Principles of Immunofluorescence

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Immunofluorescence

Immunofluorescence is a type of assay performed on biological samples to detect **specific antigens** in any biological specimen or sample and **vice-versa.**
The **specificity** of **antibodies** to their **antigen** is the **base** for **immunofluorescence**.

It was described in **1942** and refined by Coons in **1950**, which used a **fluorescence microscope** able to read the specific **immunological** reaction and cellular slide preparations.

It is an effective method for **visualizing intracellular processes**, **structures**, and conditions as well.

- In Vitro type of **Ag-Ab reaction.**
- Detects **surface antigens** or **antibodies**.
- **Fluorescent dyes** are used for the visualization of Ag-Ab reactions.

Fluorescence microscopy

- Fluorescence microscopy for biomedical research is a **light microscope technique** designed to **view fluorescence emis**sion from a **cell or tissue specimen**.

- While it can be used to **image endogenous fluorescence** from a sample, its major use is to image the fluorescence from various **exogenous probes** or **labels**.

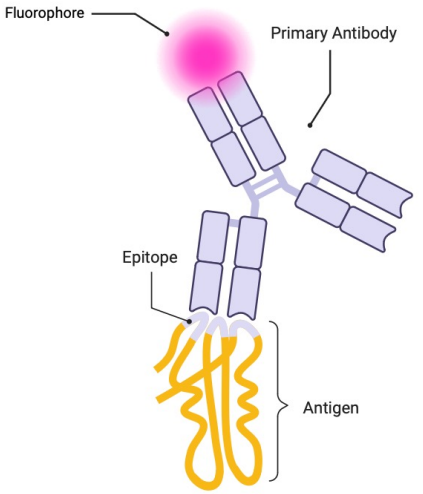
- These include dyes **staining various parts of the cell or tissue** or **fluorescently labelled antibodies for immunofluorescence or nucleotides** or **fluorescence in situ hybridization**, respectively.

- Other fluorescent reagents are employed to investigate **intracellular dynamics** of **calcium and other ions and metabolites**.

- Advanced spectroscopic techniques applied through the fluorescence microscope are used to measure translational and rotational diffusion of labelled molecules as well as proximity relationships between cellular macromolecules.

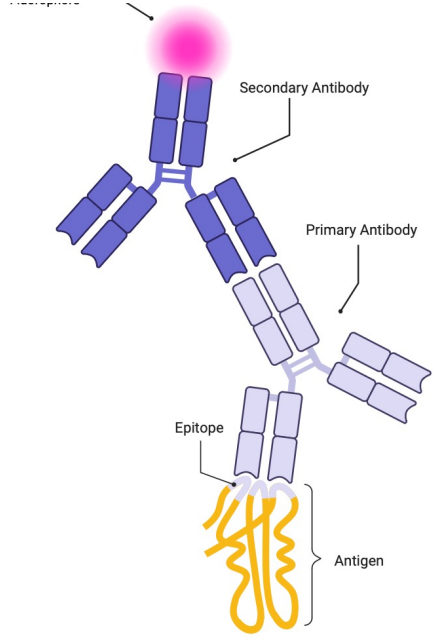
Direct Immunofluorescence Test

Single antibody i.e. primary antibody is used that is chemically linked to a fluorochrome. If the antigen is present, the primary antibody directly reacts with it and fluorescence can be observed under the fluorescent microscope.



