1. Cell Cultivation

NB 1: Always use plastic gloves and work in the sterile environment inside a LAF cabinet. Clean the cabinet with 70 % ethanol. Spray gloves, working surfaces, microscopes, centrifuges, incubators etc. with 70 % ethanol if you suspect contamination.

NB 2: Cell suspensions are to be thoroughly mixed before any transfer where a correct cell concentration is of importance.

NB 3: Always pre-heat media before adding cell suspensions for cultivation.

1.1 Subcultivation

Materials:

- 1×PBS + EDTA
- Trypsin (1×)
- cell growth media

NB: The volumes below are for 10 cm Petri dishes. Double the volumes when using 15 cm dishes.

1. Remove the medium from the Petri dish using a stripette or the VacuBoy.
2. Add 10 ml PBS + EDTA.
3. Remove the PBS + EDTA after ~ 5 minutes.
4. Add 1 ml Trypsin (1x).
5. Incubate in 37 ˚C and 5.2 % CO_2. for 3 min (+ 1 minute if needed).
6. Inspect the Petri dish to ensure that the cells are detached.
7. Add 1 ml of medium to the dish and separate the cells by pipetting (1 ml pipette).
8. Add 10 ml cell growth medium to a new Petri dish.
9. Add the detached cells to the new Petri dish.
   1:5 400 µl cell suspension
   1:7.5 267 µl cell suspension
   1:10 200 µl cell suspension
   NB: If the cells are to be split less than 1:5, the trypsin needs to be removed. Pellet the cells by centrifugation (1700 rpm, 3 min), remove the supernatant and reconstitute the pellet in 1 ml fresh growth media.
10. Swirl the Petri dish gently and inspect it in the light microscope.
11. Cultivate in humified air at 37 °C (5.2% CO_2).

2. Plate Preparation

NB 1: Always use plastic gloves and work in the sterile environment inside the LAF cabinet. Clean the cabinet with 70 % ethanol. Spray gloves, working surface, microscope, centrifuge, incubator etc. with 70 % ethanol if you suspect a minor contamination.

NB 2: Cell suspensions are to be thoroughly mixed before any transfer where a correct cell concentration is of importance.

2.1 Coating Plates

Materials:

- fibronectin, 100 µg/ml (mix carefully)
- 1×PBS
- glass bottomed 96-well plate

1. Dilute the fibronectin to 12.5 µg/ml (4.2 ml PBS + 0.6 ml fibronectin for one plate). Store undiluted fibronectin at -20 °C.
2. Add 40 µl to each well.
3. Incubate for 1 hour at room temperature or at 4°C for storage up to two weeks.

Table 1. Recommended volumes

No. of plates	1	2	3	4	5	6
Fibronectin	0.6	1.1	1.6	2.1	2.6	3.1
PBS	4.2	7.7	11.2	14.7	18.2	21.7

2.2 Cell Counting

Materials:

- Neubauer counting chamber
- Millipore Scepter with 60µl tip
- manual counter
- cell growth media or 1×PBS

2.2.1 Manual Counting: Neubauer Counting Chamber

1. Dilute a small volume of the cell suspension (5x – 20x dilutions will usually yield the optimal 30 - 100 cells/Neubauer square) with 1×PBS or cell medium.
   Example: Dilute 50 µl cell suspension with 200 µl growth media (5× dilution).
2. Put small drops of the dilution on the counting chamber mounting support and mount the cover slip (make sure there are visible liquid-ring patterns).
3. Add the sample (will be absorbed by capillary force).
4. Count the cells in square number 1-4 (and 5 if necessary) using a manual counter.
5. Calculate the average number of cells per square and multiply by 10 000 and the dilution factor to yield the cell concentration in cells/ml.

