Antibody screening by Immunofluorescence

YCharOS Standard Operating Procedure

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1. Introduction

1.1. Objectives

This protocol is used to monitor and compare the performance of a large number of commercial antibodies in an immunofluorescence experiment. The use of knockout cells allows to unambiguously distinguish specific signals from non-specific signals[1].

1.2. Protocol overview

Immunofluorescence is a variable application that requires specialized knowledge and training to avoid user-dependent bias. Critically, to reduce imaging/analyses bias, wildtype (WT) and knockout (KO) cells are plated as a mosaic with different colored fluorescent cell dyes used to differentially label WT and KO cell lines. The WT and KO cells are then plated in a 1:1 ratio. Immunostaining is performed, and WT and KO cells are imaged on the same coverslip/well[1]. The immunofluorescence protocol was inspired by several available protocol from laboratories[1,2] and from antibody suppliers[3-5].

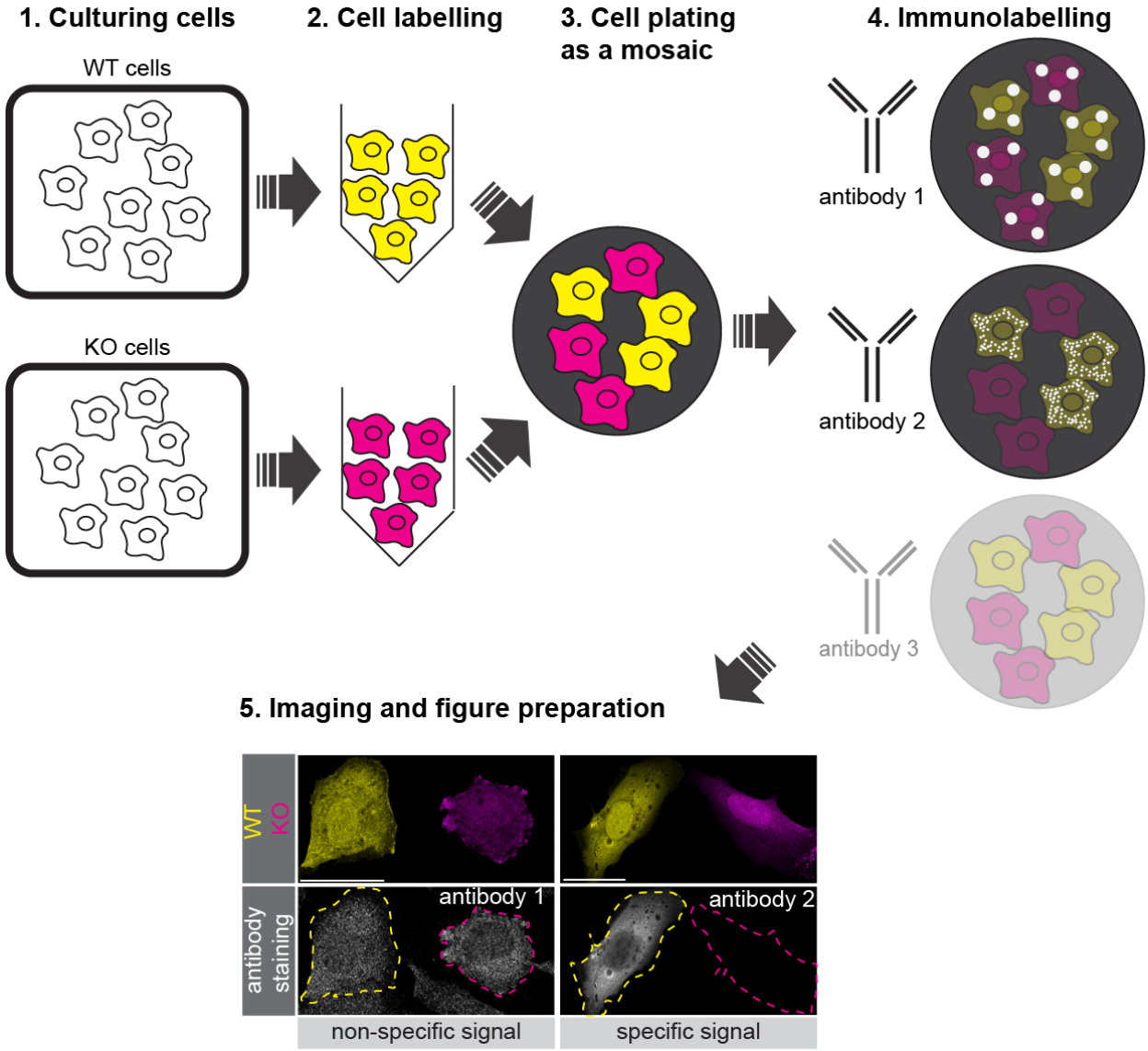

Figure 1: Schematic representation of the antibody screening strategy for immunofluorescence.

1.3. Technical and safety considerations

The following information should be read before starting the procedure:

• Ensure that the cultured cells are mycoplasma-free.

• Ensure sterile measures for handling the cells to avoid contamination; use sterile tubes, plates, pipets and tips.

• Cell lines in culture must be handled in a Class II biosafety laminar flow cabinet to create a sterile environment for cells and to protect the user from possible biohazards. Recommended institutional Environmental Health and Safety (EHS) office regulations must be followed.

• Personal protective equipment, including a lab coat, face mask and disposable gloves must be worn while handling cells.

• Cells should be maintained at 37°C with 5% CO_2.