Indirect Immunofluorescence Test

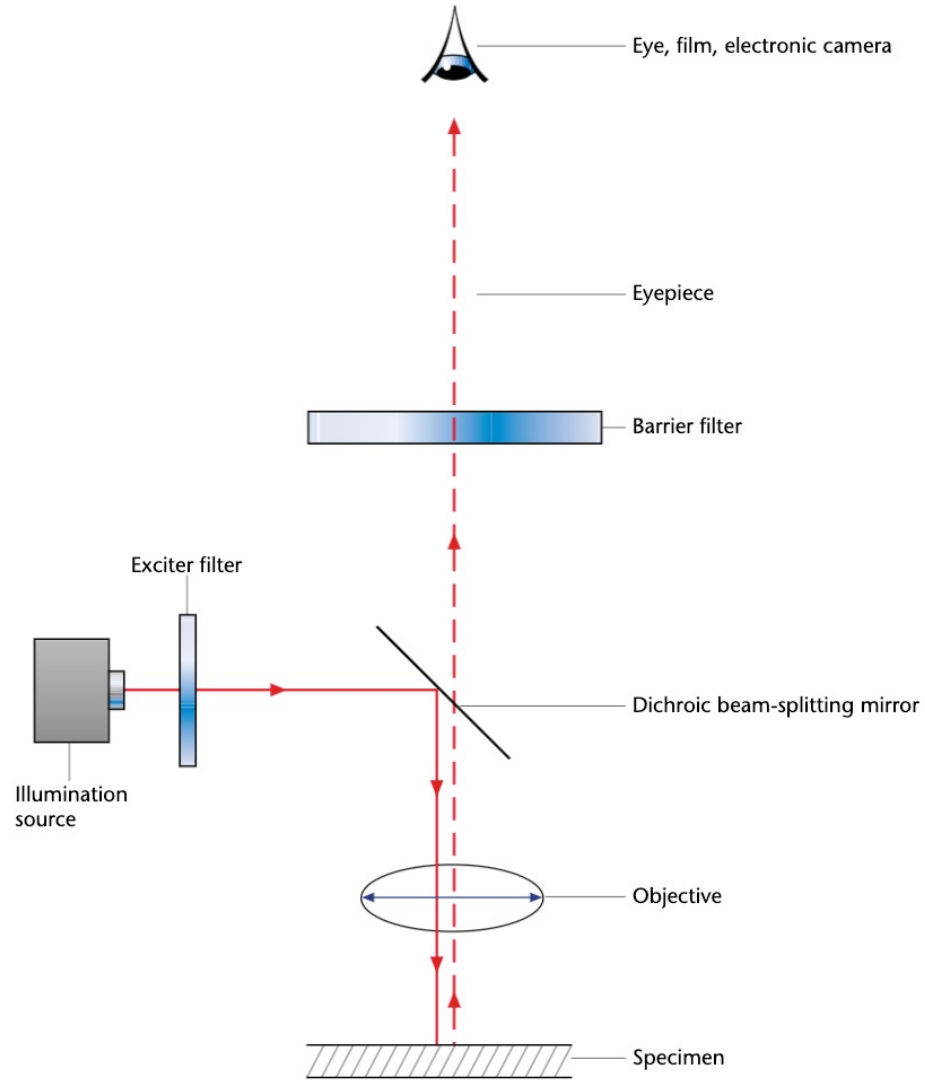
Double antibodies are used i.e. primary and secondary antibodies. The primary antibody is not labeled and a fluorochrome-labeled secondary antibody is used for detection. The antigen used is known and it binds to the specific primary antibodies of interest in the sample. The secondary antibody then binds to the Fc region of the primary antibody.



Fluorescence microscopy

- The **resulting fluorescence** is then **collected by the objective**, and **light forming the image passes through** the **dichroic mirror** to either the **eyepieces or a camera**.

- The **dichroic mirror** has the property that it will **reflect the shorter- wavelength exci**tation light and **transmit the longer- wavelength emitted light**.
- Thus, the intense excitation light is directed away from the eye or other detector so that it does not have to be filtered out of the fluorescent image reaching the eye or other detector. The most important optical elements of the microscope are the objective lens, as it determines the resolution and brightness of the image, the filters and the dichroic mirror.

