

CHARACTERISATION of CELLS

Laboratorio 2

CHARACTERISATION of CELLS

CELL staining is a technique that can be used to better **visualize cells and cell components** under a microscope, also various activities and structures of a cell can be targeted for staining with fluorescent compounds

Most stains can be used on fixed, or non-living cells, while only some can be used on living cells; some stains can be used on either living or non-living cells.

The most commonly stained cell components are cell membranes, mitochondria, and nucleus.

How Are Cells Stained and Slides Prepared?

Cell staining techniques and preparation depend on the type of stain and analysis used.

Fixation - serves to "fix" or preserve cell morphology through the preparation process. This process may involve several steps, but most fixation procedures involve adding a chemical fixative that creates chemical bonds between proteins to increase their rigidity. Common fixatives include **paraformaldehyde, ethanol, methanol**.

Permeabilization - treatment of cells, generally with a mild surfactant, which dissolves cell membranes in order to allow larger dye molecules to enter inside the cell. Two general types of reagents are commonly used: **organic solvents**, such as methanol and acetone, and **detergents such as saponin, Triton X-100 and Tween-20**.

The organic solvents dissolve lipids from cell membranes making them permeable to antibodies. Because the organic solvents also coagulate proteins, they can be used to fix and permeabilize cells at the same time.

Saponin interacts with membrane cholesterol, selectively removing it and leaving **holes in the membrane**. The disadvantage of detergents such as Triton X-100 and Tween-20 is that they are non-selective in nature and may extract proteins along with the lipids.

Staining - application of stain to a sample to **color cells, tissues, components, or metabolic processes**. This process may involve immersing the sample (before or after fixation or mounting) in a **dye solution** and then rinsing and observing the sample under a microscope.

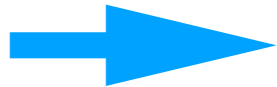
Mounting - involves attaching samples to a glass microscope slide for observation and analysis. The main purpose of mounting media is to **physically protect the specimen**; the mounting medium **bonds specimen, slide and coverslip** together with a clear durable film. The medium is important for the image formation as it affects the specimen's rendition.

CHARACTERISATION of CELLS
(staining for mitochondria, cytoskeleton and nucleus)



Material you need:

- cells (sheep skin fibroblasts)
- around 20 ml of PBS (in 50 ml tube)
- 1ml of 4% Paraformaldehyde (transfer into eppendorf)
- 1ml of 0.1% Triton X- 100 (transfer into eppendorf)



- 1ml of Mitotracker Green solution (transfer into eppendorf and cover with aluminium foil) - for mitochondria
- 1ml of Phalloidin solution (transfer into eppendorf, cover with aluminium foil) - for cytoskeleton
- 1ml of Hoechst solution (transfer into eppendorf, cover with aluminium foil) - for nucleus

- Pasteur pipet
- Piece of aluminium foil
- trash container

- Needle
- Pincette
- 1 big glass slide



Procedure

1. Prepare work place - take all needed materials.
2. Observed cells under inverted microscope.
3. Discard culture medium using Pasteur pipet.
4. Add 1ml of PBS.
5. Wash cells with PBS then aspirate and trash
6. Add 1ml of medium contained Mitotracker Green (50 nM working solution) (**FOR MITOCHONDRIA STAINING**)
7. Incubate 15 min in 37°C covered from light (light sensitive).
8. Discard medium contain Mitotracker Green.
9. Add 1ml of PBS and wash cells for 2-3 min. Repeat 2 time.
10. Add 1ml of 4% paraformaldehyde (PFA). **Careful - PFA is toxic.**
11. Incubate 10 min at 37°C (incubator).
12. Carefully aspirate paraformaldehyde and trash.
13. Add 1ml of PBS - wash for 3 min.
14. Repeat washing 3 times.
15. Add 1ml of 0.1% Triton - X - 100 incubate at room temperature (RT) for 10 min (cover from light).
16. Aspirate 0.1% Triton - X- 100 and trash.
17. Add 1ml of PBS - wash for 3 min.
18. Repeat washing 3 times.
19. Add 1ml of medium contains 500ng/ml of Phalloidin conjugated with FITC (green) (**F-actin staining**).
20. Incubate at room temperature for 30 min (covered from light).

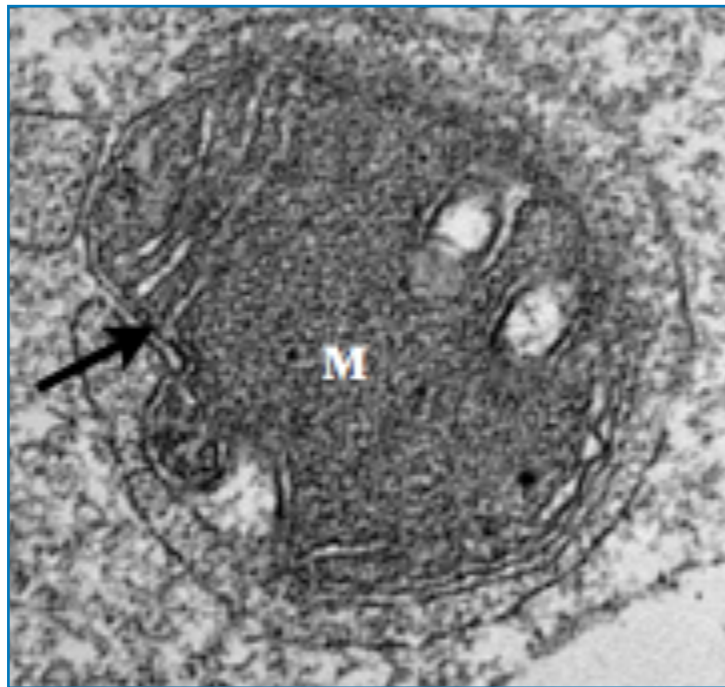
Mitochondria Staining

Mitochondria exist in most eukaryotic cells and play a very **important role** in **oxidative metabolism by generating ATP as an energy source**. The average number of mitochondria per cell is from 100 to 2,000. Though the typical size is about 0.5-2µm the shape, abundance, and location of mitochondria vary by cell type, cell cycle, and cell viability.

Therefore, visualization of mitochondria is important. Since mitochondria have **electron transport systems**, they can be stained with **various redox dyes**. MitoRed and Rh123 readily pass through cell membranes and accumulate in mitochondria.

The fluorescence intensity of Rh123 reflects the amount of ATP generated in mitochondria

Mitochondria



- “powerhouse” of the cells;
- involved in programmed cell death, innate immunity, autophagy, redox signalling, calcium homeostasis and stem cells reprogramming;
- inadequate or perturbed mitochondrial functions may adversely affect cellular growth

Nucleus Staining

Fluorescent dyes with aromatic amino or guanidine groups, such as propidium iodide (PI), ethidium bromide (EB), acridine orange (AO), and Hoechst dyes, interact with nucleotides to emit fluorescence.

Hoechst dye molecules attach at the minor groove of the DNA double helix.

These fluorescent dyes, except for the Hoechst dyes, are impermeable through the cell membranes of viable cells, and can be used as fluorescent indicators of dead cells. **Hoechst dyes are positively charged under physiological conditions and can pass through viable cell membranes.**

Cytoskeleton Staining

Phalloidin is a highly selective bicyclic peptide that is used for staining actin filaments (also known as F-actin).

It binds to all variants of actin filaments in many different species of animals and plants. It functions by **binding and stabilizing filamentous actin** (F-actin) and effectively prevents the **depolymerization of actin fibers**.

Typically, it is used conjugated to a fluorescent dye, such as FITC, Rhodamine, TRITC or similar dyes.

Phalloidin can be used with sample types such as formaldehyde-fixed and permeabilized tissue sections, cell cultures and cell-free experiments. It can also be used in paraffin-embedded samples that have been de-paraffinized.

Phalloidin belongs to a class of toxins called phallotoxins, which are found in the death cap mushroom.



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24 h before the experiment we need to plate our cells

Subculturing

Remove spent media from the culture vessel.

