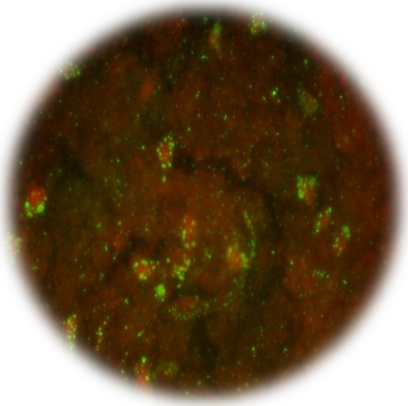
WORLD ORGANISATION FOR ANIMAL HEALTH

Direct Fluorescent Antibody (DFA) Test



Sharada, R.* and Kavitha, G.

Assistant Prof. & Quality Manager

KVAFSU-CVA Rabies Diagnostic Laboratory

OIE Reference Laboratory for Rabies

Dept. of Microbiology, Veterinary College, KVAFSU

Hebbal, Bengaluru, India

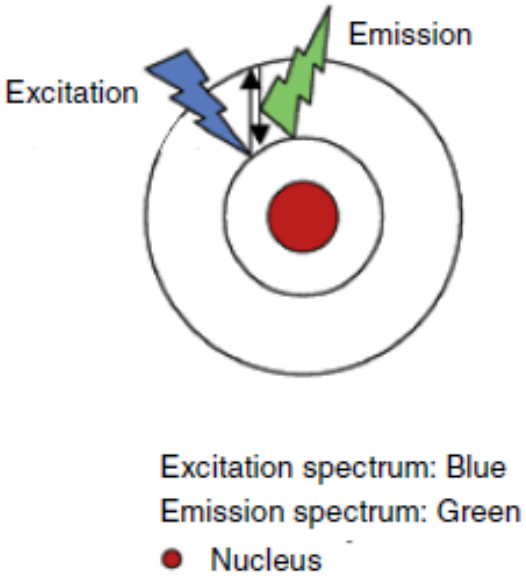
Fluorescent microscope

- **Coons *et al.* (1941/2)** - method of conjugating the antibody with fluorescein isothiocyanate (FITC) and first localized the pneumococci in tissues of mice

Principle of fluorescence

- **Fluorochromes / Fluorophores**

dye molecules : absorb light of a particular λ & energy, becomes excited / unstable



So emits light of longer λ & lesser energy to reach a stable state - **Fluorescence**

Fluorochromes

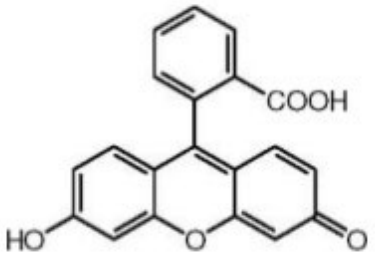
- **Acridine orange, Auramine, Fluorescein iso thiocyanate (FITC)**

  Need blue excitation light

  Excitation filter selects λ 450-490

  Barrier filter λ 515

Fluorescein

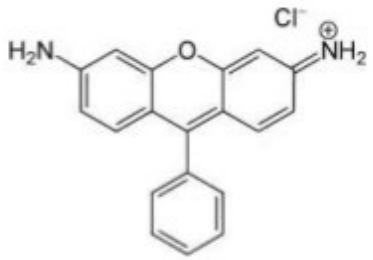


- **Calcofluor white**

  uses violet excitation light λ 355-425

  Barrier filter λ 460

Rhodamine



Fluorescence	Ex(nm)	Em(nm)	Emission Color
Alexa Fluor 350	346	422	Blue
FITC	495	519	Green
Alexa Fluor 488	495	519	Green
Cy3	514,552	570	Orange
Alexa Fluor 555	556	572	Orange
Rhodamine B	540	625	Orange
PE	496,564	578	Orange
PE-Cy3	488,532,561	578	Orange
Alexa Fluor 594	590	621	Red
APC	650	660	Red
Alexa Fluor 647	647	666	Red
Cy5	649	670	Red
PE-Cy5	488,561,649	667	Red
Cy5.5	675	694	Red
PE-Cy5.5	488,561,675	694	Red
Cy7	710,743	767	Far Red
PE-Cy7	488,561,743	785	Far Red