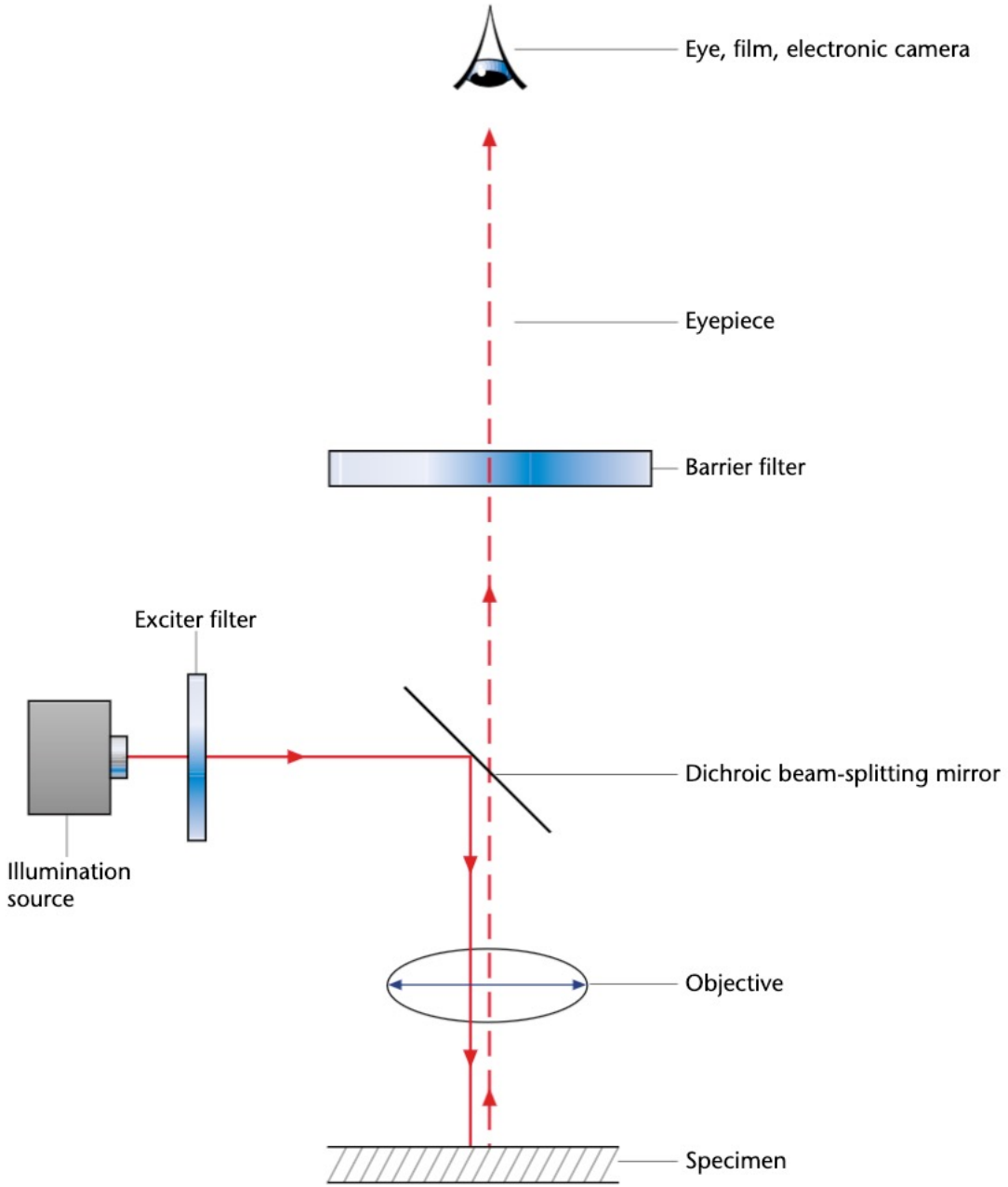


Schematic diagram of the basic fluorescence microscope. —, exciting light; – – –, fluorescent light.

Fluorescence microscopy

- The microscope A fluorescence microscope is basically a **filter fluorometer.**
- With desired spectral range (band) of **excitation wavelengths**, **typically produced by a mercury or xenon arc lamp**, is isolated using a **narrow-band exciter filter**, and the emission is detected through an **emission or barrier filter** that **blocks all wavelengths below the emission band**.

