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We use this protocol and it's working

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Preparation of cells for live-cell imaging of phosphoinositide reporters by total internal reflection fluorescence (TIRF) microscopy

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ABSTRACT

This protocol provides detailed instructions for transient expression of PIP_3/PI(3,4)P_2-selective PH domain-based reporters for subsequent live-cell imaging by total internal reflection fluorescence microscopy. Instructions are also provided for execution of perturbation experiments using growth factors or inhibitors that act through the PI3K signalling pathway. The protocol was originally developed with advice from Dr York Posor (Vanhaesebroeck Lab, University College London), Dr James Burchfield (University of Sydney) and Dr Alison Kearney (David James Lab, University of Sydney).

The protocol is routinely used in our lab for experiments with HeLa cells. It has also been used with mouse embryonic fibroblasts and lung adenocarcinoma A549 cells, however the complete medium (CM), seeding densities and transfection conditions differ for each cell line.

We culture our cells without antibiotics except during live-cell imaging as indicated.

For subsequent data analysis, please refer to the pipeline provided on the Open Science Framework under doi: 10.17605/OSF.IO/4F69N.

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GUIDELINES

- Use filter tips for all cell culture and molecular biology workflows.
- Ensure that cells are healthy and have been passaged at least once since thawing prior to using them for experimentation
- Ensure that all solutions are pre-warmed to room temperature before they come in contact with cells
- Ensure that all plasmids used for transfection are of high purity and without endotoxins (we advise additional validation by restriction enzyme digests and Sanger sequencing after plasmid prepping)
- We recommend making 50 ml aliquots of stock media solutions (e.g., complete medium and live-cell imaging); if kept in the fridge, we use these for up to 1 month.
- Some cell lines (e.g., human pluripotent stem cells) may not grow well in the glass bottom Ibidi dishes even if these are coated; we would instead recommend the use of the equivalent dishes with Ibidi polymer coverslips with IbiTreat (Ibidi #81156)

MATERIALS

For cell seeding and transfection

- 25 Culture-Inserts 4 Well for self-insertion (Ibidi #80469; area of each well: 0.35 cm^2)
- µ-Dish 35 mm, high Glass Bottom (Ibidi #81158)
- Growth Factor-reduced Matrigel (Corning #354230)
- DMEM high-glucose with L-Glutamine and Sodium Pyruvate (Thermo Fisher Scientific #41966-029)
- L-Glutamine (Sigma Aldrich #G7513)
- Fetal Bovine Serum (FBS; Pan-Biotech #P30-8500)
- Opti-MEM Reduced Serum Medium (Thermo Fisher Scientific #31985070)
- 1X TrypLE Express (Thermo Fisher Scientific #12604021)
- DPBS (Sigma Aldrich #RNBH8966)

Plasmid constructs

- MB35_pNES-EGFP-C1-PH-AKT2-WT (wildtype PH domain of AKT2, tagged C-terminally with EGFP and a nuclear export sequence) - diluted to 100 ng/µl in nuclease-free H2O.
- MB35mut_pNES-mCherry-C1-PH-AKT2-MUT (R>A phosphoinositide binding-deficient version of the AKT2 PH domain, tagged C-terminally with EGFP and a nuclear export sequence) - diluted to 100 ng/µl in nuclease-free H2O.
- MB33_pUC19_NEB_version (used as a carrier plasmid) - diluted to 100 ng/µl in nuclease-free H2O.

Note that the same experiments can be carried out with other phosphoinositide PH domain-based reporters, provided that they come in orthogonally-tagged wildtype-mutant pairs as the mutant PH domain reporter is used as an internal control for specificity and for normalisation.

