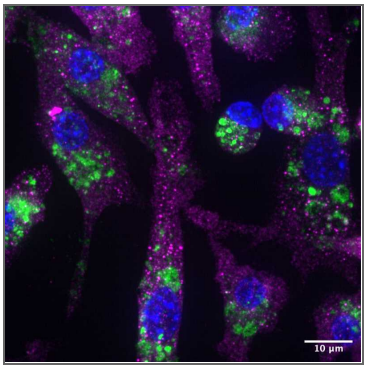

LRRK2 and LAMP1 immunofluorescence staining in various cell lines

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DISCLAIMER

ABSTRACT

This protocol is being used to test the antibody Recombinant Anti-LRRK2 antibody [MJFF2 (c41-2)] (ab133474) **Abcam**, as well as Anti-LAMP1 antibody [1D4B] (ab25245) **Abcam**. Please note that after multiple rounds of optimization, anti-LRRK2 antibody generates significant non-specific signals in the LRRK2 KO cell lines so this antibody may not be reliable for Immunofluorescence staining experiments. The protocol can be used for general IF experiments.

Protocol status: Other
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GUIDELINES

GDB buffer was used as blocking buffer in the optimized protocol.
This protocol uses ibidi 8 Well Chamber µ-Slides and will coat them with Fibronectin. Standard volume would be 300 uL for each well. Volumes will need altering for wells, other plates and slides.
All steps are performed at room temperature (RT) on the lab bench, except for methanol permeabilization.

MATERIALS

- (0.1M) NaPi pH 7.4
  3.1 g of NaH2PO4•H2O
  0.9 g of Na2HPO4 (anhydrous)
  distilled H2O to make a volume of 1 L
  The pH of the final solution will be 7.4. This buffer can be stored for up to 1 mo at 4°C
- GDB buffer
  30mM NaPi (sodium phosphate) pH 7.4
  0.45mM NaCl
  0.2% porcine (or fish) gelatin
  In ddH2O
- Kim wipes
- Ethanol (100%, stored in dark chemicals cupboard)
- Water (double-deionised H2O from Milli-Q, “MQ-H2O”)

SAFETY WARNINGS

N/A

BEFORE START INSTRUCTIONS

Important note:
While LAMP1 immunofluorescence staining robustly gives expected results, after multiple rounds of optimization, anti-LRRK2 antibody generates significant non-specific signals in the LRRK2 KO cell lines in our hand. This is a warning that this antibody may not be reliable for Immunofluorescence staining experiments. However, this protocol can be used widely for IF experiments.
Please check This google sheet to learn more about the cell lines and conditions tested for the antibody

Day 0: : Seed cells

1h

1 Add 300 µL of [M] 11 ug/mL fibronectin into each ibidi well. Incubate at RT for 01:00:00. 1h

2 Rinse the wells with PBS for 3 times

3 Make GDB buffer if necessary

	Stock	Amount needed	C	Final conc.	E
	(0.1M) NaPi pH 7.4	15	mL	30	mM
	(5M) NaCl	0.0045	mL	0.45	mM
	Gelatin	0.1	g	0.2	%
	H2O	34.9955	mL		
	Total	50	mL		

Recipe to make GDB buffer

4 Seed adherent cells to 40-80% confluency in each well. Incubate at 37 °C Overnight to get optimal seeding.

Note

For RAW264.7 cells, 6x10^4 in 300uL is a good starting point. Less would be needed for other typical cell lines since macrophage cells are smaller.
It is suggested to start with two different cell concentrations for the first time. Please refer to this page for more information

Day 1: Drug treatment 3h

5 Apply any drug treatments or controls and note time of additions before proceeding with fixing. As an example, 3h

Chloroquine diphosphate salt **Merck MilliporeSigma (Sigma-Aldrich) Catalog #C6628-25G**

and

Leu-Leu methyl ester hydrobromide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #L7393-500MG**

can be added at desired concentrations for 03:00:00

	Drugs	MW - g/mol	mM	weight in 1 mL	E
	LLOME	339.27	1000	339.27	mg/1mL
	CQ	515.86	100	51.586	mg/1mL

Drug stock recipe and concentrations

In each well, add 1 in 1000 ([M] 1 millimolar (mM) for LLOME and [M] 0.1 millimolar (mM) for CQ)

Staining

25m

6 Put 100% ethanol on ice before proceeding with next steps.

7 Bring GDB buffer to Room temperature . Prepare and prewarm fixation buffer (4% sucrose, 3% PFA in 1xPBS) at 37 °C

Note

3% PFA is preferred from 4% as it reduces autofluorescence
Need 375 µL
Pierce™ 16% Formaldehyde (w/v) Methanol-free **Thermo Fisher Scientific Catalog #28906**
and 0.08 g Sucrose **Merck MilliporeSigma (Sigma-Aldrich) Catalog #S0389**,
dissove with 2 mL PBS.