$Example$: $\tilde{x} = 50\,;\, d = 5$ $c = \tilde{x} \cdot d \cdot 10\,000 = 2\,500\,000\ cells/ml$

2.3 Cell Seeding

Materials:

- 1×PBS + EDTA
- Trypsin (1×)
- fibronectin coated glass bottomed 96-well plate

2.3.1 Diluting the Cell Suspension

1. Detach and centrifuge the cell suspension to remove the trypsin (1700 rpm, 3 min) and resuspend the pellet in medium.
2. Determine the concentration by cell counting (section 2.2).
3. Calculate the volume of cell suspension needed i.e. v_1.

   a) Number of cells per well
   b) Number of cells per plate (i.e. c_2v_2)
   c) Medium per plate (i.e. v_2) 80 µl × 125 wells = 10 000 µl
   d) Calculating v_1
      - equation: $c_1v_1 = c_2v_2$

      - v_1 *(ml)* = $\frac{c_2v_2}{c_1} = \frac{2\ 500\ 000}{c_1}$ (example: NIH-3T3)

4. Mix v_1 ml of cells with (10 - v_1) ml medium.

2.3.2. Manual Cell Seeding

1. Remove the fibronectin coating with a multi pipette or the 8-channel VacuBoy.
2. Add 80 µl of cell suspension to the center of each well without touching the bottom of the well with the pipette tip.
3. Remember to mix the cell suspension by pipetting before adding it to the wells as the cells can form aggregates and/or sediment.
4. Avoid shaking or tilting the plate and inspect it in the microscope to confirm even distribution of the cells.
5. Incubate in humidified air (37 °C, 5.2 % CO_2).

3. Primary Antibody Dilution

Materials per portion:

- 95 HPA antibodies
- 9.6 ml 1×PBS
- 400 µl FBS (1:25)
- 10 µl mouse anti-tubulin (cytoskeleton marker) (1:1000)
- 12,5 µl chicken anti-calreticulin (ER marker) (1:800)
- 96-well PCR plate

NB: The HPA antibodies are diluted in 1×PBS (4 % FBS) containing the anti-tubulin and anti-KDEL markers (a.k.a. stock solution).

4. Immunostaining

4.1 Day 1

Materials:

- 1×PBS
- PFA (4 %)
- 1×PBS + 0,1 % Triton X-100
- primary antibody template dilution

1. *Fixation*
   a) Remove growth medium i.e. aspirate ~ 100 µl from all wells.
   b) Wash the cells once with 40 µl 1×PBS.
   c) Fix the cells by incubating in 40 µl ice cold PFA for 15 minutes.

2. *Permeabilization*
   a) Remove the PFA and permeabilize the cells by incubating with 40 µl 1×PBS + 0.1 % Triton X-100 for 3 × 5 minutes.
   b) Wash the cells once with 40 µl 1×PBS.

3. *Primary Antibody Incubation*
   a) Add 40 µl primary antibody and incubate over night at 4 °C or in room temperature for 2 h.

4.2 Day 2

Materials:

- 1×PBS
- blocking buffer (1×PBS + 4 % FBS with secondary antibodies)
- DAPI
- glycerol (5 % 10×PBS)
- 96-well PCR plates

1. *Secondary Antibody Incubation*
   a) Remove the primary antibody and wash the cells with 40 µl 1×PBS for 4 × 10 minutes.
   b) Add 40 µl blocking buffer with secondary antibody and incubate for 90 minutes.

2. *DAPI Nuclear Staining and Mounting*
   a) Remove the blocking buffer and incubate in 40 µl DAPI for 10 minutes.
   b) Wash the cells with 40 µl 1×PBS for 4 × 10 minutes and mount with ~350 µl glycerol/10×PBS.
   c) Adjust the level of glycerol if needed and seal the plate with foil cover.

5. Buffers and Solutions

NB 1: Always use pressure safe 1l bottles when vacuum filtering buffers. The filtering pressure flow is to be turned on before attaching the tubing to the filtering casette.

5.1 PBS

10×PBS

- 160 g NaCl
- 4 g KCl
- 28.8g Na_2HPO_4
- 4.8 g KH_2PO_4
- Dissolve in 1600 ml Milli-Q. Adjust pH to 7.2 and add Milli-Q up to two liters, vacuum filtrate and autoclave the buffer.

1×PBS

- Mix 100 ml 10×PBS with 900 ml Milli-Q. Autoclave.

1×PBS + EDTA

- Mix 0.37 g EDTA with 100 ml 10×PBS and 900 ml Milli-Q. Autoclave the buffer.

5.2 Fibronectin

NB: The following steps are to be performed in a LAF cabinet.