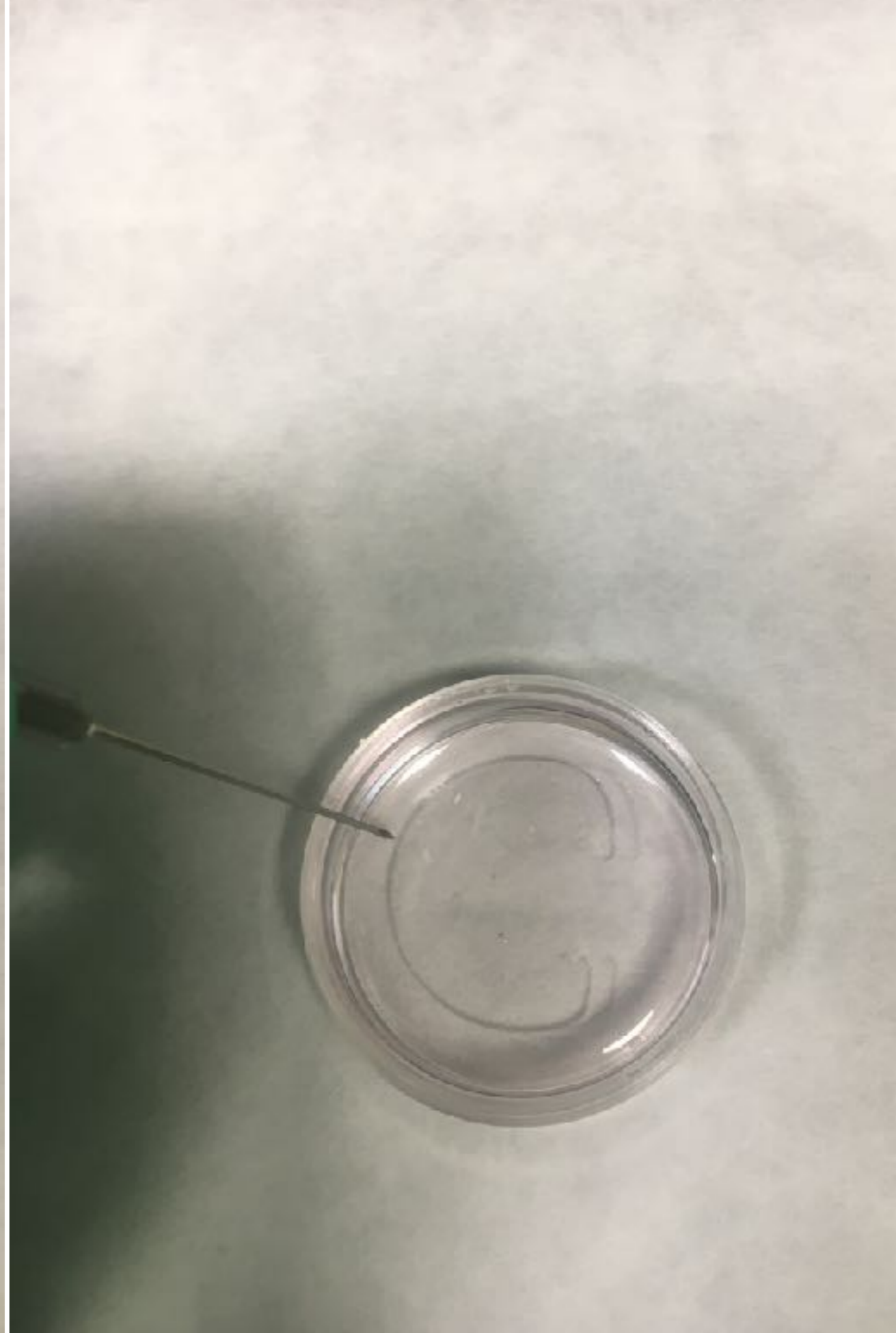
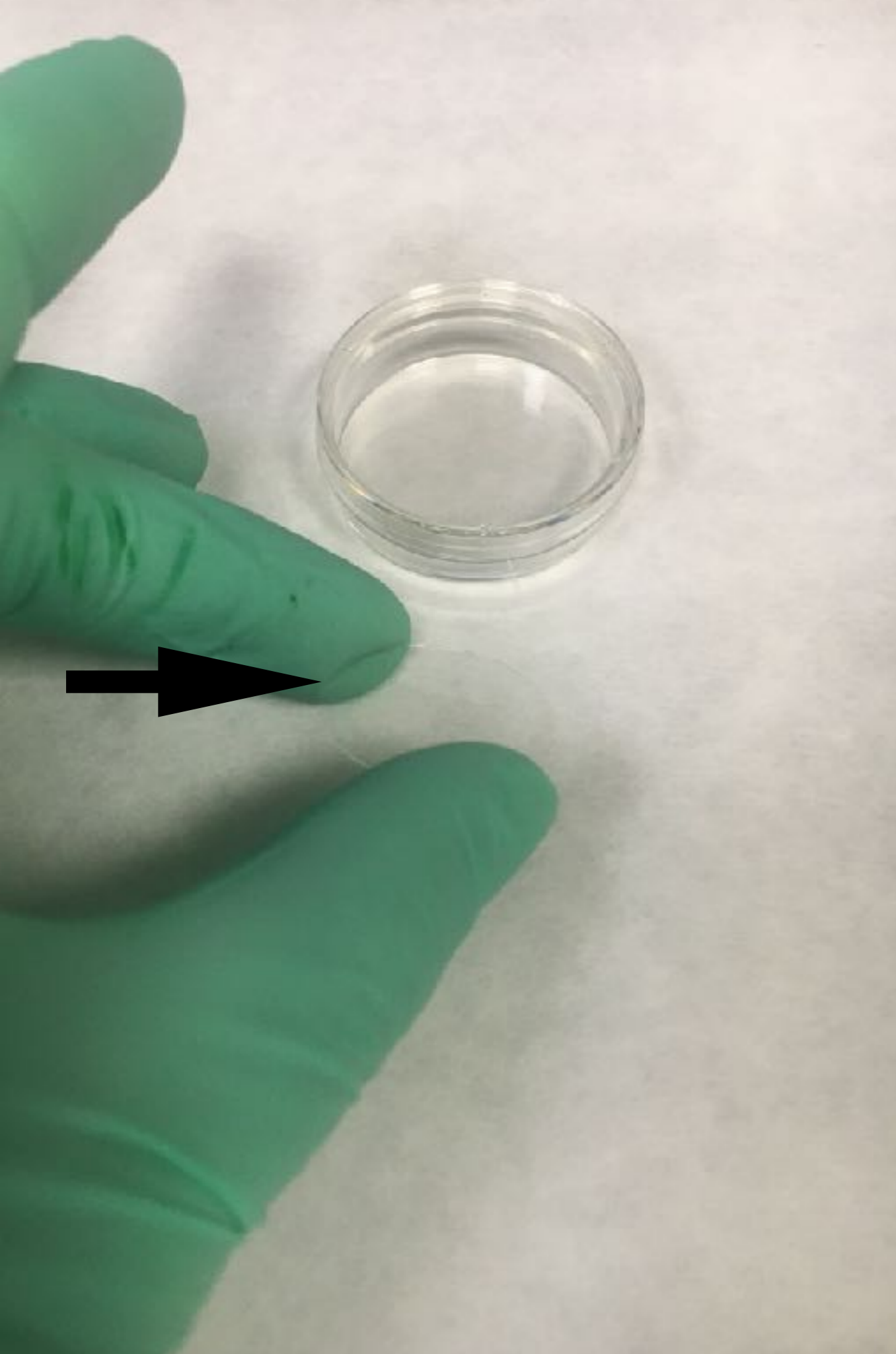
Add the pre-warmed dissociation reagent such as trypsin. Gently rock the container to get complete coverage of the cell layer.

Incubate the culture vessel at room temperature for approximately 2 minutes.

Add equivalent of 2 volumes of pre-warmed complete growth medium. Disperse the medium by pipetting over the cell layer surface several times.

Then split the cells into 2 or 3 flasks containing complete media.

Incubate the cells.



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- Needle
- Pincette
- 1 big glass slide