For treatment solutions

- Human IGF1 (Peprotech #100-11; reconstituted at 100 µM in non-DEPC, nuclease-free H2O from Ambion #9937 and stored in 15 µl aliquots at -80 degrees Celsius) - aliquots are freeze-thawed a maximum of 1 times and are kept for up to 1 year
- Human EGF (Peprotech #AF-100-15-1mg; reconstituted at 1 mg/ml or 161.3 µM in non-DEPC, nuclease-free H2O from Ambion #9937 and stored in 15 µl aliquots at -80 degrees Celsius) - aliquots are freeze-thawed a maximum of 1 times and are kept for up to 1 year
- BYL719 (SelleckChem #S2814-10mM in DMSO) - diluted to 1 mM in DMSO and stored at -80 degrees Celsius long-term (up to 2 years) or -20 degrees Celsius short-term (up to 3 months)

For assembly of fluidics system for drug delivery

- PFTE tubing (Diba Omnifit Tubing, PTFE, 1/16" (1.6 mm) OD x 0.5 mm ID; 20 m/pk, Cole-Parmer #WZ-21942-70)
- Pharmed tubing (Biorad #7318208)
- Luer Lock Connector, Female (Ibidi #10825)

For live-cell perturbation experiments

- Fluorobrite DMEM (Thermo Fisher Scientific #A1896701)
- Penicillin-Streptomycin (Sigma Aldrich #P4333)

Other

- 1.5 ml Eppendorf tubes
- 5 ml Eppendorf tubes
- 15 ml Falcon tubes
- Filter Tips (Starlab or equivalent; 10/20 µl, 200 µl, 1000 µl)
- Non-filter tips for aspiration
- Serological stripettes (5 ml, 10 ml, 25 ml; Corning Costar or equivalent)
- Integra aspiration system or equivalent
- Aspiration stripettes
- Cell culture plates or flasks (e.g. Corning 25cm² Rectangular Canted Neck Cell Culture Flask with Vent Cap #430639)
- Trypan Blue 0.4 % solution (Thermo Fisher Scientific #15250-061)

- 3 ml syringes (e.g. Terumo Syringe without needle #MDSS03SE)
- 70 % ethanol and industrial methylated spirit
- MilliQ H2O
- Haemocytometer
- Calculator
- White Blu-Tack (only use the original product and the white colour to avoid interference during imaging)

SAFETY WARNINGS

- Take care when working with the flame when making holes in the lids for insertion of the tubings.
- Take appropriate precaution when working with lasers.

BEFORE START INSTRUCTIONS

- Prepare coating solution: thaw Matrigel stock aliquot on ice, then dissolve 400 µl in 19.6 ml of cold OptiMEM
- Prepare autoclaved forceps
- Prepare complete medium (CM) in advance, e.g. for HeLa cells (see materials for individual items): DMEM with L-Glutamine and Sodium Pyruvate (500 ml) + L-Glutamine (5 ml) + FBS (50 ml)
- Prepare live cell imaging (LCI) medium: 49 ml Fluorobrite DMEM + 500 µl L-Glutamine + 500 µl Penicillin-Streptomycin
- Make sure that the microscope, including objectives, holders and oil are warmed up to 37 degrees Celsius for least 3 hours prior to set up for imaging
- Assemble the fluidics system by threading the end of a 40-50-cm-long piece of PFTE tubing into a 1.5-2 cm-long piece of Pharmed tubing with a luer lock at the other end. Roll the tubing and store away in a 150 cm putri dish when not in use. When in use, the end of the PFTE tubing that is free must be threaded through an appropriately sized hole in the lid of an imaging dish - this hole can be made using a flame to heat a needle, then melting the plastic in the lid until the required size has been reached.

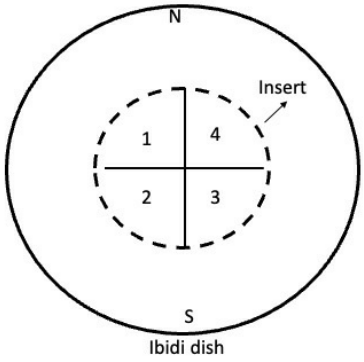
Dish assembly and coating

1h

1 Take the required number of Ibidi dishes that will be needed for the experiment, along with the equivalent number of inserts. (NB: you can reuse inserts once provided that they have been sterilised in 70% ethanol and dried since the last use; we do this by passing them through PBS once, then 2x across two distinct 70% ethanol aliquots in 50 ml Falcons, then transfer to dry in a closed Petri dish).

