each gene, 4 housekeeping genes, and 3 genes-of-interest:

Reagent	10 µL reaction	Final concentration	Example for 1 gene for 4 samples + non template control + 10%
DNA template	1 µL	variable	Added later
Forward Primer	0.25 µL	0.25 µM	4.25 µL
Reverse Primer	0.25 µL	0.25 µM	4.25 µL
Luna Universal qPCR Master Mix	5 µL	1×	85 µL
Nuclease-free water	3.5 µL	–	59.5 µL

    Note: Do not yet add DNA template.

    g. Distribute master mix into 384 well plate.
    h. Add DNA samples.
    i. Seal the plate with a thin, topically transparent film.
    j. Spin plate for 1 min at 500 g.
64. Perform quantitative real time PCR (qRT-PCR).
    a. Program light-cycler:

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	60 s	1
Denaturation	95°C	15 s	40–45 cycles
Annealing/Extension	60°C	30 s	
Hold	4°C	forever	

Table 5. Reaction for no RT-control

Reagent	20 μL reaction	Final concentration
No-RT Control Mix (5×)	4 μL	1×
RNA Sample	15 μL	Up to 1 μg
Nuclease free water	1 μL	N/A

b. Start the light-cycler.
c. After completion of the run, select the "Second Derivate Maximum Method" to determine Cq-values and export the data as .xlsx file.

65. In total, repeat the assay 3–5 times using microglia-like cells harvested from independent differentiations.

Analysis

Timing: 1 h

With this step you will analyze the fold changes in inflammatory gene expression levels of treated and untreated microglia-like cells.[45]

66. Export Cq values from the Lightcycler 480 software into an excel file.
67. Calculate the mean Cq values from the three technical replicates for each gene (Equation 1).

$$Cq(mean) = \frac{Cq\ (1) + Cq\ (2) + Cq\ (3)}{3} \quad \text{(Equation 1)}$$

68. Calculate the standard deviation for the mean Cq values for each gene.
69. Remove technical replicates with a standard deviation larger than 0.5.
70. Calculate the geometric mean of the four reference genes (Equation 2).

$$Cq\ (REF\ mean) = \sqrt[4]{Cq\ (REF1) * Cq\ (REF2) * Cq\ (REF3) * Cq\ (REF4)} \quad \text{(Equation 2)}$$

71. Calculate ΔCq values (Equation 3): normalize mean Cq values of all genes of interest to the geometric mean of the reference genes measured within the same sample.

$$\Delta Cq = Cq\ (\text{geometric mean of reference gene}) - Cq\ (\text{gene of interest}) \quad \text{(Equation 3)}$$

72. Calculate ΔΔCq values (Equation 4): normalize ΔCq values of treated samples to untreated control condition within each experimental repetition.

$$\Delta\Delta Cq = \Delta Cq\ (\text{Treated}) - \Delta Cq\ (\text{Untreated}) \quad \text{(Equation 4)}$$

Table 6. Reaction for no template control

Reagent	20 μL reaction	Final concentration
LunaScript RT SuperMix (5×)	4 μL	1×
Nuclease free water	16 μL	N/A

Table 7. Thermocycler conditions

Steps	Temperature	Time	Cycles
Primer Annealing	25°C	2 min	1
cDNA Synthesis	55°C	10 min	
Heat inactivation	95°C	1 min	
Hold	4°C	Forever	

73. Transform ΔΔCq values from log2-scale (Equation 5) to describe fold changes between the treated and untreated group.

$$Fold\ change\ (gene\ expression) = 2^{-(\Delta\Delta Cq)} \quad \text{(Equation 5)}$$

74. Use e.g., the software *R* to visualize and analyze the data.
75. To compare the stimulated condition with its untreated control, perform one-sample t-test and adjust for multiple testing using the Benjamini-Hochberg method.

EXPECTED OUTCOMES

Here, we outline four protocols that allow the identification and functional characterization of microglia.

Part 1: Immunostaining of microglia marker

The morphological shape of the human iPSC-derived microglia-like cells is detectable with IBA1. The expression of the transcription factor PU.1 (Figure 6A) will overlap with the nuclei-dye Hoechst. P2Y12 visualizes microglial morphology (Figure 6B).

Part 2: Beads-based phagocytosis assay

Methods video S1 shows a live cell imaging of the phagocytic activity of hiPSC-derived microglia-like cells. Tomato-lectin labeled microglia (green) gradually take up magenta-labeled beads (Figure 6C). The uptake of the beads can be quantified over time (Figure 6D).