- piece of aluminium foil

- around 20 ml of PBS

- Trash container

- Pasteur pipet

- 4% Paraformaldehyde

- 0.1% Triton X- 100

- Needle

- Pincette

- 1 big glass slide

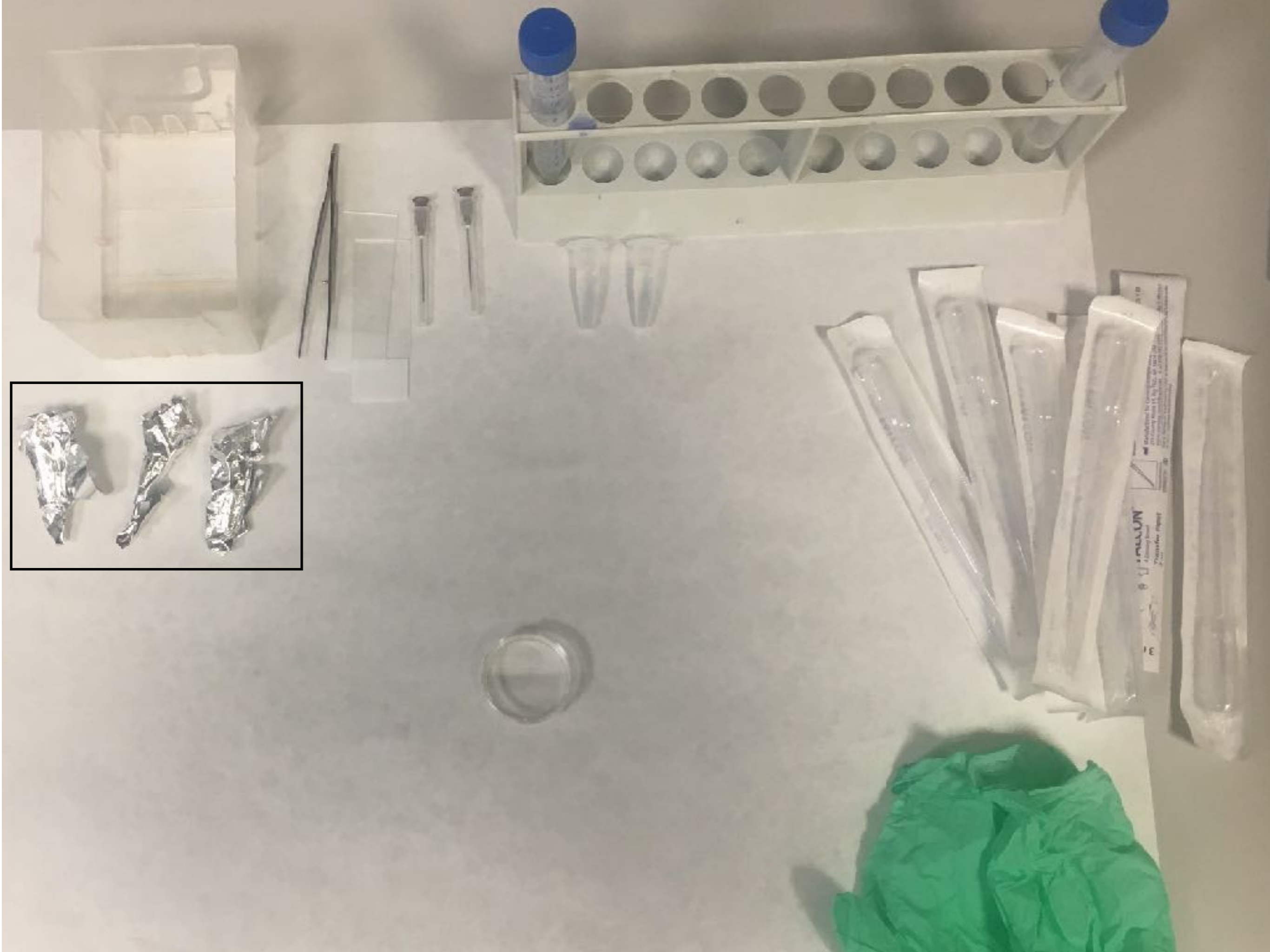
- Mitotracker Green

- Phalloidin

- Hoechst

- cells

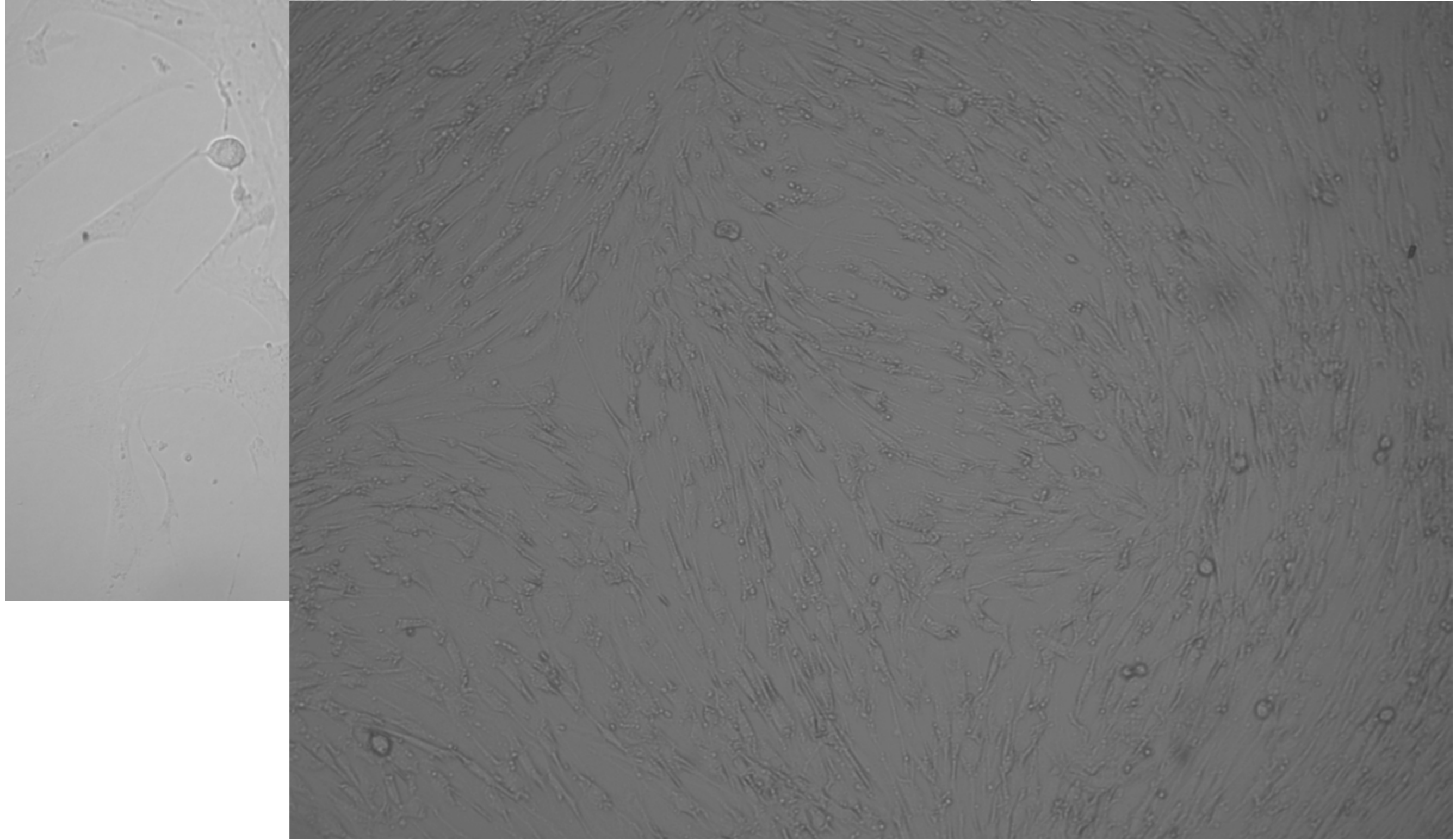
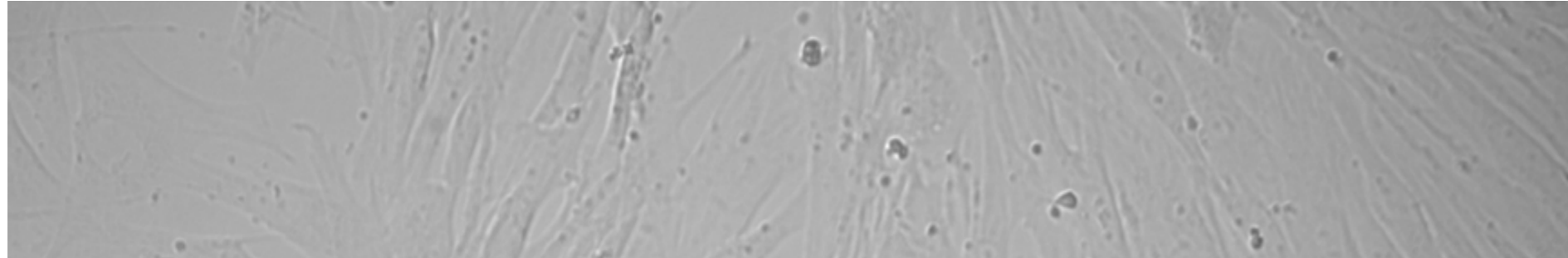
PREPARE WORK PLACE