- In this setup, **the exciting light** is reflected into the back aperture of the objective (which acts as a condenser) by a **dichroic beam splitting mirror**.



Schematic diagram of the basic fluorescence microscope. —, exciting light; – – –, fluorescent light.

Procedures of parasites staining

Immunofluorescence in *E. invadens* staining was performed according to Siegesmund (2011) and Samanta *et al* (2018).

- Briefly, Poly- prep slides were prepared and wiped with acetone.
- Cells were iced for 10 minutes, harvested at 400 g for 5 minutes and washed with PBS.
- Cells were resuspended in incomplete media and attached on acetone wiped poly-L-lysine coated slides for 10- 30 minutes.
- Afterward, cells were fixed with PFA 4% for 45 minutes and rehydrated with 1x PBS for 30 minutes.
- Trophozoites were permeabilized with Triton 100x 0.2% -1xPBS at room temperature for 1 hour and cysts were permeabilized overnight at 4 °C.
- Cells Blocked with BSA 2% at room temperature for 2 hours followed by incubation with anti-HA primary antibodies (1:500) in Triton X100 0.2%, Sod. Azide 0.02% and 2% BSA, for 1 hour at room temperature and washed twice with 1x PBS-Triton 0.2% for 3-5 minutes.
- In the dark, slides were incubated with secondary antibodies conjugated with Alexa flour 488 (1:500) in Triton 0.2%, Sod. Azide 0.02% and 1% BSA in 1x PBS, for 1 hour at room temperature.
- Then, slides were washed with Triton 0.2% - BSA 0.5% in 1x PBS, 3 times 5 minutes each, mounted with Fluoro-shield mounting medium contains DAPI.
- Slides were covered immediately with long coverslips, sealed with nail polish and stored at 4 °C in the dark until microscopic examination.