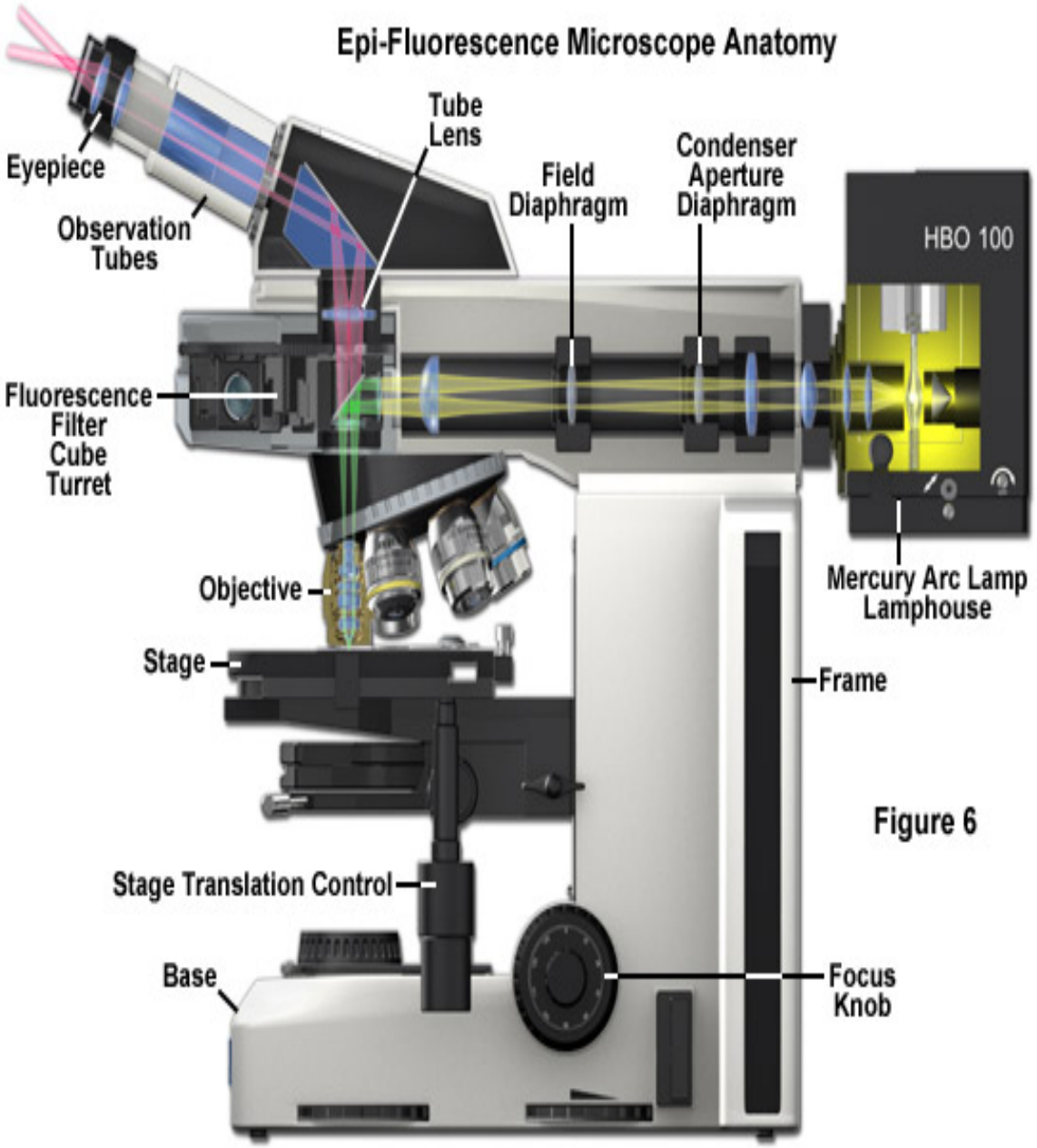


Figure 6

Light source

Infra red / heat filter

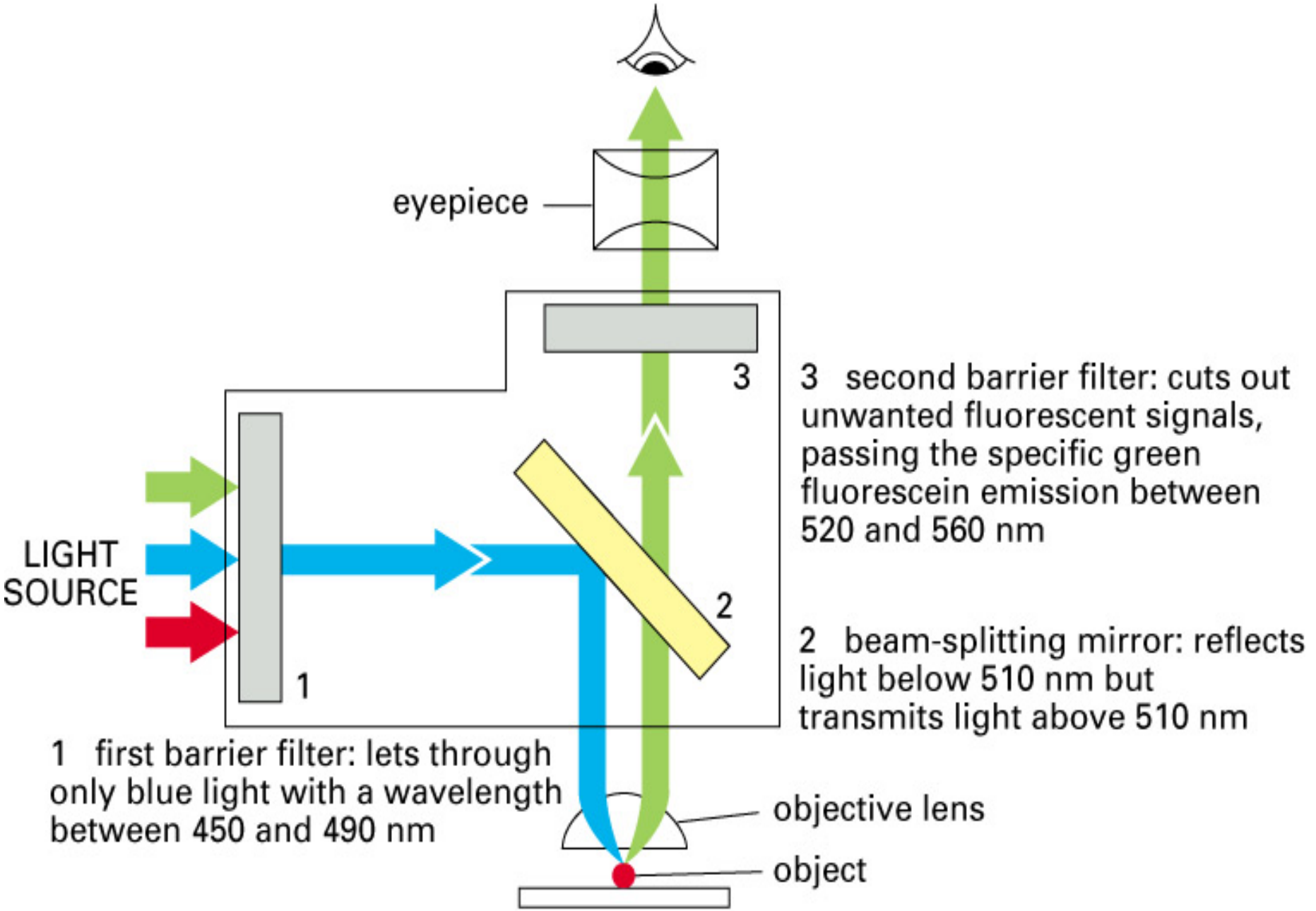
Excitation filter

Dichroic / Beam splitting mirror

Dark field condenser

Emission / Barrier filter

Objective lens


eyepiece
3