Procedure

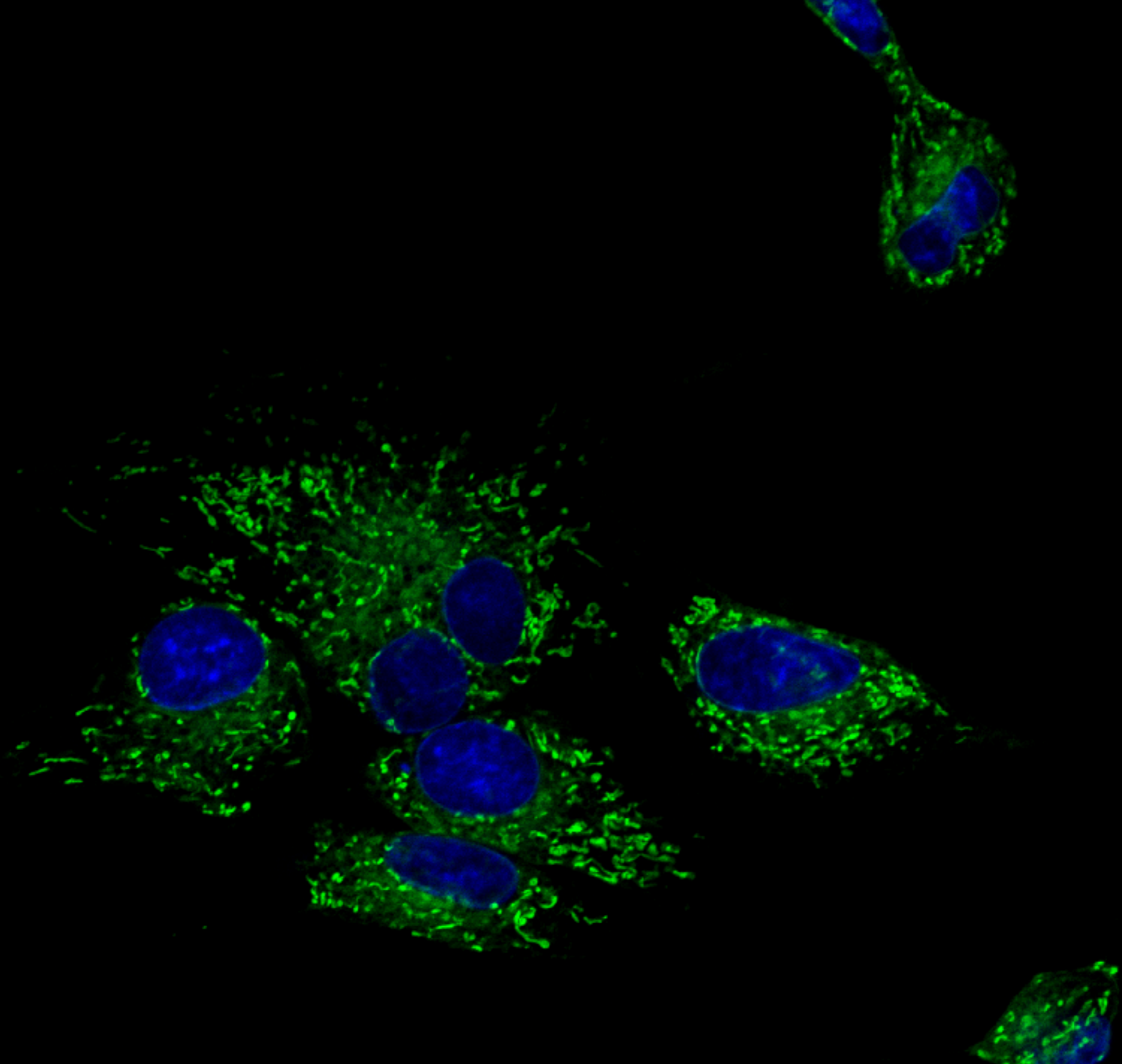
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2. Observed cells under microscope - perfect confluency

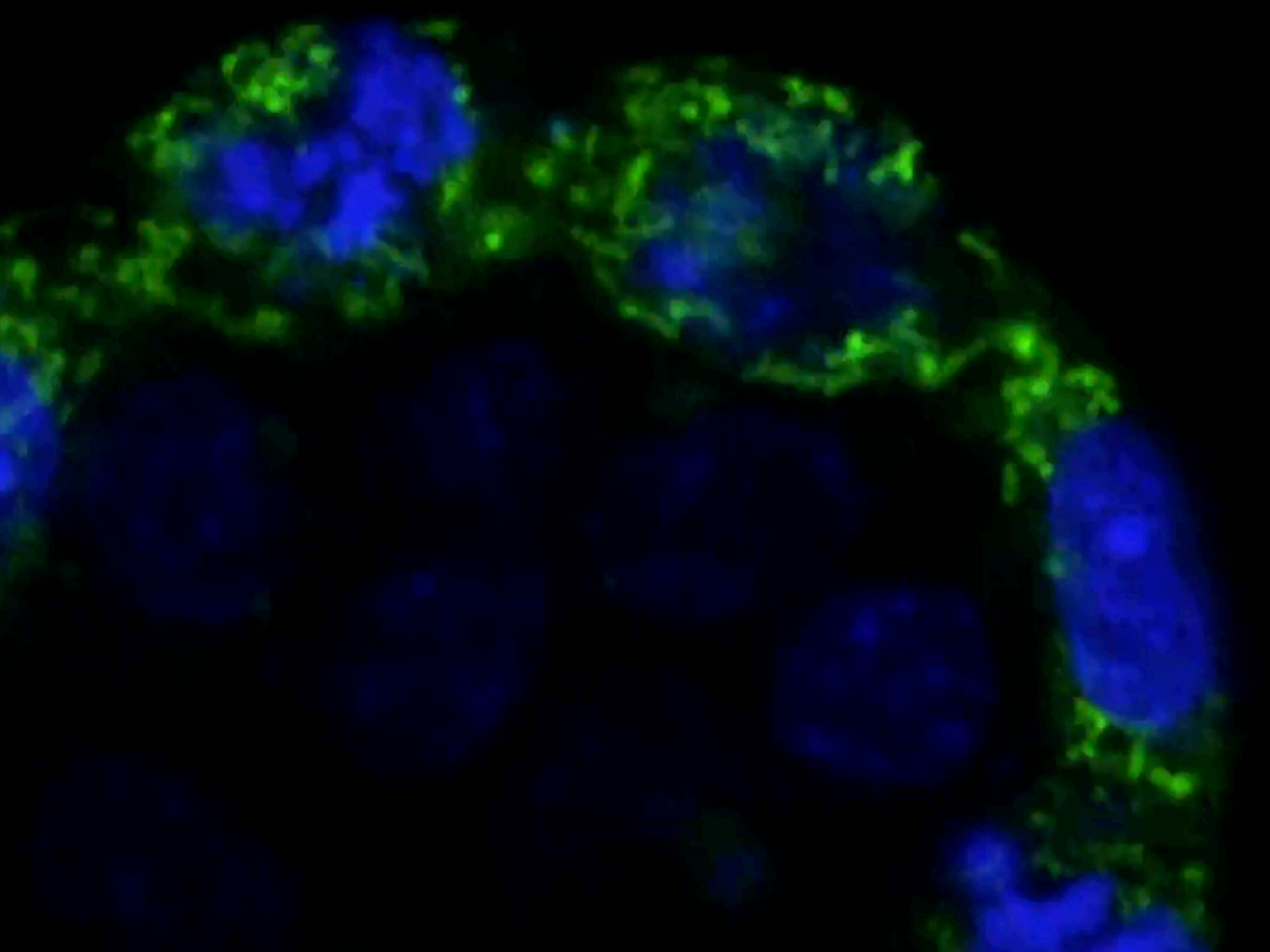


STEP 1. MITOCHONDRIAL STAINING

1. Discard culture medium using Pasteur pipet.
2. Add 1ml of PBS.
3. Wash cells with PBS then aspirate and trash
4. Repeat washing 3 times
5. Add 1ml of medium contained Mitotracker Green/Red (50 nM working solution) (**FOR MITOCHONDRIA STAINING**)
6. Incubate 15 min in 37°C covered from light (light sensitive).