1.1 Using autoclaved forceps, pick up the individual inserts and place an insert in each dish. Invert the dish once to check that the insert is sticking to the bottom of the dish.

1.2 Label the lid of the dish with the well layout. To ensure that you can keep track of the orientation even when you remove the lid, label both lid and base with "N" (north) and "S" (south) in the equivalent positions (see attached schematic).



Schematic of Ibidi dish and suggested labelling to ensure correct orientation. N and S must be added both to the lid and the base of the dish in exactly the same positions to ensure correct alignment.

2 Using ice-col Matrigel solution (1:50 dilution in OptiMEM, prepared in advance), coat each well with 100 µL.

2.1 5 mL Note the coating time and transfer the dishes to the incubator for a minimum of 00:45:00 (NB: we usually seed within 1-2h of coating). 45m

2.2 Make sure to keep the Matrigel solution on ice and return to the fridge when done.

Cell seeding (the given volumes for splitting are for HeLa cell...

1h

3 Take the cells for processing out of the incubator and confirm healthy state by light microscopy examination. The cells should be 80-90% confluent.

4 Remove the spent medium and wash the cells with 5 mL DPBS.

5 Remove the DPBS and add 0.75 mL TrypLE Express.

6 Incubate the cells for 00:08:00 at 37 degrees Celsius.

8m

6.1 If you are processing more than one cell line (e.g. different HeLa mutants), proceed with the next, ensuring that you stagger individual cultures 2 minutes apart. DO NOT process multiple cell lines for splitting the same time as this poses a cross-contamination risk.

7 Once the cells have dissociated, add 3.3 mL of complete medium (CM), resuspend the cells and transfer to an appropriate labelled 5 ml Eppendorf tube. The final volume should be approximately 4 ml.

7.1 Immediately after transfer, mix 20 µL of the cell suspension with 20 µL Trypan Blue.

8 Count the cells using a haemocytometer (NB: we find this method to be the most reliable compared to automated cell counting)

9 Dilute the cell suspension to 30000 cells/ml (NB: ensure sufficient mixing at all stages, including of the stock cell suspension) in a final volume of 1 mL (use 1.5 ml Eppendorf tubes).

10 Take out the coated dishes from the incubator.

10.1 For each well to be seeded, take the respective cell suspension and mix 2-3 times with a P1000 Gilson pipette set at 500 µl.

10.2 Immediately after mixing the cell suspension, aspirate the coating solution from the well to receive cells using non-filter tips (be careful not to let the wells dry out, yet also take care to observe sterile techniques!).

10.3 Transfer 100 µL (3000 cells) of the mixed cell suspension to each well. Make sure not to tilt the dish at any point after cell seeding. (NB: if you left the mixed cell suspension sit for more than 1-2 minutes, mix again before seeding though be mindful of time as the wells dry out once the coating has been removed).

10.4 Repeat this procedure for the remaining cell suspensions.

10.5 Once you have seeded all wells, leave the dishes in the tissue culture cabinet for 15-20 minutes before transfer to the cell culture incubator. This ensures a more even seeding distribution.

11 Leave the cells in the cell culture incubator for 20-28 hours after seeding. 1d

Reporter plasmid transfection 1d

12 Pre-equilibrate FuGENE to room temperature and vortex vigorously before use.

13 Prepare the transfection mastermix as follows (example given for 8 transfections + 2 extra)

	A	B	C
		1X (25 ng of each PH domain construct, 20 ng carrier plasmid)	10X
	Opti-MEM	9.09 µl	90.9
	MB35 (100 ng/µl)	0.25 µl	2.5
	MB35mut (100 ng/µl)	0.25 µl	2.5
	MB33_pUC19 (100 ng/µl)	0.20 µl	2
	FuGENE	0.21 µl	2.1
	Total V	10 µl	100
	Total DNA amount	0.07 µg	
	DNA concentration	0.007 µg/µl	

Transfection mix preparation. Make sure that FuGENE is added last and does not touch the sides of the tube. The ratio of FuGENE volume (µl) to DNA mass (µg) is 3:1.