• Diluted paraformaldehyde solution (4%) may be used on the benchtop in small quantities.

• Wear standard nitrile laboratory gloves, chemical splash goggles, face shield, and lab coat when handling 4% formaldehyde.

• Formaldehyde spills must be cleaned properly by trained personnel who are not sensitive to formaldehyde. All other persons should leave the area.

Table 1: Abbreviation list

BSA	Bovine serum albumin
cm	Centimeter
DAPI	4′,6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
EHS	Environmental Health and Safety
FBS	Fetal bovine serum
h	Hour
IF	Immunofluorescence
KO	Knockout
L	Liter
MeOH	Methanol
ml	Milliliter
µl	Microliter
µM	Micromolar
min	Minute
M	Molar

NGS	Normal goat serum
PFA	Paraformaldehyde solution
PBS	Phosphate buffer saline
PMT	Photomultiplier tube
RT	Room temperature
WT	Wildtype

2. Materials

Refer to the product datasheet from the supplier for further details on storage and preparation instructions. It is important to highlight that the lot number for some reagents can vary and the use of reagents coming from different lots might affect the efficiency of the protocol.

Table 2: Labware

Labware	Supplier
Bottle top filter 0.2 µm	Thermo Scientific
150 mm cell culture dish	Corning, cat. number 430599
Cell culture dish, 4-well	Thermo Fisher Scientific, cat. number 176740
Cell culture dish, 24-well	VWR, cat. number 734-2325
Conical tube, 1.5 mL	Sarstedt, cat. number 72.706
Conical tube, 15 mL	VWR, cat. number 89039-664
Conical sterile tube, 50 mL	VWR, cat. number 89039-656
Cover glasses	Fisher Scientific, cat. number 12-545-81
Cryovials	Fisher, cat. number 09-761-71
Cryobox	Thermo Scientific, cat. number 5115-0012
Dual-chamber counting slides	Biorad
Parafilm	VWR, cat. number 52858-032
Pipet tips, P200	VWR, cat. number 14229-872
Pipet tips, P1000	Sarstedt, cat. number 70.3050.205
Plastic serological pipet, 5 mL	VWR, cat. number 89130-896
Plastic serological pipet, 10 mL	VWR, cat. number 89130-898