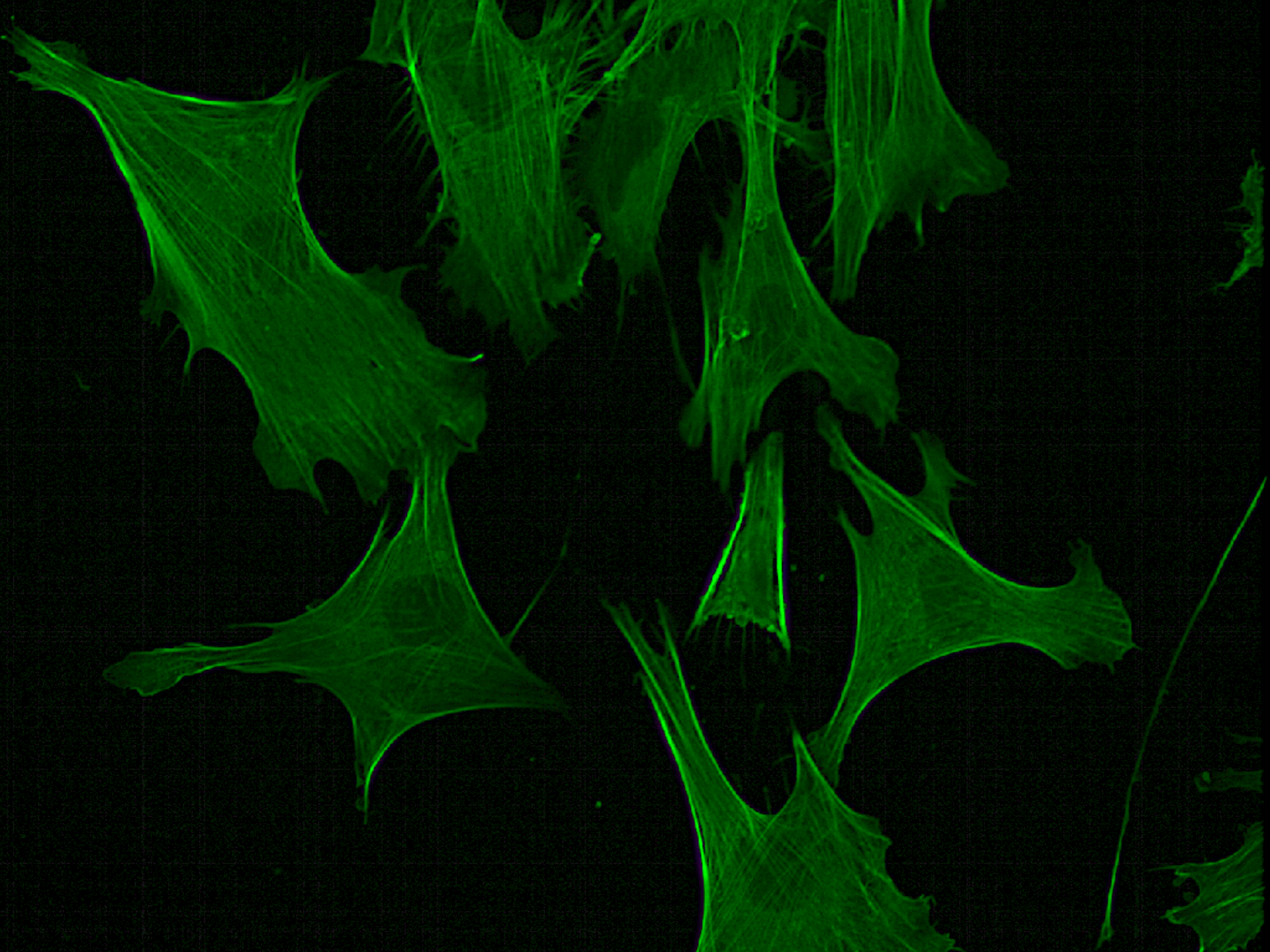




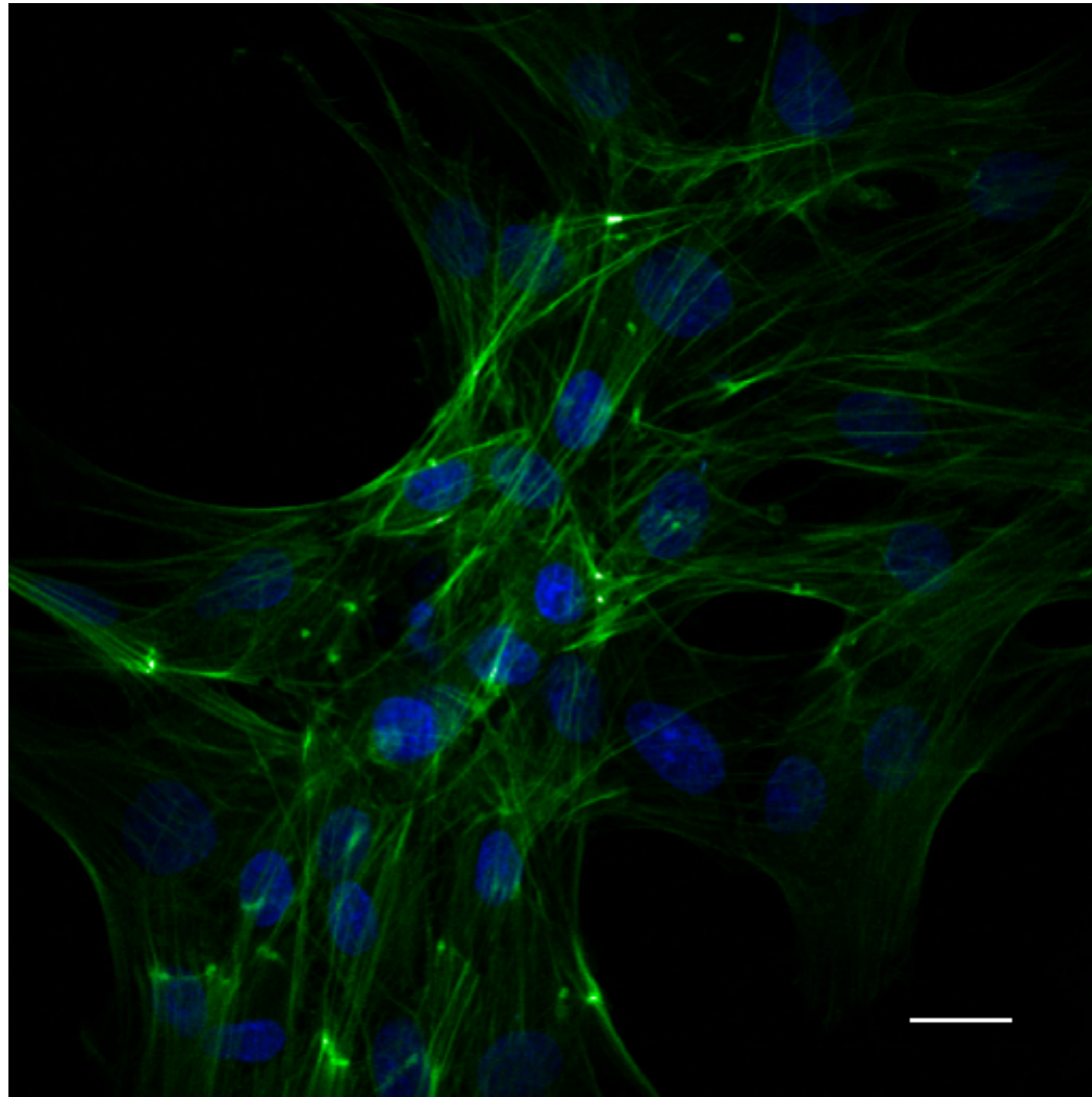
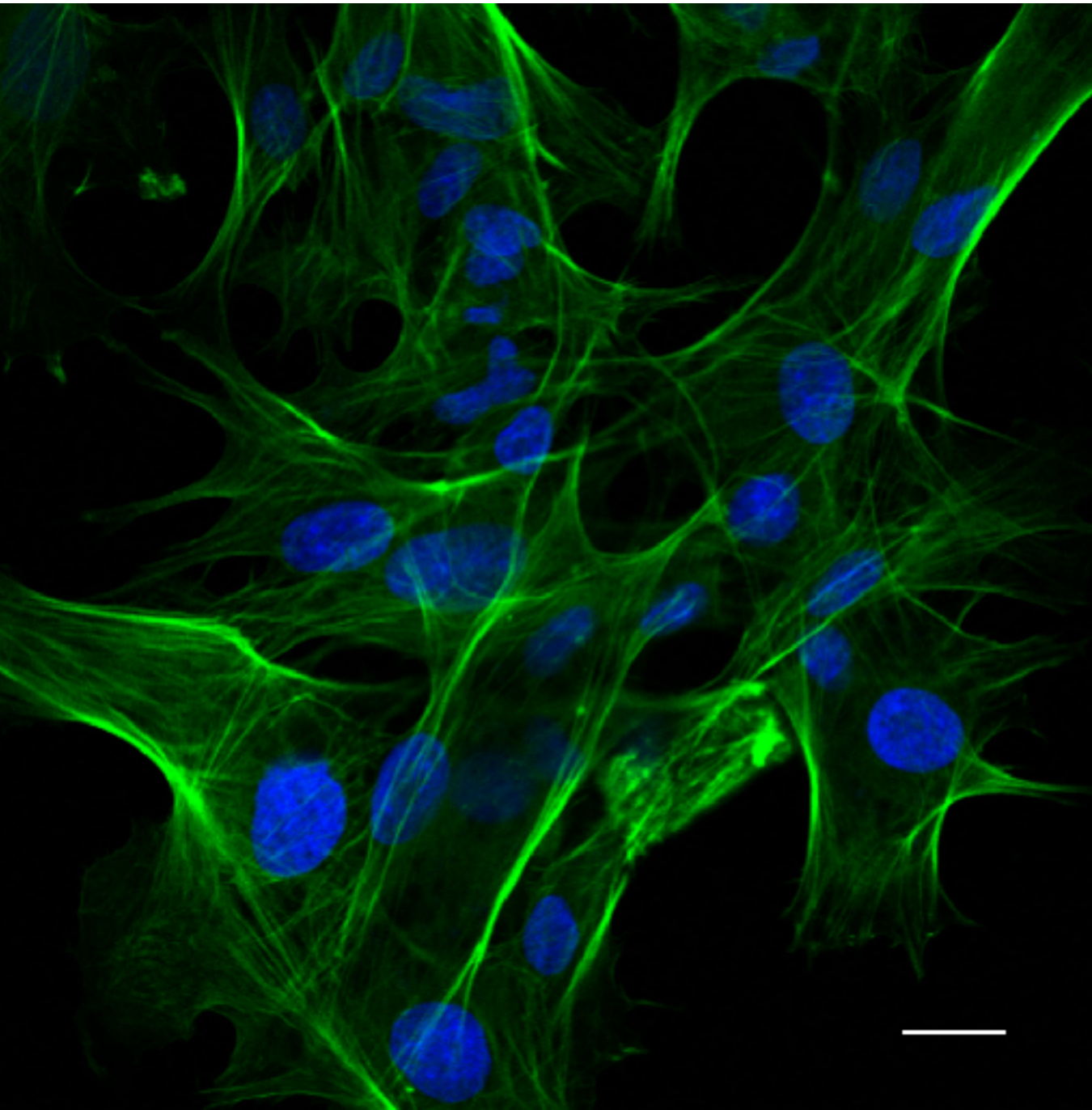
STEP 2. CYTOSKELETON STAINING