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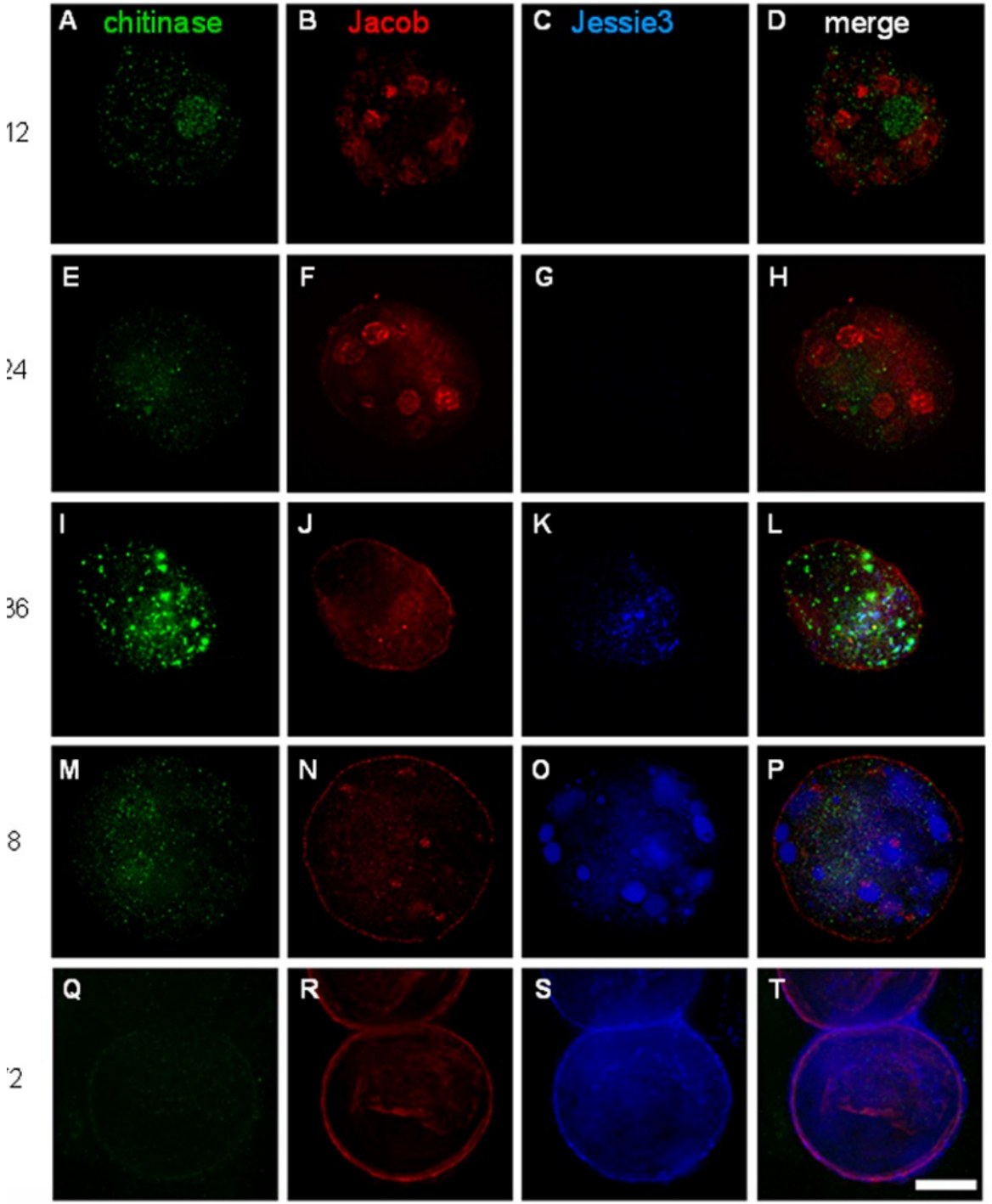
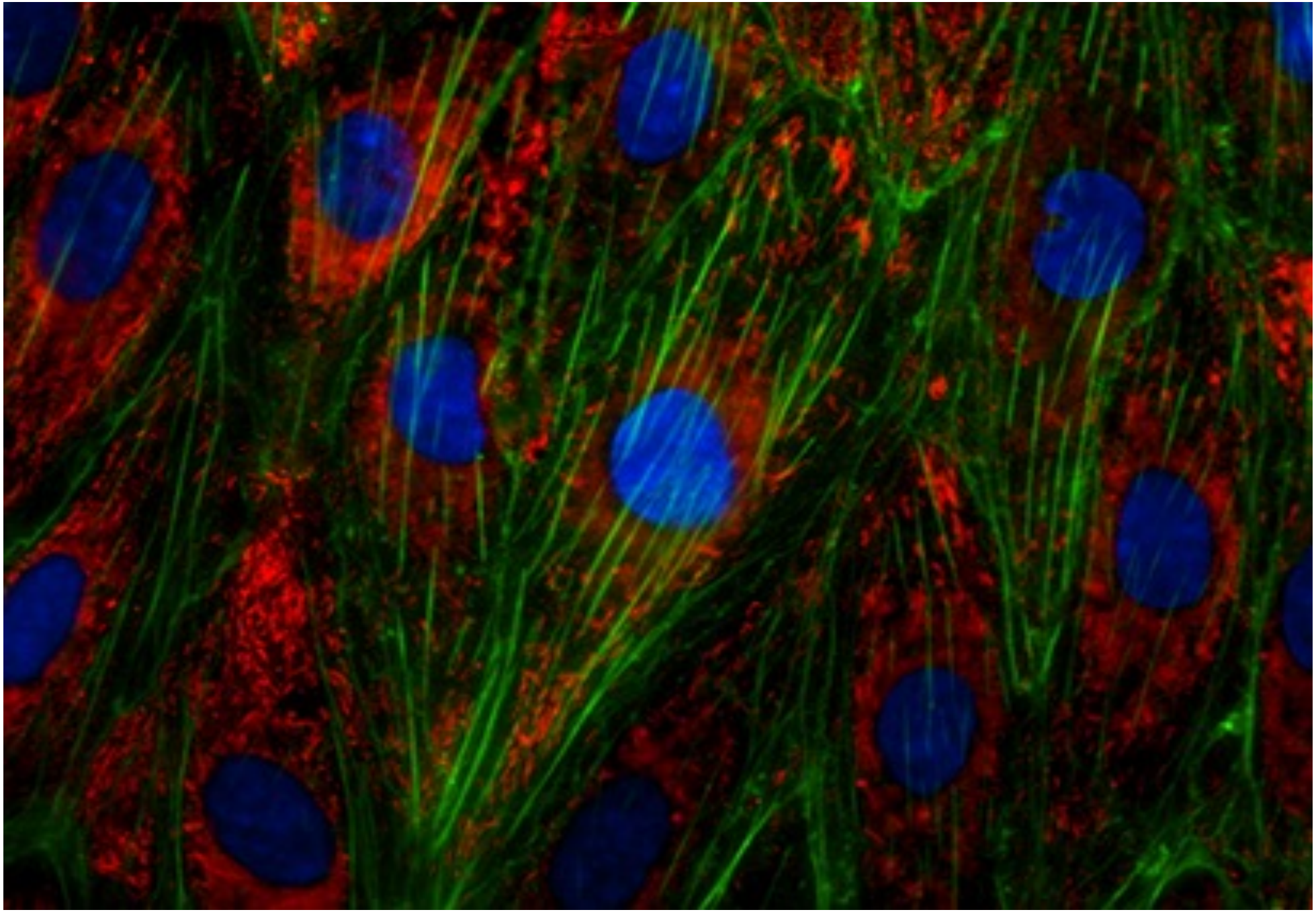
Procedures of Mammalian cells staining