Plastic serological pipet, 25 mL	VWR, cat. number 89130-900

Table 3: Reagent

Reagent	supplier
Boric acid powder	Fisher Scientific, cat. number A76-3
Bovine serum albumin	Wisent, cat. number 800-095
CellTracker Green CMFDA Dye	Thermo Fisher Scientific, cat. number C2925
CellTracker Deep Red Dye	Thermo Fisher Scientific, cat. number C34565
Dako Mounting Medium	Dako, cat. number S3023
DAPI	Thermo Fisher Scientific, cat. number D3571
Dimethyl sulfoxide (DMSO)	Sigma Aldrish, cat. number D8418
Goat anti-mouse Alexa 555	Invitrogen, cat. number A21424
Goat anti-rabbit Alexa 555	Invitrogen, cat. number A21429
Goat serum	Gibco, cat. number 16210-064
Fetal Bovine Serum (FBS)	Wisent, cat. number 080450
HyClone DMEM	Fisher Scientific, cat. number SH30081.01
Sodium Hydroxide, 10.0 N	VWR, cat. number BDH3247-4
Sodium Chloride	VWR, cat. number CA97061-266
Sodium Phosphate monobasic anhydrous	Thermo Fisher Scientific, cat. number BP329-1
L-glutamine	Wisent, cat. number 609-065-EL
PFA (4%) in PBS	Thermo Fisher Scientific, cat. number J61899
PBS	Wisent, cat. number 311-010
Penicillin-Streptomycin	Wisent, cat. number 50-201-EL
Phosphate-Buffered Saline	Wisent, cat. number 311-010-CL
Poly-L-lysine	Sigma Aldrish, cat. number P9155-5MG
PH 4 buffer solution (red)	Fisher Scientific, cat. number SB101-500
PH 7 buffer solution (yellow)	Fisher Scientific, cat. number SB107-500
PH 10 buffer solution (blue)	Fisher Scientific, cat. number SB115-500

Triton X-100	Fisher Scientific, cat. number BP151-500
Trypsin/EDTA	Wisent, cat. number 325-542-EL

Table 4: Equipment

Equipment	Supplier
Class II Biosafety Cabinet	Thermo scientific 1300 series A2
Co_2 water jacketed incubator	Forma scientific
Isotemp 220 cell Culture water bath	Fisher Scientific, cat. number FS-220
Inverted light Microscope	Motic AE2000
Microcl 21 centrifuge	Thermo electron corporation
PH meter	Fisher Scientific
TC20 Automated Cell Counter	Biorad
Microscope	Zeiss LSM-880 inverted microscope

Table 5: Software

Software	supplier
Zen2 black for imaging	Zeiss
Adobe Illustrator	Adobe Inc.
Zen Lite V 2.3 for image processing	Zeiss

3. Protocol

3.1. Preparation of required reagents and buffers

3.1.1. Preparation of poly-L-lysine stock solution

a) Resuspend 5 mg of poly-L-lysine with 4 ml of ddH_2O to make a stock at 1 mg/ml.
b) Complete to 5 ml with ddH_2O.
c) Dilute the poly-L-lysine stock 1:100 with borate buffer pH, 8.4 to generate a final concentration at 10 µg/ml.
d) Sterilize by filtration using a 0.2 µm filter unit.
e) Store at room temperature.

Recipe: 0.15 M Borate buffer

Procedure for 500 ml

a) Weigt 4.64 g of boric acid powder.

b) Add the boric acid to a glass beaker containing 450 ml of ddH_2O.
c) Stir using a magnet until the powder is completely dissolved.
d) Adjust the PH to 8.4 using 10N NaOH.
e) Complete to 500 ml with ddH_2O.

3.1.2. Poly-L-lysine coating of coverslips

Coating is done in a Class II biosafety cabinet with sterile condition.

a) Add 500 µl of poly-L-lysine solution into each well of the 4-well or 24-well culture plates containing one previously autoclaved coverslip.
b) Incubate 1 h.
c) Aspirate the poly-L-lysine and wash the wells with sterile ddH2O.
d) Store sterile poly-L-lysine coated coverslips until needed.

3.1.3. PBS (5X)

Recipe: 770 mM NaCl, 288 mM Na_2H2PO_4 monobasic

Procedure for 1 L:

a) Add in a 1 L glass beaker 800 mL of ddH_2O.
b) Add 45 g of NaCl to the ddH2O.
c) Add 34.5 g of Na_2HPO_4 to the solution.
d) Adjust solution to pH ≈ 7.4.
e) Complete to 1 L with distilled water.