1. Remove PBS from dish with cells
2. Add 1ml of 4% paraformaldehyde (PFA). **Careful - PFA is toxic.**
3. Incubate 10 min at 37°C (incubator).
4. Carefully aspirate paraformaldehyde and trash.
5. Add 1ml of PBS - wash for 3 min.
6. Repeat washing 3 times.
7. Add 1ml of 0.1% Triton - X - 100 incubate at room temperature (RT) for 10 min (cover from light).
8. Aspirate 0.1% Triton - X- 100 and trash.
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12. Incubate at room temperature for 30 min (covered from light).

<https://www.youtube.com/watch?v=gqcjfrJ7Ko>

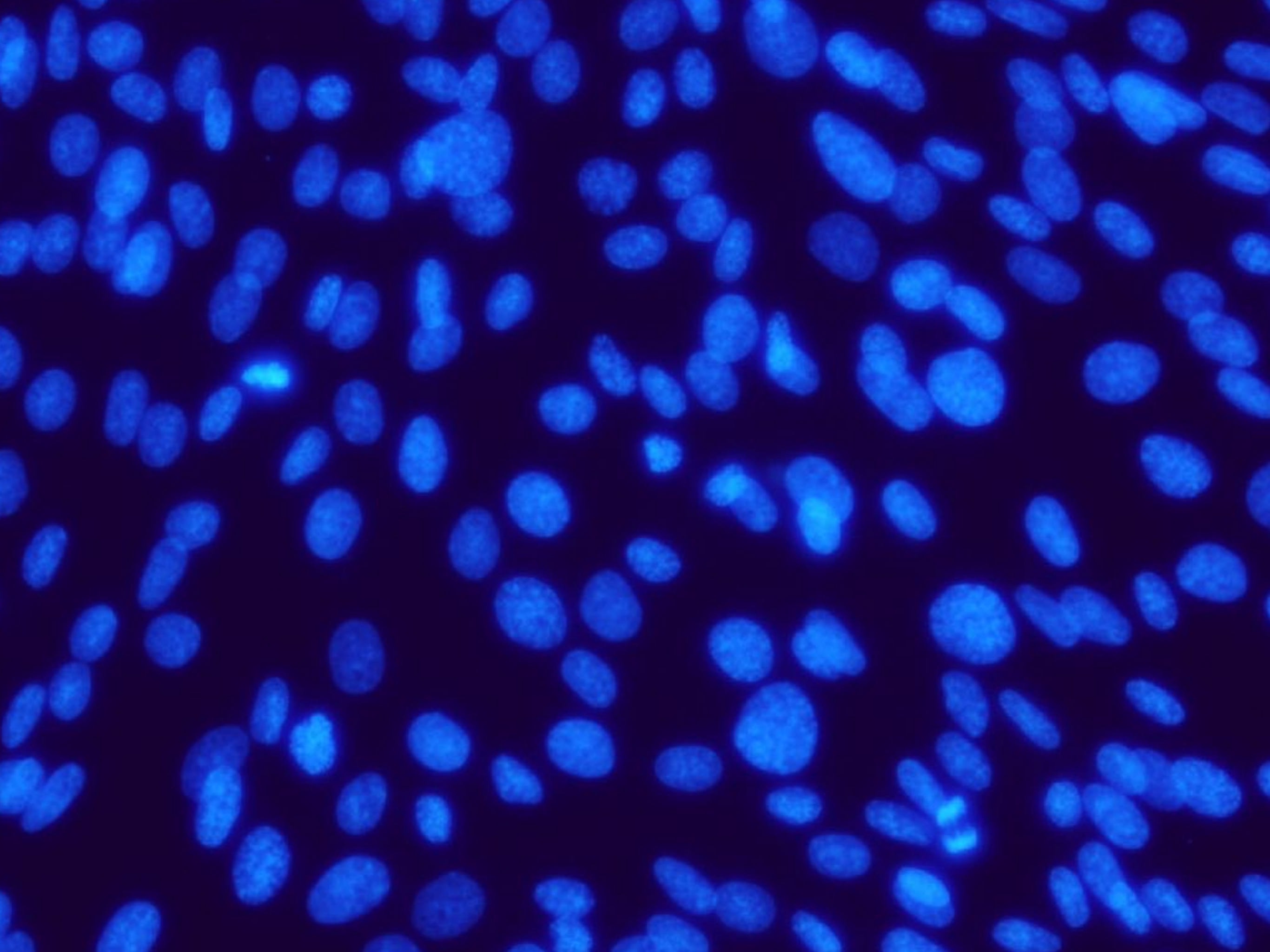


Falloidin staining - ACTINA



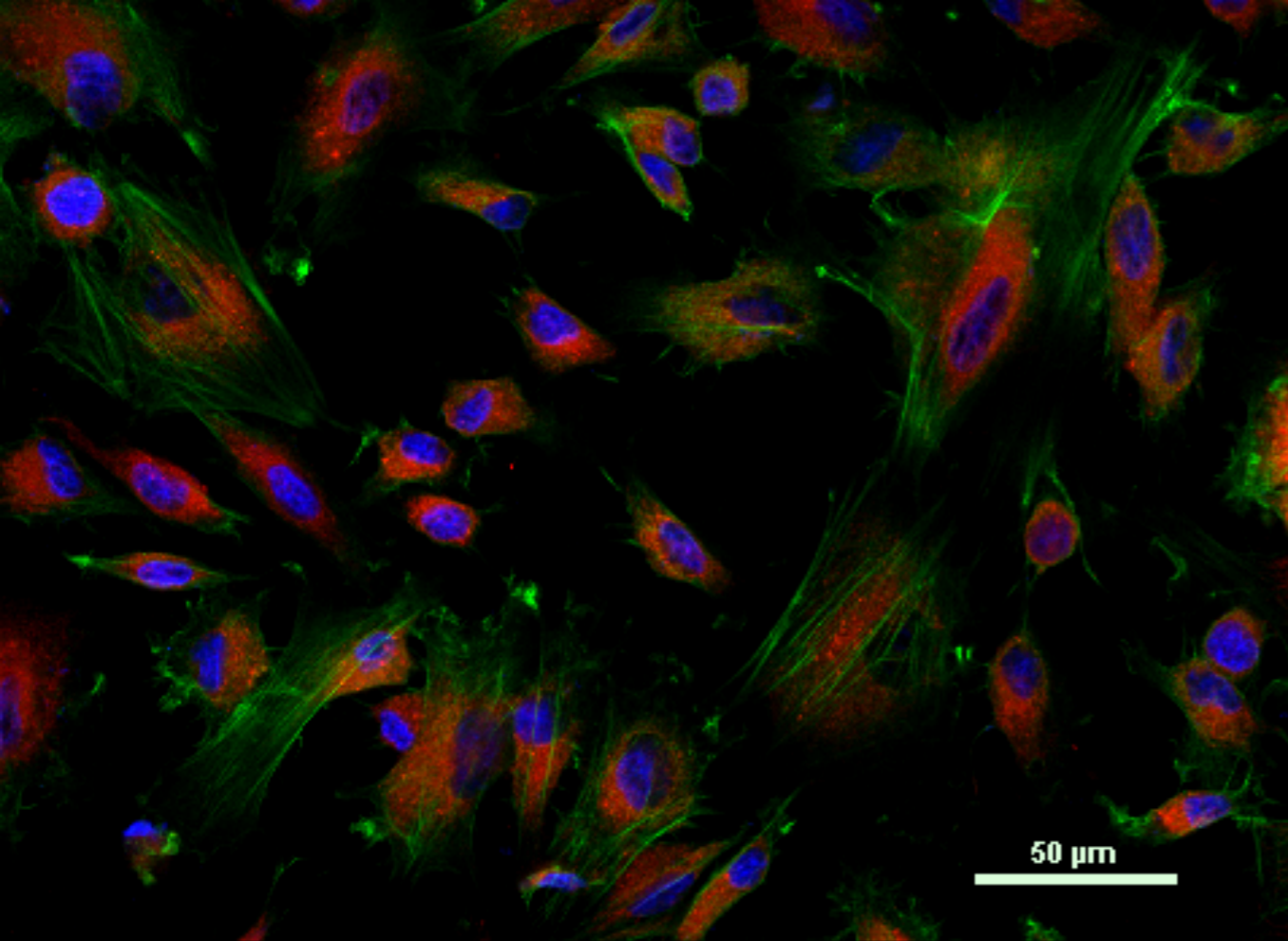
STEP 3. STAINING Of The NUCLEUS

1. Discard medium with Phalloidin.
2. Add 1ml of PBS - wash for 3 min.
3. Repeat washing 3 times.
4. Add 1ml of PBS contains 5ug/ml of Hoechst (**NUCLEAR STAINING**)
5. Incubate for 5min cover from light.
6. Aspirate PBS containing Hoechst.
7. Add 1ml of fresh PBS
8. Wash quickly 2 times.