Part 3: Imaging calcium activity after ATP stimulation

Here, we labeled hiPSC-derived microglia-like cells with the Ca^{2+}-sensitive fluorescent dye Fluo-4 (Figure 7A). First, we imaged the fluorescent intensity during baseline activity and then we applied

Table 8. List of real-time quantitative PCR (RT-qPCR) primers

Target	Primer name	Primer pair	Primer sequence 5′-3′
Housekeeping genes	OAZ1	Forward	AGGACAGCTTTGCAGTTCTC
		Reverse	CGGTTCTTGTGGAAGCAAATG
	GAPDH	Forward	GTCTCCTCTGACTTCAACAGCG
		Reverse	ACCACCCTGTTGCTGTAGCCAA
	ACTB	Forward	CACCATTGGCAATGAGCGGTTC
		Reverse	AGGTCTTTGCGGATGTCCACGT
	RPL27	Forward	ATCGCCAAGAGATCAAAGATAA
		Reverse	TCTGAAGACATCCTTATTGACG
Genes of interest	TNFα	Forward	GCACTTTGGAGTGATCGG
		Reverse	TTCGAGAAGATGATCTGACTGC
	IL1β	Forward	ATGATGGCTTATTACAGTGGCAA
		Reverse	GTCGGAGATTCGTAGCTGGA
	IL6	Forward	GGCACTGGCAGAAAACAACC
		Reverse	GCAAGTCTCCTCATTGAATCC