3.1.4. Permeabilization buffer

Recipe: PBS/0.1% Triton X-100

Procedure:

a) Add 500 ml PBS to a 500 ml glass bottle.
b) Add 500 µl Triton X-100
c) Mix well
d) Store at 4 °C

3.1.5. PBS/0.01% Triton X-100

Procedure for 500 ml:

a) Add 500 ml of PBS to a 500 ml glass bottle.
b) Add 50 µl Triton X-100
c) Mix well
d) Store at 4 °C

3.1.6. Blocking buffer, prepare fresh

Recipe: PBS/0.01% Triton X-100 + 5% BSA + 5% NGS[a]

Procedure for 40 ml:

a) Add 30 ml of PBS/0.01% Triton X-100 to a 50 ml conical tube.
b) Add 2 g BSA
c) Add 2 ml NGS
d) Rock slightly at 4°C until the BSA is dissolved
e) Complete to 40 ml with PBS/0.01% Triton X-100
f) Keep on ice

[a] Use 5% serum from the species the secondary antibody was raised in.

3.1.7. IF buffer, prepare fresh

Recipe: PBS/0.01% Triton X-100 + BSA (5%)

Procedure for 40 ml:

a) Add 30 ml of PBS/0.01% Triton X-100 to a 50 ml conical tube.
b) Add 2 g BSA
c) Vortex until dissolved
d) Complete to 40 ml with PBS/0.01% Triton X-100
e) Keep on ice

3.2. Cell preparation for immunolabeling

IMPORTANT: All cell manipulations must be done in a Class II biosafety cabinet with sterile media and solutions prewarmed to 37°C.

WT and KO cells are cultured separately in a 15 cm dish containing 25 mL of media in a 37°C incubator with 5% CO_2. The composition of the complete culture media depends on the cell line used. Follow the recommendation of the suppliers.

3.2.1. Cells labeling with fluorescence dyes

a) From a 15 cm dish with confluent adherent cells, wash cells twice with 15 ml of sterile PBS. Discard the PBS.
b) Add 5 mL of Trypsin to both the WT and the KO cell plates. Incubate both plates in the 37°C/5% CO_2 incubator until cells have detached from the cultured plate. Incubation time depends on the cell line used.
c) Verify under microscope that the cells have detached and then inactivate trypsin by adding 5 mL of complete DMEM.
d) Collect both WT and KO cells in a separate 15 ml canonical tube. Centrifuge at 1.2 x g for 3-5 min to pellet the cells. Discard the supernatant.
e) Label both the WT and KO cells as follow:
    a. Resuspend the WT cell pellet with 2 ml of the cell culture media containing 5% FBS together with 5 µM of CellTracker Green CMFDA Dye.
    b. Resuspend the KO cell pellet with 2 ml of the cell culture media containing 5% FBS together with 1 µM of CellTracker Deep Red Dye.
f) Incubate the 15 ml tubes in the incubator at 37°C/5% CO2 for 30 min with the lid slightly open. Gently tap the bottom of each tubes every five min to put the cells back in suspension.
g) Centrifuge both tubes at 1.2 g for 5 min.
h) Discard the supernatant.
i) Resuspend each cell pellet with complete culture media and count cells.
j) Plate appropriate numbers of cells as a mosaic of WT and KO cell at a 1:1 ratio on poly-L-lysine treated coverslips in 24- or 4- well dishes.
k) Transfer the plates to the 37°C/5% CO2 incubator for overnight incubation.

3.2.2. Cell fixation (PFA)

a) Aspirate the culture media.
b) Add 0.5 ml of PFA (4.0%) in PBS and incubate for 15 min at RT.
c) Aspirate and wash 3x with 0.5 ml PBS at RT.
d) Coverslips can be stored at 4°C when protected from light. Avoid evaporation from the plate using parafilm.

3.2.3. Cell fixation (MeOH)

In the case where no antibodies show specific signal using the PFA fixation protocol in 3.2.2, all antibodies are screened again using the following MeOH fixation protocol:

a) Aspirate the culture media.

b) Add 0.5 ml of 100% MeOH (chilled at -20°C) and incubate on ice for 5 min.
c) Aspirate and wash 3x with 0.5 ml PBS at RT

The permeabilization step of MeOH-fixed cells is not required. Directly proceed with the blocking step (3.2.4c).