3 second barrier filter: cuts out unwanted fluorescent signals, passing the specific green fluorescein emission between 520 and 560 nm
LIGHT SOURCE
2
2 beam-splitting mirror: reflects light below 510 nm but transmits light above 510 nm
1
1 first barrier filter: lets through only blue light with a wavelength between 450 and 490 nm
objective lens
object

Staining techniques

- Based on composition of fluorescent stain reagents

Fluorochroming

only dye is used

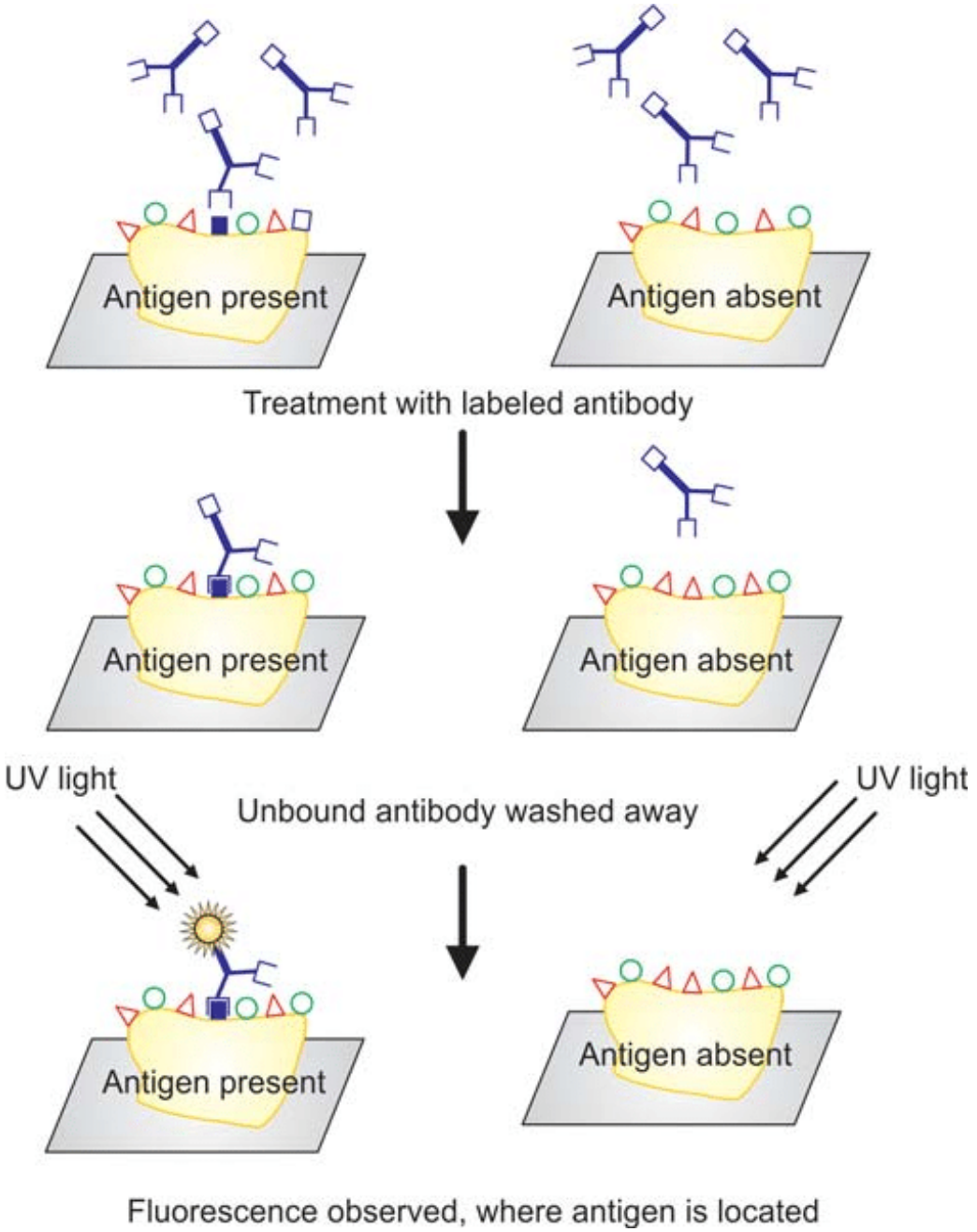
- Acridine orange – binds to Nucleic acid
- Auramine-Rhodamine – Mycolic acid
- Calcofluor white – cellwall of fungi