8 Get cells, aspirate media and immediately add prewarmed fixation buffer. Incubate for 00:10:00 at 37 °C 10m

9 Aspirate PFA, rinse 2x with PBS and wash two times with PBS for 5 minutes each, 00:10:00 in total 10m

10 Quenching: Only necessary when staining the day of fixation. Incubate 3 times of 10 minutes (00:30:00 in total) using 0.4% Ammonium chloride **Merck MilliporeSigma (Sigma-Aldrich) Catalog #254134** ([M] 75 millimolar (mM)) 30m

11 Optional step: For half of the samples, choose to add another permeablisation step with 300 µL 100% ethanol at -20°C for 00:20:00 . Leave the rest at Room temperature in GDB buffer. 20m

> **Note**
>
> This step helps to increase contrast when imaging Recombinant Anti-LRRK2 antibody [MJFF2 (c41-2)] (ab133474) **Abcam** , but doesn't help with the issue of non-specific signals when LRRK2 KO cell lines are used.

> **Note**
>
> ethanol/methanol permeablisation at -20 °C will disrupt the microtubule staining. Apply this step with caution when other antibodies are used.

12 Apsirate ethanol, wash 2x with PBS.

13 Prepare GDB + 0.05% Triton X-100 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #X100** freshly. Add 300 µL for 00:10:00 . 5 µL in 10 mL GDB buffer. Can be stored at 4 °C for a couple of days. 10m

14 Aspirate Triton X-100, wash 2x with GDB.

15 Block cells with 300 µL GDB for 00:30:00 at Room temperature 30m

16 During blocking, prepare final concentration of [M] 1 ug/mL for both Recombinant Anti-LRRK2 antibody [MJFF2 (c41-2)] (ab133474) **Abcam** and Anti-LAMP1 antibody [1D4B] (ab25245) **Abcam** solution with GDB and 50000 rpm, 4°C, 00:10:00 - each ibidi dish well requires 150 µL 10m

Note

150 µL is the minimum required amount. 200 µL would be sufficient and optimal to cover the entire well

17 Take most of the supernatant and place in new tube. Mix to get even concentration (due to Gelatin **Merck MilliporeSigma (Sigma-Aldrich) Catalog #G2500** in there, there will be a small clear precipitate)

18 Aspirate blocking solution and incubate cells with 150 µL of primary antibody solution Overnight at 4 °C on a table-top shaker

Note

Primary antibody incubation can be as short as 2 hours without affecting the final outcome

Note

When incubating overnight, consider wrapping up the dish with parafilm and/or put the dish in a humidity chamber to prevent the well from drying up

Day 2 15m

19 Bring GDB to Room temperature. Aspirate antibody solution and rinse 2x with GDB.

20 Wash 3x 5min Room temperature RT with GDB, 00:15:00 in total 15m

21 Prepare 1:500 Alexa-flour conjugated secondary antibody solution with GDB - each ibidi well requires 200 µL.
When LRRK2 and LAMP1 are co-stained,
Goat Anti-Rabbit IgG H&L (Alexa Fluor® 647) (ab150079) **Abcam** and
Goat Anti-Rat IgG H&L (Alexa Fluor® 488) (ab150157) **Abcam** are used at final concentration of [M] 4 ug/mL. Lower concentration did not help with the issue of non-specific LRRK2 signal.

22 Incubate cells with 200 µL of secondary antibody solution for 01:30:00 at Room temperature and protect from light with an ice box. 1h 30m

23 15 minutes before the incubation is finished, add DAPI **Thermo Fisher Scientific Catalog #D1306** to a final concentration of [M] 1 ug/mL and incubate until the last step finishes.

24 Rinse cells with 1xPBS for 5 times

25 Apply 2-4 drops of FluorSave™ Reagent **Merck MilliporeSigma (Sigma-Aldrich) Catalog #345789** hard mounting media in each well and swirl to make sure the bottom is fully covered.

26 Allow to air-dry for 00:10:00 at Room temperature . 10m

27 Image within 48 h of mounting or the sample will begin to deteriorate (bright debris impeding imaging) and visibly autofluoresce in red.