3.2.4. Immunolabelling procedure

a) Incubate coverslips for 10 min at RT with 0.5 ml of permeabilization buffer.
b) Wash coverslips 3x with 0.5 ml of PBS.
c) Incubate coverslips for 30 min at RT with 0.5 ml of blocking buffer.
d) Incubate overnight at 4°C with primary antibody (starting with a dilution at 1.0 µg/ml) in 0.3 ml of IF buffer.
e) Incubate one coverslip with the IF buffer without primary antibody overnight at 4°C (no primary antibody control).
f) Wash coverslip 3 times for 5 min with 0.5 ml of IF buffer.
g) Incubate coverslips with 1.0 µg/ml of goat anti-mouse or goat anti-rabbit coupled to Alexa 555 in 0.3 ml of IF buffer for 1h at RT.
h) Wash coverslips 3x for 5 min with 0.5 ml of PBS.
i) When needed, add DAPI at concentration of 5 nanogram/ml in the last wash for 3 min.
j) Mount on microscopy slide using DAKO as a mounting medium.

3.2.5. Cell imaging

Microscope: imaging was done on the Zeiss LSM 880 microscope using the manual confocal mode. Please refer to Table 6 and Table 7 for appropriate set-up of the microscope.

Table 6: Microscope set up

Channel	**Laser for excitation**	**Detector**
DAPI	-Solid state laser 405nm, 30 mW -Laser set up at 1-2%	PMT
Alexa 488	-458/488/514nm, 25 mW -Laser set up at 1-30% depends on signal strength	PMT
Alexa 555	-Solid state laser 561nm, 30 mW -Laser set up at 1-30% depends on signal strength	PMT
Alexa 633	-Solid state laser 633nm, 5 mW -Laser set up at 1-30% depends on signal strength	PMT

Table 7: Imaging setup and microsope settings

Objective lens	Plan-Apo 63x/1.40 Oil
Mode of acquisition	Sequential scan mode
Image bit depth	8 bits
Scan speed	Adjust the scan speed to achieve pixel dwell time of ~1 µs/pixel
Frame size	1024 × 1024 pixels for full frame and/or zoom into a smaller region of interest.

Pinhole diameter	1 Airy Unit
Averaging	Averaging over 4 frames
Image volume	Images were acquired as a single optical section through the cells
Image saving	CZI file format
Gain and laser power	Please refer to section 3.2.5. Excess of gain or laser power can led to excitation of fluorophore(s) outside the wanted channel.

3.2.6. Imaging consideration[6]

a) Ensure the use of coverslips with 170-µm-thick glass (#1.5) for cell imaging to prevent refractive index (RI) mismatch that could result in spherical aberrations.
b) Choosing fluorophores with separate excitation and emission spectra is necessary.
c) Sequential imaging setup is preferred to avoid any bleed-through between channels.
d) To control for bleed-through when imaging 3 or 4 channels, four different controls are needed for each imaging experiment as the following:
    a. Prepare the experimental sample with all labeling (fluorescent cell dyes, labelled target and DAPI).
    b. Prepare coverslips with single-labeled controls for each fluorophore.
    c. Use the fully labeled sample to adjust image acquisition parameters for each fluorophore separately, including illumination intensity, laser power and scan speed.
    d. Adjust acquisition parameter as the following: start with a low laser power to avoid photobleaching, increase the detector gain until an image is visible, then increase laser power as needed while avoiding saturation. The optimal setting button ensures the Nyquist sampling for pixel size and numbers is preserved given the current objective lens and zoom factor.
    e. Use the same setting for imaging the control samples.
    f. Image the single labeled control with all filters sets and carefully analyze potential bleed-through in the unlabeled channels.
    g. Adjust the emission spectra for each channel so that there is no/minimal bleed-through into the unlabeled channels.
    h. To control for auto fluorescence, image unlabeled sample gone through the staining protocol with each filter set.

3.2.7. Figure preparation

Images are processed using the Zen Lite 2.3 as the following:

a) Apply Gaussian filter with sigma set to 1.0.
b) Contrast and brightness were adjusted for better signal visualization.
c) Images were cropped and exported as tiffs.

Adobe Illustrator was used to prepare the figures.

4. References

1 Laflamme, C. *et al.* Implementation of an antibody characterization procedure and application to the major ALS/FTD disease gene C9ORF72. *Elife* **8**, doi:10.7554/eLife.48363 (2019).

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