Immunofluorescence

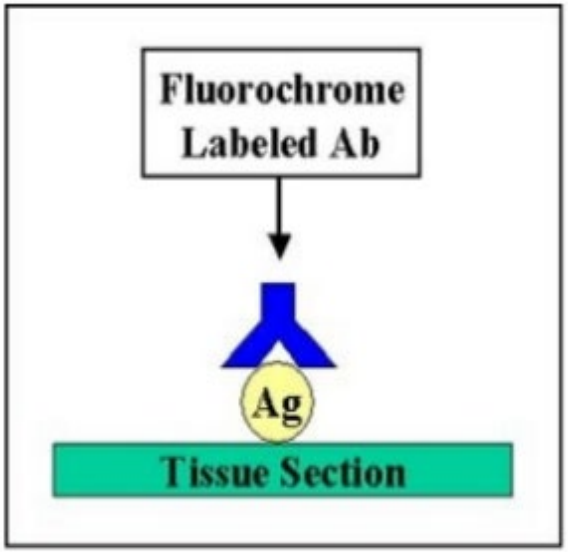
Dye conjugated with specific antibodies is used

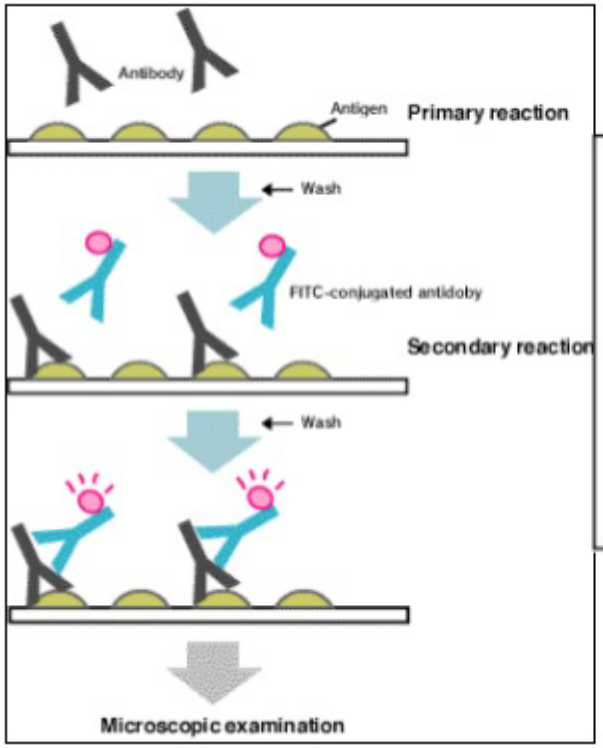
- FITC – to identify pathogens in cultures, tissues

