DAY 1: PLATING ADHERENT CELLS ON COVERSLIPS FOR IF PROTOCOL

To be performed in biosafety cabinet using sterile technique. Get all the reagents ready before you start.

MATERIALS (Supplier, Catalogue Number)

- LLC-PK1 cells (ATTC)
- Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, 11965-092)
- Sterile PBS 1X (Invitrogen, 10010-049)
- Trypsin EDTA 0.25% (Invitrogen, 25200-056)
- Heat inactivated fetal bovine serum (FBS) (Invitrogen, 12484-028)
- T25 sterile tissue culture treated flasks
- 6-well plates
- 25mm glass coverslips, #1.5 thickness - 0.17mm (Electron Microscopy Sciences, 72225-01)

PREPARATION

- Warm up sterile PBS, trypsin-EDTA and DMEM+10% FBS to 37°C.
- Using sterile forceps place one sterile 25mm coverslip in each well of a 6-well plate.
- Coverslips can be sterilized by autoclaving or by storing in 70% ethanol (UV irradiation is NOT sufficient for sterilization).
    - Some cells do not adhere well to glass coverslips, so acid etching and/or coating (i.e. with poly-L-lysine, collagen, matrigel) is necessary**.**

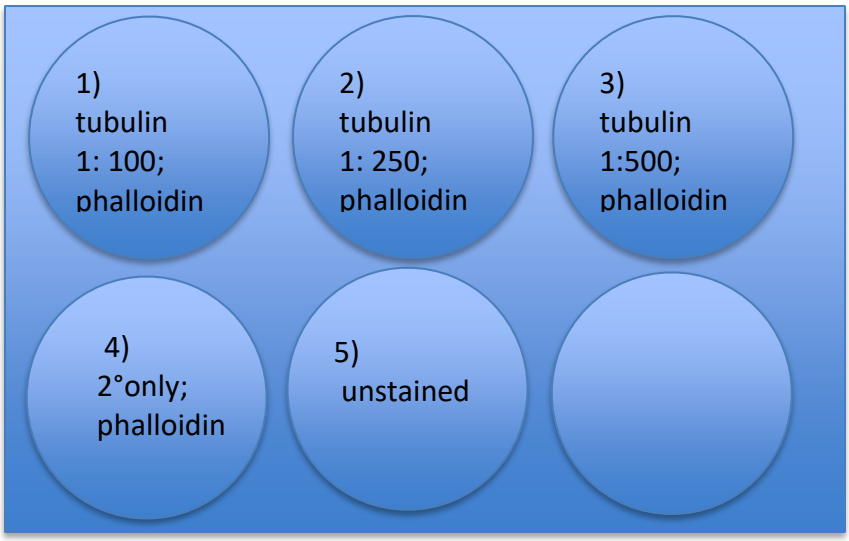
PROCEDURE

Starting with confluent 6cm dish.

- Wash cells 2 X with 5 ml sterile PBS.
- Add 1.0 mL trypsin-EDTA to the cells and return to the incubator for 5-15 minutes.
- Examine T25 flask under light microscope to see that cells are detached an in single cell suspension.
- Add 9 mL DMEM + 10% FBS to cell suspension (total 10 mL).
- Pipet gently up and down.
- Add 2mL of cell suspension per well of the 6-well plate.
- Gently swirl the cells around to mix and place in the incubator.

DAY 2: SINGLE INDIRECT STAINING OF TUBULIN (WITH F-ACTIN AND DNA STAIN) PROTOCOL

Conditions



MATERIALS (Supplier, Catalogue Number)

- HBSS (Invitrogen, 14025-092)
- 16% parafolraldehyde (Canemco, 017B)
- 10 X PBS (Invitrogen, AM9624)
- Glycine (Bioshop, GLN001)
- BSA-fraction V (Bioshop, ALB001)
- TritonX-100 (Sigma, X100)
- Mouse anti-alpha tubulin antibody (Invitrogen, 32-2500)
- Donkey anti-mouse CF ™488 (Biotium, 20014)
- CF ™555 labeled phalloidin (Biotium, 00040)
- Hoescht (Invitrogen, N21485)
- DAKO™ mounting medium (Aglient, S3023)
- Curved fine forceps (E.g. Medstore, 7720-A10-708)
- Parafilm
- Kimwipes
- Glass slides

DILUTIONS

Mouse anti-alpha tubulin antibody

STOCK = 0.5 mg/mL
1: 100 = 5 µg/mL
1: 250 = 2.5 µg/mL
1: 500 = 1 µg/mL

CF™555 labeled phalloidin

STOCK = 300 U / 1.5 mL = 0.2 U / µL
FINAL = 0.5 U (2.5 µL) / 100 µL

Donkey anti-mouse CF™488

STOCK = 2 mg / mL
FINAL = 5 µg (2.5 µL) / mL

BUFFERS

Fixation Buffer:

-3 mL 16% paraforladehyde
-9 mL HBSS
Note: This must be made fresh for each experiment.
Note: 16% PFA, after opening ampule store at 4°C for a maximum of 10 days

1X PBS:

-100 mL 10X PBS
-900 mL dH_2O

Glycine Inactivation Buffer:

-0.375 g glycine
-fill to 50 mL with PBS
-store at 4°C
Note: Prepare fresh as this buffer can get contaminated very easily

Blocking Buffer (3% BSA in PBS):

-1.5 g BSA-fraction V
-fill to 50 mL with PBS
-store at 4°C

10% Triton X-100 stock solution:

-1 ml Triton X-100
-9 mL dH_2O
-put on shaker to dissolve (this may take a couple of hours, so prepare ahead of time)
-store at R.T. to avoid precipitaion of detergent

NOTE: Triton X-100 is difficult to pipet. Soluiton: Pipet 1ml of water into an empty 15 cc tube and carefully mark the level, then I remove all the water. Cut the end of a 1mL pipe tip and use this to carefully pipet 1mL of Triton X-100 to marked level.