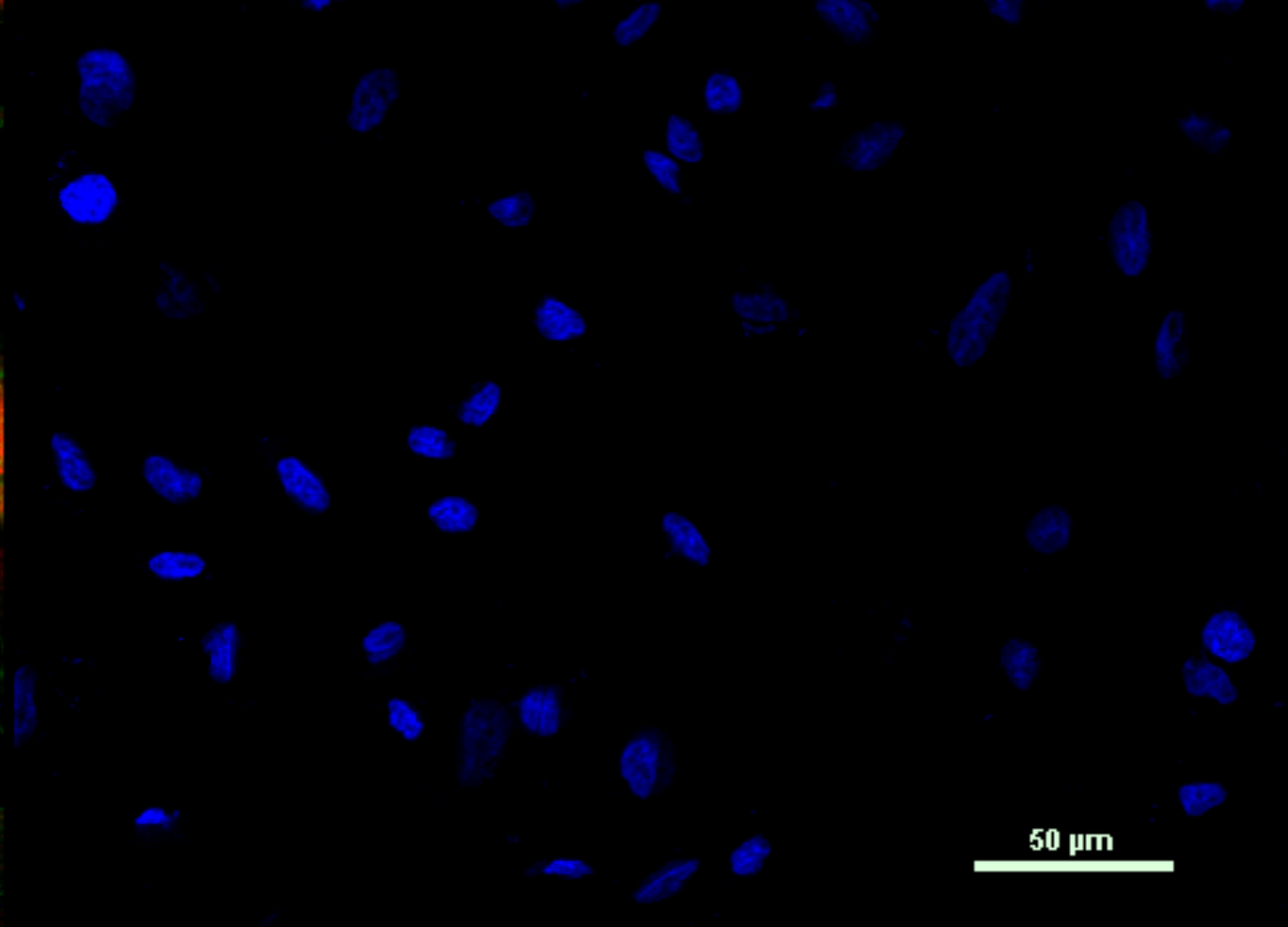


STEP 4. SLIDES PREPARATION

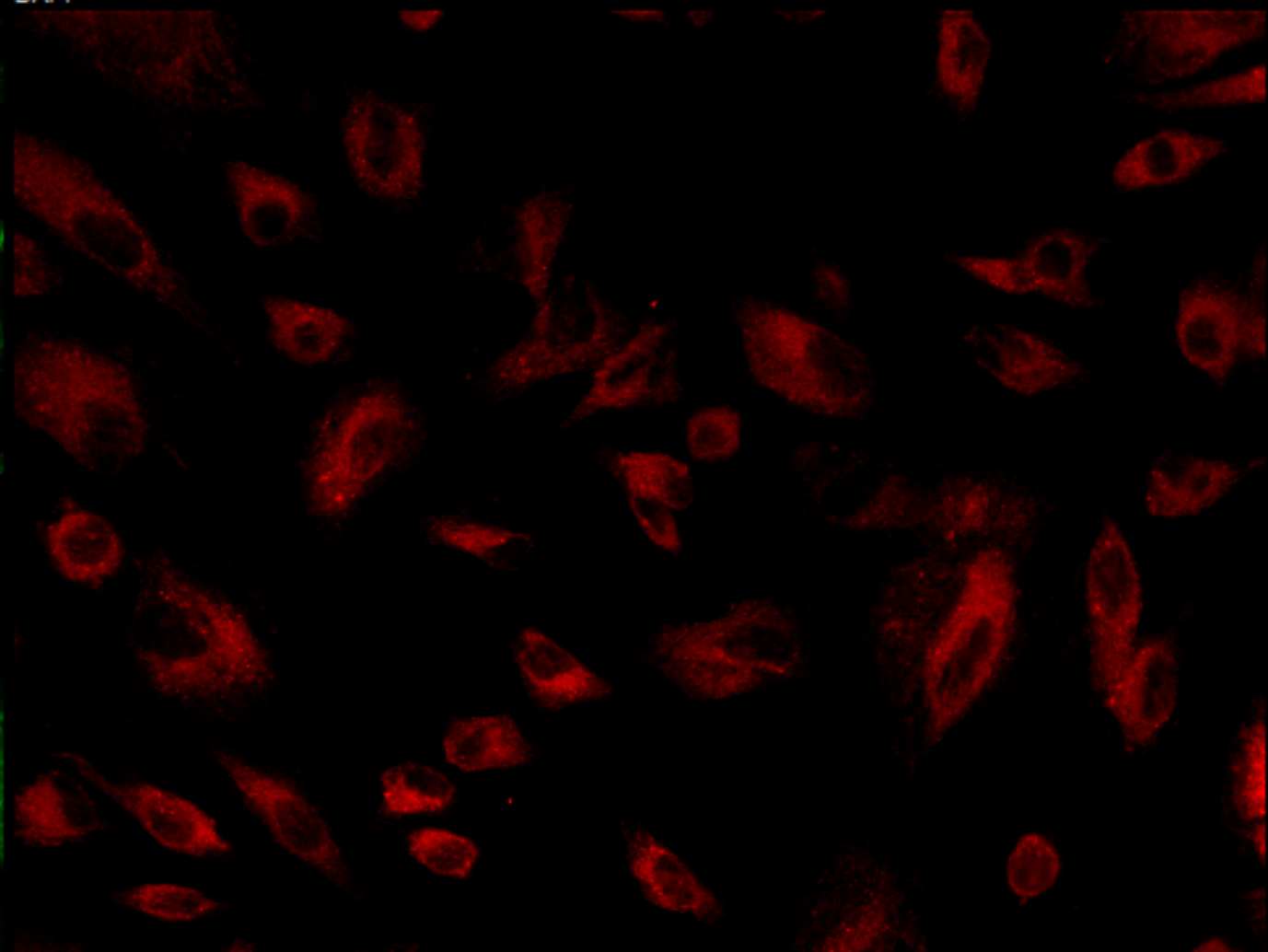
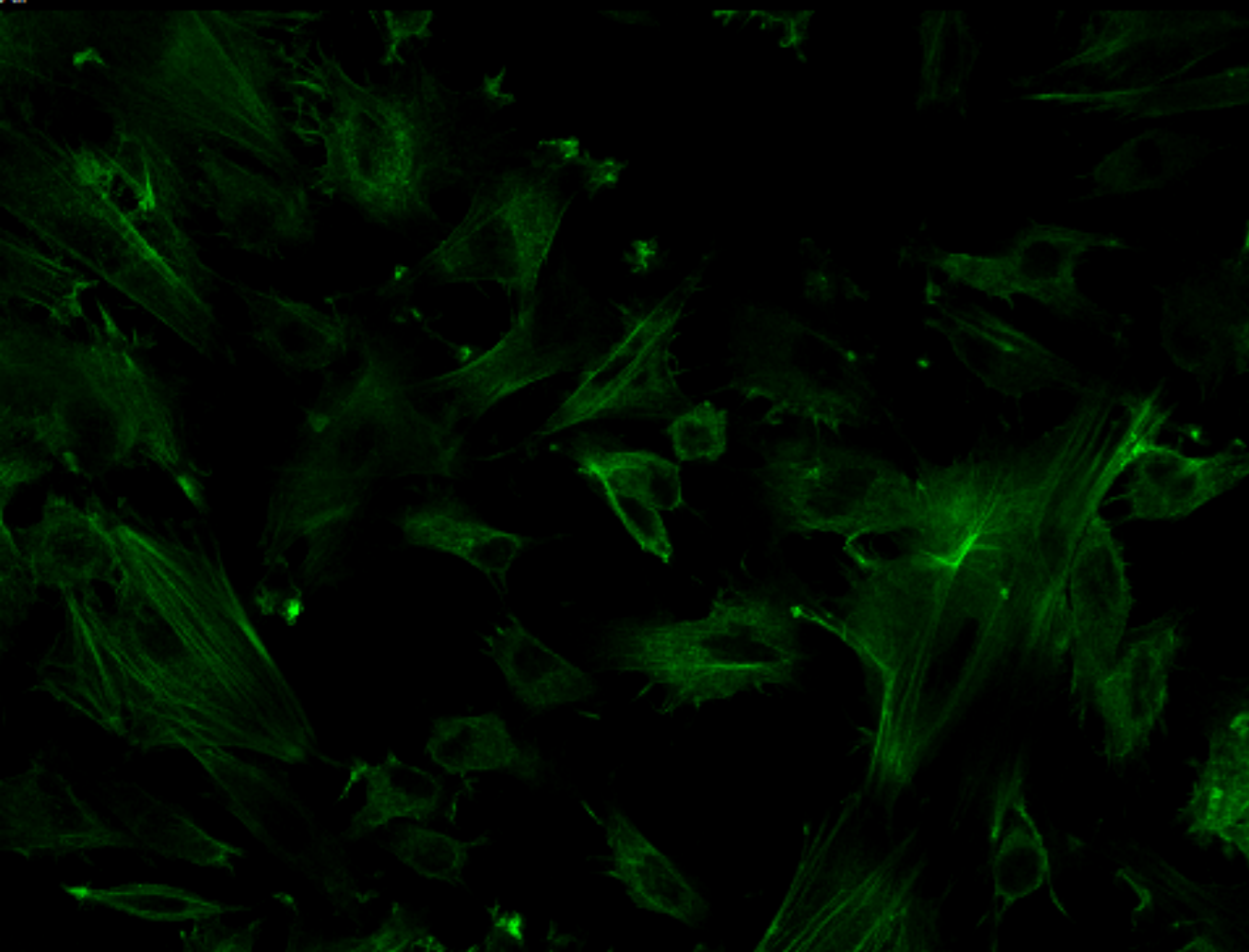
2. Take big glass slide.
3. Wash glass with 70% ethanol
4. Add small drop of mounting medium in the centre of glass slide.
5. Take out cover glass with cells (use needle and/or pincette) and put on the drop of the mounting medium