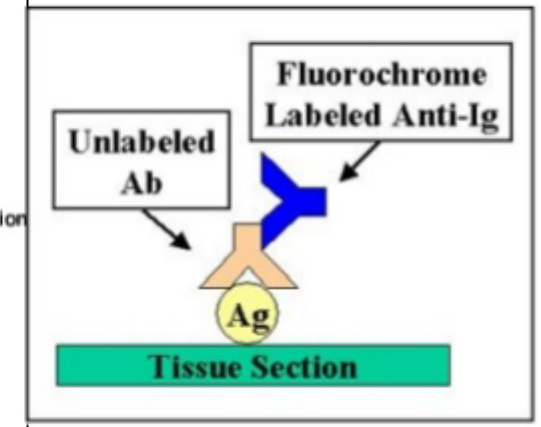
Immunofluorescence



Direct and Indirect Fluorescent Antibody Assay



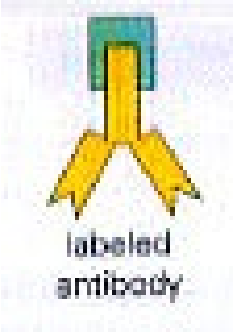




Direct Fluorescent Antibody Test for Rabies Diagnosis

- Preparation of DFA slides from brain sample
- DFA testing for Rabies virus antigen
- Observation and interpretation of results

Conjugate titration



To optimize the working dilution of the conjugate

1. Prepare serial **two-fold dilutions** (eg., 1:10, 1:20, 1:40 etc) in PBS.
2. The impressions from **known rabies positive** (minimum two) and **known negative brains** (minimum two) are made in duplicates for each of the dilutions
3. Fix in chilled acetone at -20 ° C for an hour
4. Stain the slides as per the standard DFA protocol
5. Each of the stained slides is to be **read by at least two persons** independently

6. The last **dilution showing crisp and high fluorescent staining** with minimal background fluorescence is considered as **the end-point dilution** of the reagent

7. Determine **a more precise working dilution**

   preparing limited dilutions of the conjugate (around the end-point dilution determined)

   For example, if the end-point dilution of the titration is 1:80, the conjugate should be retested at dilutions of 1:70, 1:80, 1:90, 1:100 and 1:110

8. Repeat steps 2-6

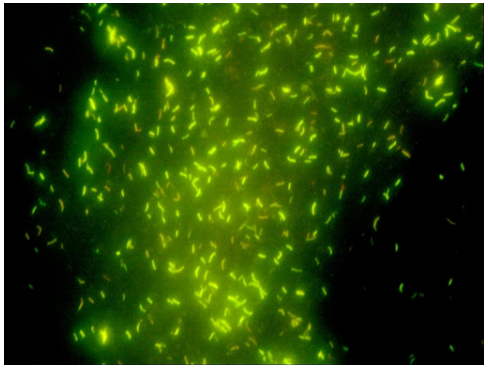
9. The working dilution should be **two steps more concentrated** than the first dilution at which a **fall-off** in staining is observed

   For example, if the fall-off in staining or the intensity is diminished at a 1:110 dilution, the working dilution of the conjugate should be 1:90

- **Working solution of conjugate** is prepared at the optimum dilution, filtered using 0.45µm filters and stored for use at 4 ° C.