13.1 Immediately after adding FuGENE, mix the suspension by pipetting multiple times, then leave to incubate for 10-15 minutes.

13.2 Five minutes before the transfection mix incubation is complete, take the seeded cells out of the incubator and inspect under the light microscope to check cell health, then transfer to the tissue culture cabinet.

14 After incubation, mix the transfection mix 1:10 with complete medium (e.g. mix 100 µl transfection mix with 1000 µl complete medium, for a final volume of 1100 µl)

15 Working with maximum two wells at a time, aspirate the spent medium solution from the cells using non-

filter tips and quickly albeit gently, add 110 µL transfection mix to each well, taking care not to touch the cell monolayer

- NB: change filter tips in between each addition to minimise cross-contamination risk.
- NB: if you are too slow at this stage, the cells will not transfect properly as the cell monolayers dry very quickly once the medium is removed from the small wells.

16 Return the transfected cells to the incubator for 20-28 hours. 1d

Serum-starvation 10m

17 Approximately 24 hours following transfection, inspect the cells under the light microscope to check health, then transfer to the tissue culture cabinet.

18 Using autoclaved forceps, remove the insert from each dish (NB: either discard if it has been used once before, or sterilise as described in Step 1).

19 Using non-filter tips, aspirate the spent medium from the cells from the area where the insert boundary is visible (do not touch the cells).

20 Gently add 3 mL of live cell imaging (LCI) medium to the dish, taking care to cover the inner circle containing the cells. This is a wash step to ensure phenol red and serum removal.

21 Remove the LCI wash and replenish the cells with exactly 2 mL LCI, then return to the incubator f 2h 02:00:00 prior to microscopy set-up.

21.1 When imaging, you can only work with one dish at the same time for fast acquisition, therefore make sure that you do not serum-starve the cells in multiple dishes at the same time. Instead, stagger starvation timings to ensure that cultures are not starved for more than 3 hours at the time of

imaging start (2 hours in incubator + 1 hour at the microscope while setting up)

21.2 Remember to switch on the microscope temperature unit on at 37 °C at least 2h in advance of initiating imaging, and ensure that all components (including lens and dish holders) are heated up.

Preparation of treatment solutions

15m

22 During the 2 hour wait, prepare the treatment solutions in LCI following the example tables below for 100 nM final stimulations with IGF1 and EGF (note that the solutions are prepared at a higher concentration for final dilution to the required 1X amount once added to the cells in volumes of 500 µl). Make sure to mix all stock solutions prior to final pipetting.

	A	B	C
		Solution: IGF1 (5X = 500 nM)	Soution: IGF1 (1X)-BYL719 (6X - 3000 nM)
	LCI	597 µl	597.6 µl
	IGF1 (100 µM)	3 µl	0.6 µl
	BYL719 (1 mM)		1.8 µl
	Final V	600 µl	600 µl

Example table for preparation of IGF1/BYL719 treatment solutions for a final addition of 100 nM IGF1 and 500 nM BYL719. Each treatment required is added in volumes of 500 µl, therefore you can scale the master mix volume according to the number of dishes to treat, then aliquot separately for each treatment. Note that the first IGF1 solution is prepared at 5X stock concentration because it is diluted 5X when adding 500 µl to 2 ml of the medium that is already on the cells. The subsequent IGF1-BYL719 solution is added to 2.5 ml medium that already contains IGF1 but does not have BYL719, therefore the latter is prepared at 6X the required concentration due to a final 6X dilution when 500 µl are added to 2.5 ml.

	A	B	C
		Solution: EGF (5X = 500 nM)	Solution: EGF (1X)-BYL719 (6X - 3000 nM)
	LCI	1196	1196 µl
	EGF (161.3 µM)	3.7 µl	0.74 µl

	A	B	C
	BYL719 (1 mM)		3.6 µl
	Final V	1200 µl	1200 µl

As above but for EGF; the higher final volumes ensure that no volume to be pipetted falls below 0.5 µl.