- Twenty-four hours after transfection, cells grown on glass coverslips were fixed with 4% PFA in 1x PBS (pH 7.4)**,** permeabilized with 0.2% Triton X-100, blocked with 1% BSA and incubated with primary and secondary antibodies according to Schrader *et al* (2017). Briefly:
- Culture medium removed from 24 hrs old transfected COS-7 cells grown on coverslips in 6 cm dish and washed briefly with 1× PBS.
- Cells were **fixed immediately with fresh 4% paraformaldehyde (PFA)** for 20 minutes at room temperature and **washed three times** in 1× PBS for 2–5 minutes.
- Followed by **permeabilization with 0.2% Triton X-100 in 1× PBS** for 45 minutes at room temperature and **washing three times** in 1× PBS for 2–5 minutes.
- Cells were **blocked in 1% BSA in 1× PBS for 10 minutes** at room temperature and washed three times in 1× PBS for 2– 5 minutes.
- **Primary antibodies** diluted in 1% BSA/1× PBS (rabbit anti-PEX14 polyclonal antibodies,1:1400; rabbit anti-VAPB polyclonal antibody,1:1400; mouse anti-TOM20 monoclonal antibody, 1:200), were added and incubated in humid chamber for 1 hr at room temperature.
- Cells were **washed three times in 1× PBS for 2–5 minutes** and incubated with the **secondary antibodies** (e.g., donkey anti- rabbit-**Alexa 594**, donkey anti-mouse- **Alexa 488**) diluted in 1× PBS for 1 hr at room temperature in dark humid chamber followed by washing in 1× PBS three times for 2–5 minutes.
- A drop of **Fluoro-shield mounting medium** was applied on a clean, labelled glass microscope slide. Coverslip placed (cell-side down) onto the drop of Fluoro-shield mounting medium on the glass slide. Slides were kept in a **lightproof slide box for 2–3 h until Fluoro-shield mounting medium** dry. Stored at 4 °C in the dark until microscopic examination.

Direct Immunofluorescence Test



Organells staining



Organells staining