Counter stain

- Lowers background activity of the fluorescent dye

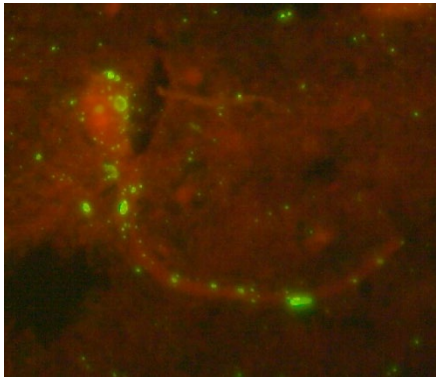


- Provides contrast
- **Evans Blue** is used as a counter stain

  stock solution (0.5% Evans Blue in PBS) is stored at 4 ° C for up to 6 months

- Add to working dilution of the conjugate

  Evans Blue is used at a final concentration of 0.00125%

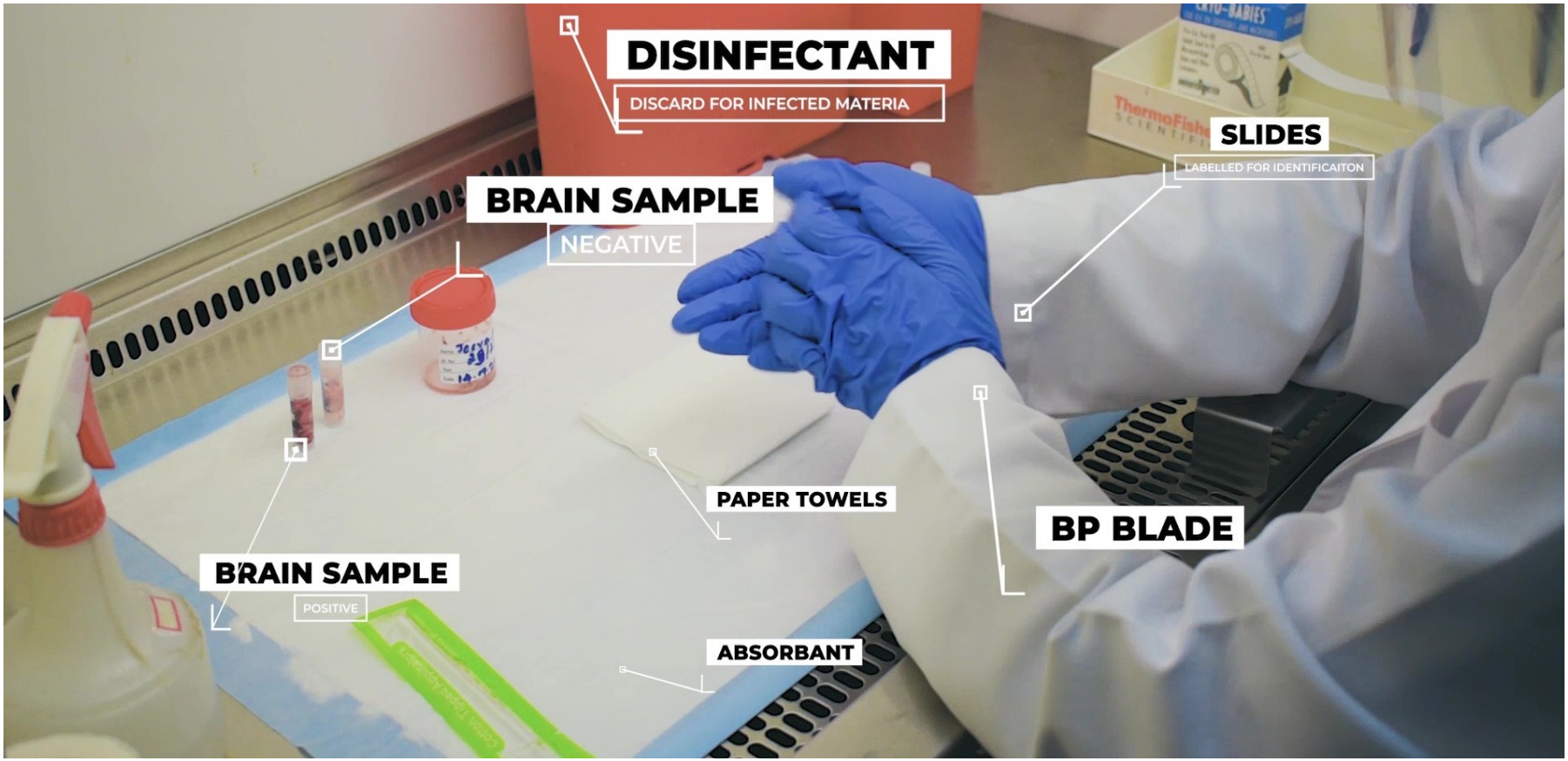


- The **tissue** when stained with counterstain **appears red**

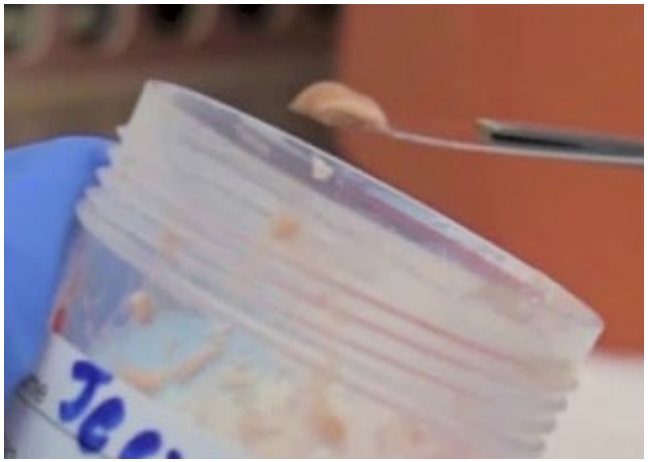