Live-cell imaging and treatment additions

2h

23 Following 2 hours of serum-starvation, bring the cells on the microscope and proceed with setting up which takes around 45 min to 1 (NB: you can only work with 1 dish at a time, therefore make sure that cells in different dishes have not had serum removed at the same time)

24 Connect the fluidics system to the dish by exchange the lids. Make sure that the fluidics system is secured (use white Blu-Tack only) and the dish is clamped in place. Ensure stable CO_2 and temperature conditions.

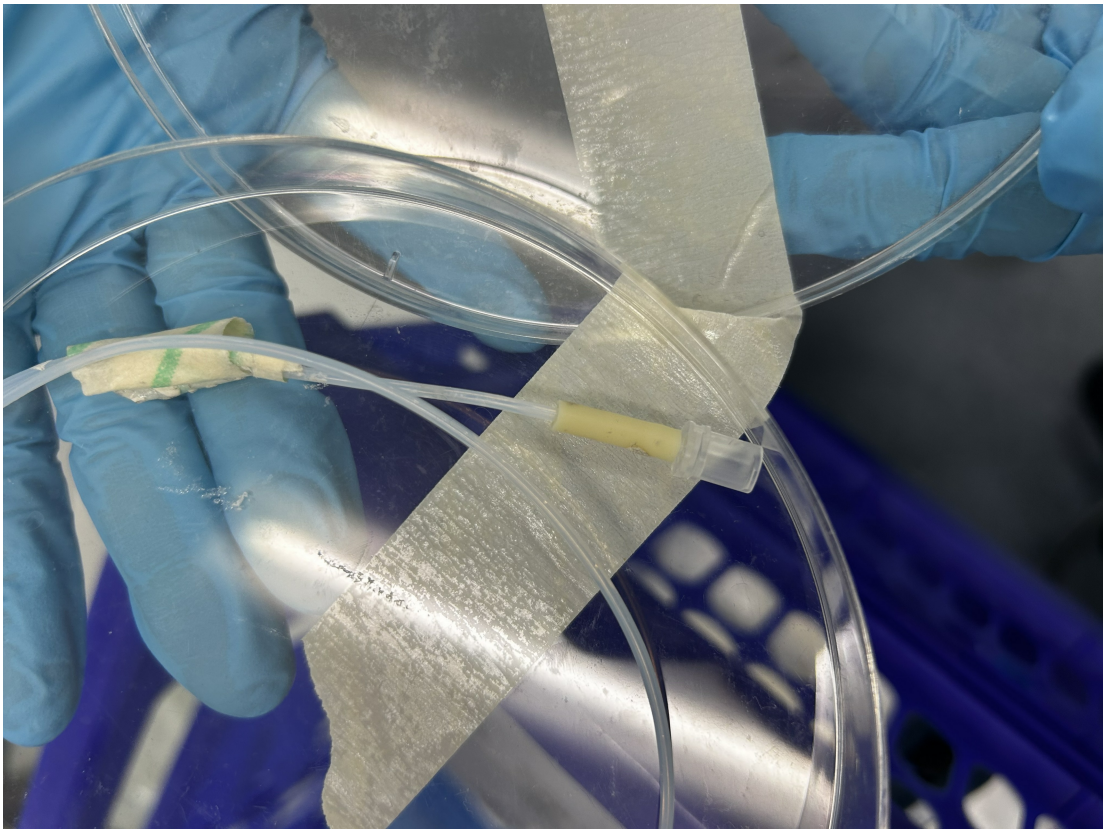


Image of the tubing assembly - this is the end that will connect to the syringe. See Guidelines section for how to prepare the tubings.