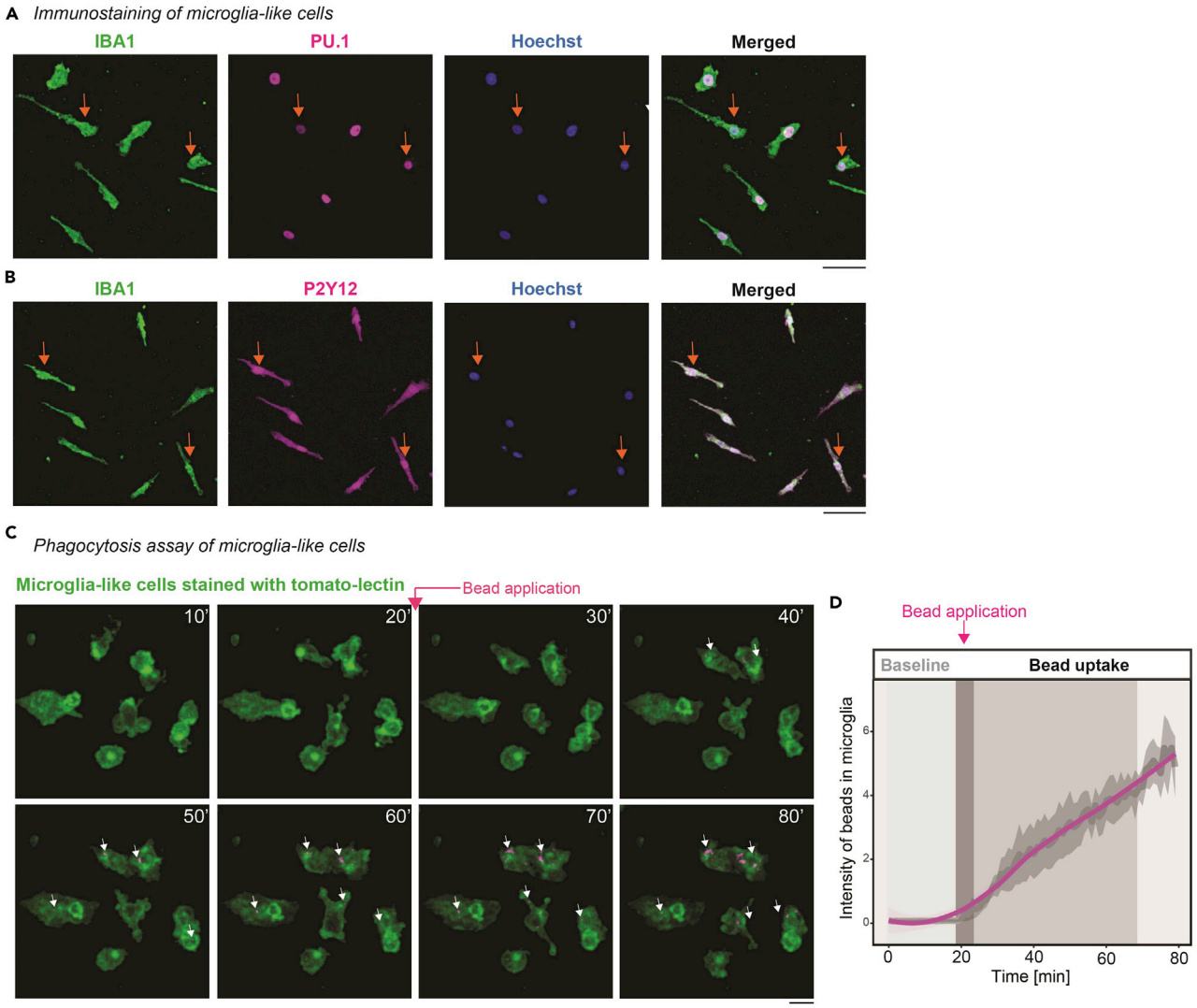

Figure 6. Expected outcome immunostaining and phagocytosis assay

(A and B) Immunostaining of hiPSC-derived microglia-like cells collected from the supernatant and seeded. IBA1 (ionized calcium-binding adapter molecule 1, green), nuclei-dye Hoechst (blue) and (A) PU.1 (hematopoietic transcription factor PU.1, magenta) or (B) P2Y12 (purinergic receptor P2Y G protein-coupled 12, magenta). Arrow: Examples of overlap. Scale bar: 50 μm.

(C and D) Phagocytosis assay using hiPSC-derived microglia-like cells collected from the supernatant and seeded. (C) Consecutive snapshots of live imaged tomato-lectin labeled microglia-like cells (green) and their uptake of fluorescent beads (magenta). White arrow: Phagocytosed beads. Scale bar: 100 μm. (D) Mean intensity increase of beads within microglia-like cells (magenta line) with 95% confidence interval band during 80 min of recording. Three biological replications. Dark grey bar: Bead application after 20 min of baseline recording. Panel (D) re-used with permission from.[1]

ATP which resulted in rapid and synchronized accumulation of Ca^{2+}-events (Figures 7A–7C) in the medium-treated control (Figures 7C and 7D, Methods video S2).

Part 4: Inflammation assay

Finally, we investigated whether hiPSC-derived microglia-like cells trigger an immune response upon stimulation. We confirm an upregulation of the inflammatory signature genes IL6, TNFα and IL1β (Smith et al.[33]) upon treatment with the combination of interferon γ (IFNγ) and interleukin 1β (IL1β), bacterial lipopolysaccharide (LPS) or poly I:C mimicking a viral infection (Figure 7E).