Permeabilization Buffer (1% BSA + 0.1% TX-100 in PBS) :

-0.5 g BSA-frcation V
-500 µL 10% TritonX-100 stock solution
-fill to 50 mL with PBS
-store at 4°C

Antibody Buffer (1% BSA in PBS):

-0.5 g BSA-fraction V
-fill to 50 mL with PBS
-store at 4°C

PREPARATION

- Prepare the following buffers.
  - 10% Triton X-100 stock – 10 mL
  - 12 mL Fixation Buffer (prepared fresh)
  - 50mL each of Glycine inactivation Buffer(prepared fresh)
  - Blocking Buffer, Permeabilization Buffer and Antibody Buffer
    - these can be made ahead of time and stored at 4°C for up to one month, examine for contamination before use
- Warm up HBSS and Fixation Buffer to 37°C
- Take out all other buffers from the refrigerator

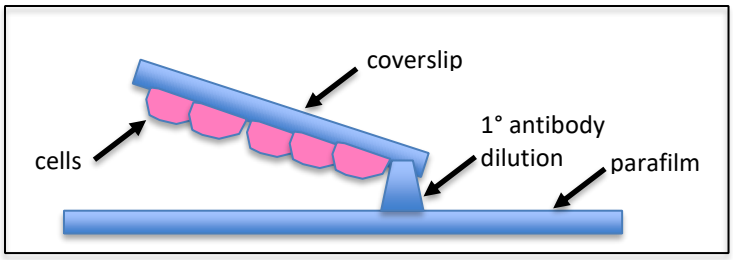
PROCEDURE

Starting with 6-well plate.

- Gently wash cells 2X with warm HBSS - 2 mL per well.
- Add 2 mL of warm Fixation Buffer per well.
- Incubate at R.T. for 30 minutes.
  Note: The rest of the procedures can be carried out at R.T.

- **CHEMICAL SAFETY**: Collect the Fixation buffer into a tube for hazardous waste pick up (do not aspirate or pour down sink)
- Wash cells 4X with PBS – 2 mL per well.
- Add 2 mL of Glycine Inactivation Buffer.
- Incubate 10 min.
- Wash cells 1X with Glycine Inactivation Buffer – 2 mL per well.
- Wash cells 1 X with PBS – 2 mL per well.
- Add 1 mL of Permeabilization Buffer per well.
- Incubate 20 min.

- Add 1-2 mL Blocking Buffer.
- Incubate 1 hour.
- During the 1 hour incubation prepare Primary antibody dilutions in eppendorf tubes.
  #1- 100µL antibody buffer + 1.0 µL mouse anti-alpha tubulin (i.e. final 5 µg/mL)
  #2- 30 µL antibody buffer + 20 µL of #1 dilution (i.e. final 2.5 µg/mL)
  #3- 40 µL antibody buffer + 10 µL of #1 dilution (i.e. final 1 µg/mL)
  #4 and #5 - 50 µL antibody buffer only
- Add primary antibody to coverslips.
  - Cut out parafilm square and label the backside with a marker.
  - Tape parafilm to flat surface.
  - Add 50 µL drop of Primary Antibody Dilution to parafilm
  - Pick up coverslip with forcep, tap coverslip to kimwipe to remove excess PBS and gently invert coverslip over the drop of Primary Antibody Dilution.
  - Do this for each coverslip.
  - Put 6-well plate lid over all coverslips (to prevent dehydration).



- Incubate 1 hour.
  Note: If you need to incubate for longer, you will need to prepared a larger volume of primary antibody dilution.
- During this time prepare secondary antibody dilution
  #1 - #4:
  400 µL antibody buffer
  + 10 µL CF555 labeled phalloidin (i.e. 2.5 µL = 0.5 U phalloidin)
  + 1 µL donkey anti-mouse CF488 (i.e. final 5 ug/mL)
  #5:
  100 µL antibody buffer only
- Gently lift coverslips from parafilm and place back into 6-well plate right side up.
- Wash cells 4X with PBS – 2 mL per well (3-5 min incubation)
- Add secondary antibody dilutions – 100 µL per well (same procedure as for primary antibody)
- Incubate 1 hour, PROTECT from LIGHT.
- Gently lift coverslips from parafilm and place back into 6-well plate right side up.
- Add 2 mL PBS containing Hoescht (1:12,000 dilution)
- Incubate 2 min
- Wash cells 5X with PBS – 2 mL per well (3-5 min incubation)
- Using forceps mount coverslips onto glass slides.
  - Places one drop of DAKO mounting medium onto glass slide.

- Pick up coverslip with forcep, tap coverslip to kimwipe to remove excess PBS and gently invert coverslip over the drop of DAKO (avoiding bubbles).
- Allow cells to dry O/N at room temperature, protected from light.
- For prolonged storage, store samples at 4°C.

PROCEED TO IMAGING!