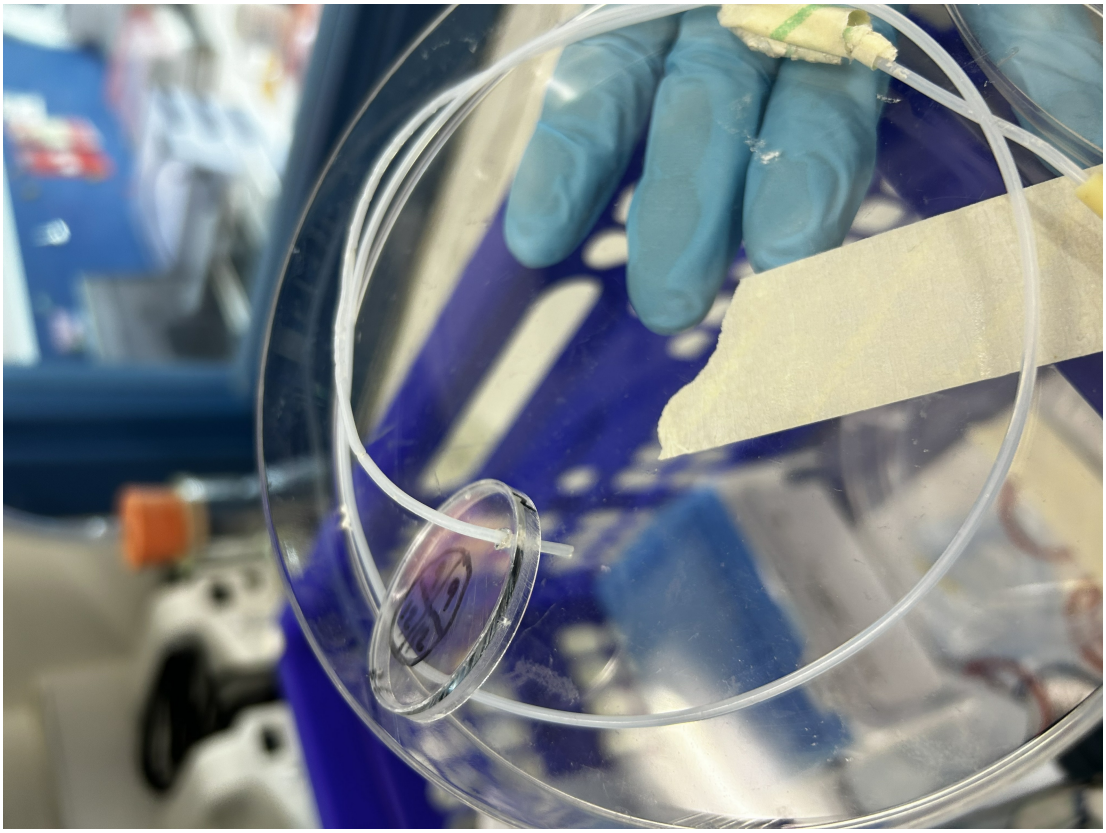


Image of the lid with a hole for the tubing end that will be inserted into the medium with cells. See Guidelines section for how to make the hole in the lid.

25 Set-up the microscope with the correct laser and exposure settings (these will be microscope-specific, but we advise careful checking of the TIRF angle to ensure that the plasma membrane is in focus, with minimal signal from the cytoplasm)

26 Select individual cells for imaging and specify positions, making sure that a cell expresses both EGFP and mCherry, i.e. both wildtype and mutant PH domain reporter.

26.1 Make sure to only select cells that do not have a saturated signal, yet have a signal that is at least twice as strong as the surrounding background. Be mindful of the fact that the signal is weaker with the mutant mCherry-tagged PH domain reporter.

27 Specify the number of time points: we usually image every 70 seconds for 60 minutes, across a total of 28 positions, with a 60X or 100X TIRF oil objective.

28 Start imaging and proceed with treatments at the required time points. We usually attach the syringes to the fluidics system in advance and 30s before treatment needs to commence, we pipette the solutions in each syringe, then push it through at the required time point, ensuring that we mix gently 3-4 times by performing several strokes of up to 0.5 ml with the syringe.

NB: Be sure to check on CO_2 levels throughout imaging. The cells and their signalling responses are very sensitive to fluctuations in CO_2 and pH. This is a common reason for inconsistent result generation.

29 Once imaging has completed, clean the fluidics system, clear the microscope out and proceed with data analysis.

29.1 To clean the tubings after each experiment, first flush with 3-4 ml 70 % ethanol (using syringe), then 3-4 ml MilliQ water, then push air through 3-4 times and leave to dry.