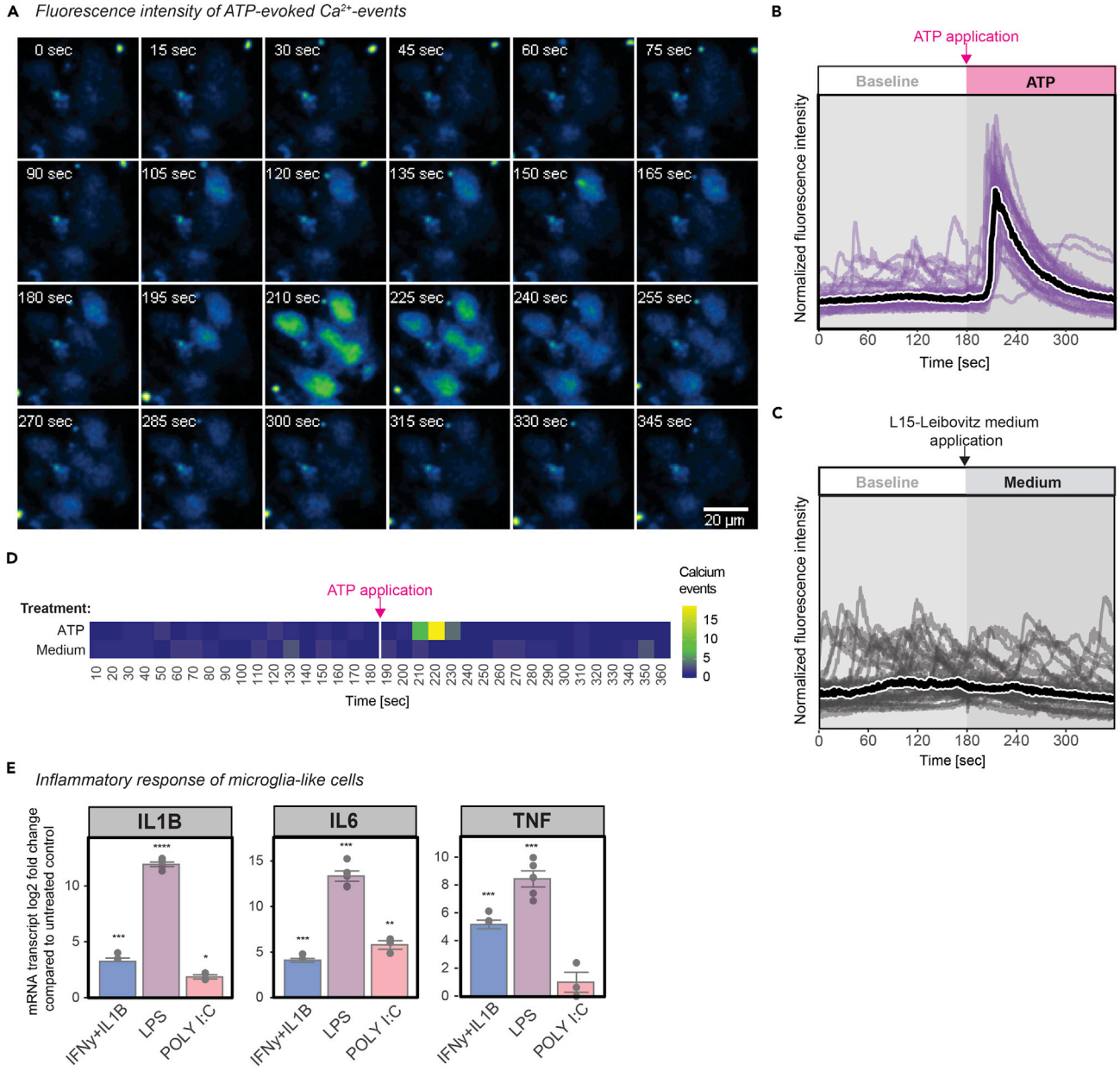

Figure 7. Expected outcome ATP-evoked Ca^{2+}-transients in and inflammatory response of microglia

(A–D) Calcium imaging of hiPSC-derived microglia-like cells collected from the supernatant and seeded. (A) Consecutive snapshots of live-imaged microglia exposed to Ca^{2+}-sensitive fluorescent dye Fluo-4. Scale bar: 20 µm. (B–D) Ca^{2+}-dependent fluorescence intensity normalized to the mean intensity of the cells throughout 360 s of recording (black line). Each curve shows the Ca^{2+} events of an individual cell. Black line: Median of 32 cells from three independent differentiations. After 180 s of baseline measurement (light gray area) either (B) ATP (1 mM final concentration) or (C) medium was applied, and recording was continued up to 360 s (dark gray area). (D) Graph shows sum of software-detected Ca^{2+}-events from all cells of ATP- and medium treated conditions across time in 10 second-bins. Ca^{2+}-dependent fluorescence is displayed through an intensity-based color code (blue-green-yellow) with blue meaning no and yellow the highest transients. White vertical line indicates drug application time point. ATP: Adenosin-tri-phosphat. Ca^{2+}: calcium.

(E) RT-qPCR for interleukin 1β (IL1β, left), interleukin 6 (IL6, middle), and tumor necrosis factor (TNF, right). Microglia-like cells were treated with a combination of recombinant interferon γ (IFN γ) and IL1β, bacterial lipopolysaccharide (LPS) or polyinosinic:polycytidylic acid (poly I:C). Bar chart: mean mRNA transcript log-2-fold changes compared to untreated control cells with SEM. Each dot represents an independent differentiation. One sample t-test. *$p < 0.05$, **$p < 0.01$, **$p < 0.001$ and ****$p < 0.0001$. SEM: Standard error of the mean. Panel (A)-(E) re-used with permission from.[1]

We have successfully applied this protocol for human iPSC-derived microglia-like cells obtained from[1] as well as for the microglia cell line HMC3,[46] a cell line that has been established from human embryonic microglial cells by transfecting a plasmid encoding for the large T antigen of SV40.[47]

LIMITATIONS

Microglia function is context-dependent and adapts quickly to changes in their environment.[48] Here, we investigate microglia cells *in vitro* and therefore their response pattern might differ in *in vivo* situation especially to microglia maturation, function and gene expression. Therefore, co-culturing of microglia with other cell types such as neurons, astrocytes, or oligodendrocytes might affect the output of the different assays.

Another limitation is the dependency of the analysis on commercial software such as Imaris or MatLab, which is provided by the ISTA Imaging and Optics Facility. Alternatively, the open-source program ImageJ can be used for parts of the analysis.