- 6.!!!!!!! **BE CAREFUL** remember that cells grow on the top of the glass. Put cover glass with cells that cells touch mounting medium - **ASK for ASSISTANCE!!**
7. Observe under fluorescent microscope.
8. Mitochondria - green “spots”, Actin - green “line”, nucleus - blue)
9. For long storage cover with nail polish. Cover from light.

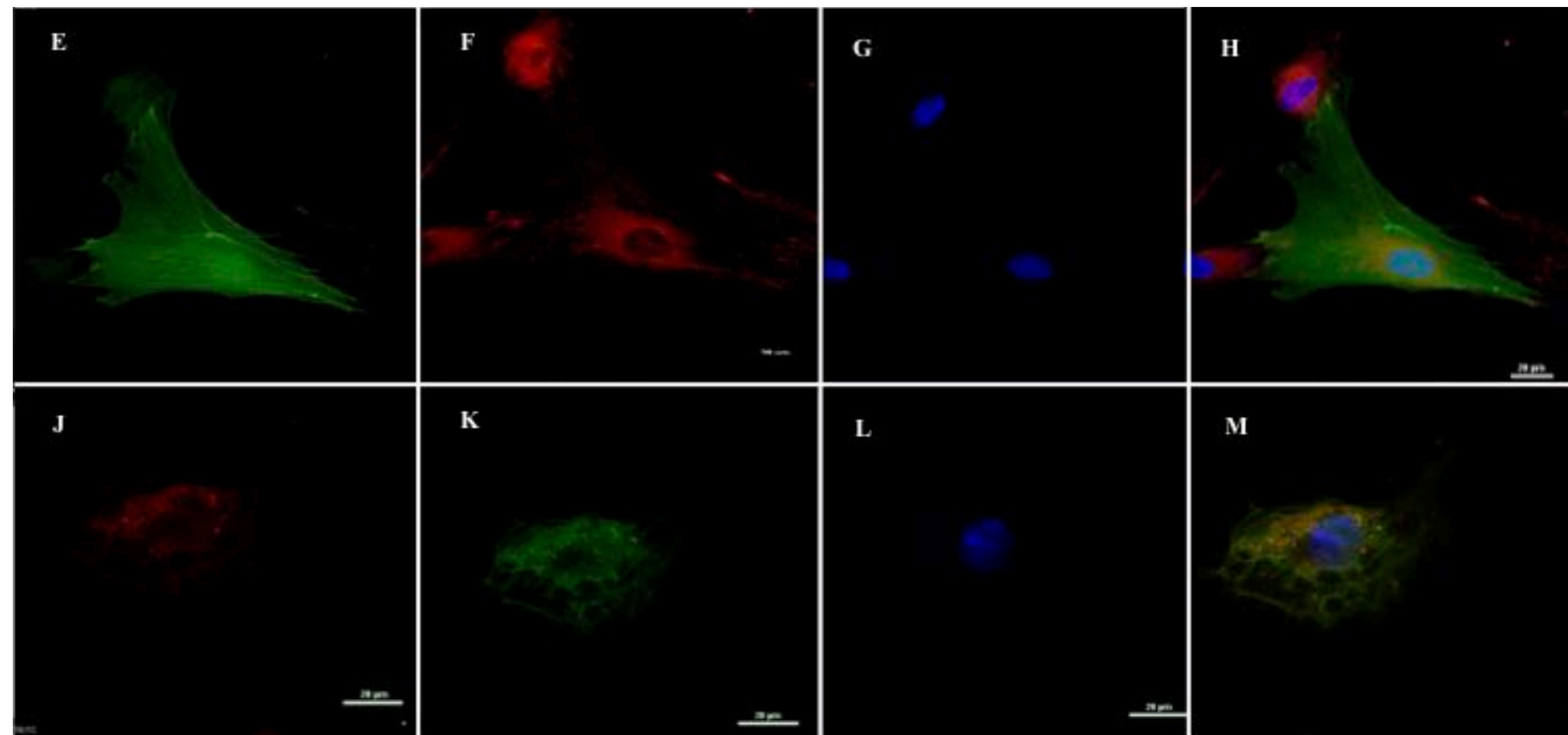


All



DAPI





QUESTIONS?