1. Equilibrate the fibronectin in room temperature.
2. Add 5 ml Milli-Q to one bottle of 5 mg lyophilized fibronectin and incubate for 30 minutes. Do not shake or mix more than absolutely necessary.
3. Add 45 ml Milli-Q and aliquot in Eppendorf tubes (1.1 ml/tube) marked with “Fn”.
4. Store the diluted fibronectin in the AC-lab freezer.

5.3 PFA (Paraformaldehyde) 4 %

NB: Remember the following safety procedures when working with PFA. Use blue gloves, lab coat and eye protection and work in a fume hood or specifically ventilated lab area. Use mouth guard when working with the powder. Residues are collected in a special container and sent for destruction. Always make larger amounts of PFA (about 200 ml) and freeze in skirted PCR plates and Eppendorf tubes.

1. Pre-heat 150 ml of PBS with 10 % FBS in a fume hood, on a hot plate magnetic stirrer to 60° C using a ≥ 250 ml beaker (set the heating plate to ~120° C).
2. Add 50 ml of the liquid PFA-stock to the warm PBS and leave stirring for ca. 20 min until the stock is mixed.
3. Add concentrated NaOH until the solution reaches pH ~11.
4. Allow the PFA to cool down to room temperature and set pH to 7.2 - 7.3. Use diluted HCl for the adjustment.
5. Transfer 50 µl, 100 µl, 150 µl and 180 µl, to microtiter plates for preparation of 1, 2, 3 and 4 plates respectively.

   N.B. If you have been forced to adjust the pH several times, discard the PFA and prepare a new solution.

5.4 PBS with 0.1 % Triton X-100

NB 1: Do not use Triton solutions older than 7 days.

NB 2, Dissolving Triton: Use the 5 ml pipette, set to maximally 3 ml, and avoid getting too much undissolved Triton into the tip (especially since this often cause a lot of foam formation).

1. Mix 50 µl Triton X-100 with 5 ml PBS.
2. Pipette carefully otherwise it will build up foam.
3. When it is dissolved add another 45 ml of PBS.

5.5 Stock Solution - Blocking Buffer (PBS + 4 % FBS) with Primary Antibodies

1. 9.6 ml PBS
2. 400 µl FBS
3. 5 µl anti-tubulin
4. 25 µl anti-KDEL

Table 2: Primary antibody details

Manufacturer	id. no.	origin	affinity	c [mg/ml]	dil. c [µg/ml]
Abcam	ab7291	Ms mAb	alpha-tubulin	1	0.5
Abcam	ab2908	Ckn pAb	Calreticulin	1	1.25
HPA	---	Rb pAb	PrEST:s	0.1 – 1.5	2 – 4

5.6 Blocking Buffer (PBS + 4 % FBS) with Secondary Antibodies

- The secondary antibodies are diluted 800x in PBS + 4% FBS.

- For one plate (4 ml) mix:
  - 3840 µl PBS +
  - 160 µl FBS
  - 5,25 µl of Goat anti Mouse Alexa 555 (Invitrogen, Ref A32727)
  - 5,25 µl of Goat anti Chicken Alexa 647 (Invitrogen, Ref A21449)
  - 5,25 µl of Goat anti Rabbit Alexa 488 (Invitrogen, Ref A11034)

NB 1: Do not mix or go down to deep with the pipette tip in the Alexa vials. After mixing the solution, aliquot 40 µl/well in a 96-well skirted PCR plate.

NB 2: The Alexa Fluor antibodies are photosensitive. Cover tubes and plates with aluminum foil and turn off the lights in the laboratory if possible.

5.7 DAPI (4', 6-diamidino-2-phenylindole)

NB: DAPI is photosensitive. Cover tubes and plates with aluminum foil and turn off the lights in the laboratory if possible.

Pre-Dilution: (only if the pre-diluted ones are out) Mix 80 µl 14.3 mM DAPI with 920 µl PBS in a 1.5 ml microcentrifuge tube. Aliquot 35 µl into 100 µl microcentrifuge tubes and store these in a 50 ml Falcon tube.

1. Mix pre-diluted DAPI with PBS.
2. Put 50/100/150 µl/well in a skirted PCR plate for preparing 1 - 5 plates.

5.8 Glycerol + 10×PBS

- For one plate:

1. 45 ml glycerol
2. 5 ml 10×PBS
3. Mix thoroughly in a 50 ml Falcon tube.