TROUBLESHOOTING

Problem 1

Low cell number when collecting hiPSC-derived microglia-like cells.

The generated microglia-like cell number is too low to perform an assay (step 6 in "before you begin").

Potential solution

The cell number can vary from cell line to cell line, the age of the differentiation, and how often cells were collected. In our experience, we could successfully collect microglia-like cells at least until day 80 of the differentiation. One possibility to maximize the number of collected microglia-like cells is to increase the interval between harvesting to allow the culture to generate sufficient number of cells. Alternatively, the differentiation can be scaled up by doubling the starting material.

Problem 2

Cells are detaching after seeding.

The cells detach from the Ibidi-chamber before fixation or during live cell imaging (step 8 in "before you begin") if they are cultured longer than 48 h.

Potential solution

In our experience, this detachment was mostly the problem in hiPSC-derived microglia than for primary mouse microglial cultures or stable macrophage-cell lines. For hiPSC-derived microglia, we varied the number of seeded cells but could not observe any changes. It is likely that certain supporting factors are missing when hiPSC-derived microglia-like cells are cultured alone. When we optimized the medium with M-CSF, the detachment was reduced. However, certain factors might be still required. We recommend to keep the time from seeding to the experiment as short as possible. If this does not help, coating the dishes e.g., with PLL or Matrigel might help. A caveat of the coating might be that it affects microglial gene expression. In this case, the consistency between replicates and appropriate controls has to be considered.

Problem 3

Immunostaining of surface markers does not work.

The staining for some microglia markers expressed on the surface e.g., CX3CR1 does not work or looks dotted (step 2 in "Immunostaining of microglia marker").

Potential solution

First, the antibody should be validated in human tissue as outlined in the notes under Table 3. Second, to distinguish the signal from background, a control with the secondary antibody alone should be included. Finally, successful immunostaining of surface proteins depends on the fixing conditions. An alternative fixative is methanol, which preserves the membrane proteins.[49] For this, 150 μL ice-cold Methanol puriss which has been pre-cooled at −20°C, were added to the cells. The cells are incubated for 10 min at 4°C. Then, aspirate the methanol and wash the cells three times with 200 μL PBS.

⚠ CRITICAL: Handle methanol solutions in a ventilated hood and collect solutions into flame-proof hazardous waste disposal container.

Problem 4

Photo bleaching during Ca^{2+}-imaging.

The Fluo-4 intensity decreases over time (step 35 in "Imaging calcium activity after ATP stimulation").

Potential solution

To avoid photo bleaching, lower the laser power and the gain and open the pinhole, which allows to reduce the laser intensity. A short exposure time can be also achieved with decreasing the resolution (e.g., to 512 × 512 pixels) and a bidirectional scan mode.

Problem 5

Microglia-like cells detach upon stimulation upon inflammatory treatment.

Upon stimulation, microglia-like cells might detach from the plate. This effect can be observed if cells are incubated for longer periods (>24 h) after seeding (step 61 in "Inflammation Assay").

Potential solution

Harvest the detached cells by centrifugation: Transfer the supernatant into an Eppendorf tube and centrifuge. Aspirate the medium and add half of the lysis buffer used for RNA isolation to the Eppendorf tube and half of the lysis buffer to the corresponding well. Then combine lysate and continue the protocol.

Problem 6

Low RNA yield.

The RNA yield can be too low due to two reasons: Either, the collected microglia cell number was not sufficient, or the microglia detached during stimulation (step 61 in "Inflammation Assay").

Potential solution

Switch to an RNA isolation protocol that is more suitable for low amounts of RNA. We experienced that the RNeasy Micro Kit from Qiagen is more suitable for RNA isolation of cell numbers less than 5×10^5.

Problem 7

Purity of RNA isolate is low.

If the A260/280 ratio is too low or too high (step 61 in "Inflammation Assay").

Potential solution

The ratio of absorbance at 260 nm and 280 nm provides information about the RNA purity and a ratio of ~2.0 is accepted as "pure" RNA. Abnormal 260/280 ratios indicate a contamination by residual phenol, guanidine, or other reagent used in the extraction protocol. In this case, increase the number of washing steps. Inaccurate ratios may also be observed at very low RNA concentrations (< 10 ng/μL). If the RNA concentration is too low, the experiment has to be repeated and the RNA re-eluted in a smaller amount of nuclease-free water.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Sandra Siegert (ssiegert@ist.ac.at).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate any unique datasets or codes.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101866.

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AUTHOR CONTRIBUTIONS

Conceptualization and writing – original draft, V.H., M.K., S.S.; Funding acquisition, S.S.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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