DFA Protocol

1. Preparation of DFA slides from brain sample

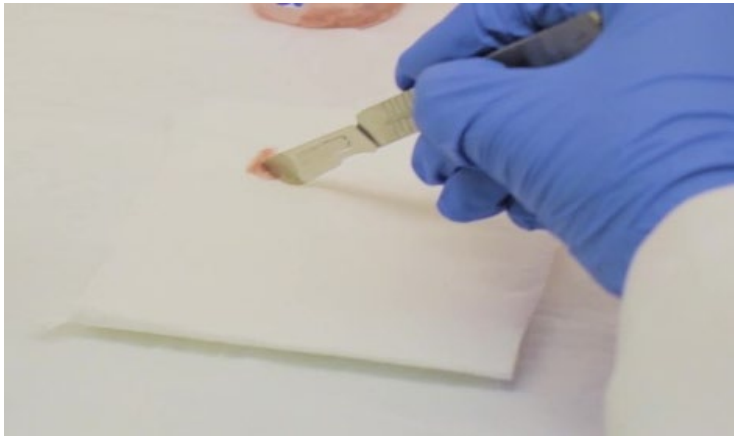
- Label the slides appropriately
- Work with one specimen at a time



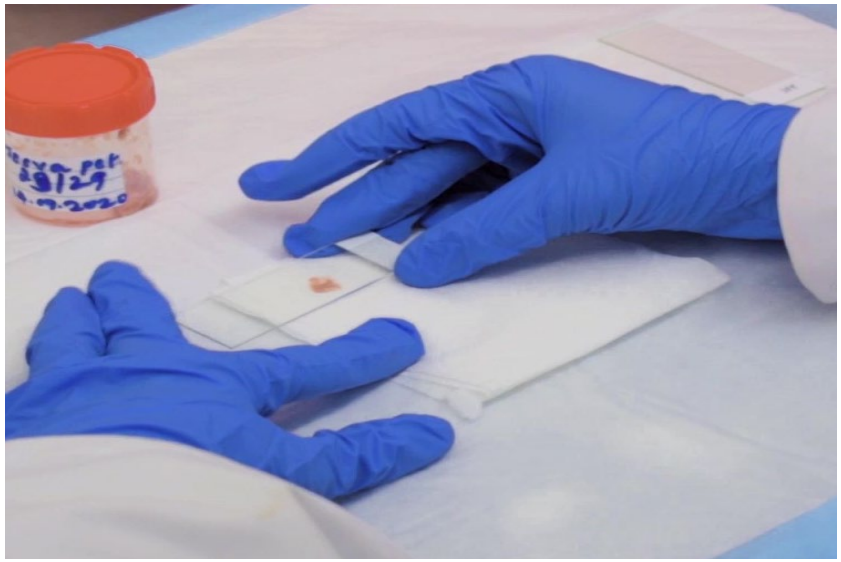
- Take a small amount of the brain tissue (pea size) from the container



- Place it on the paper towel & cut the tissue into multiple pieces



- **Make brain impressions** - gently touch the slide against the cut surface of the tissue



- For each sample, take **two impressions**

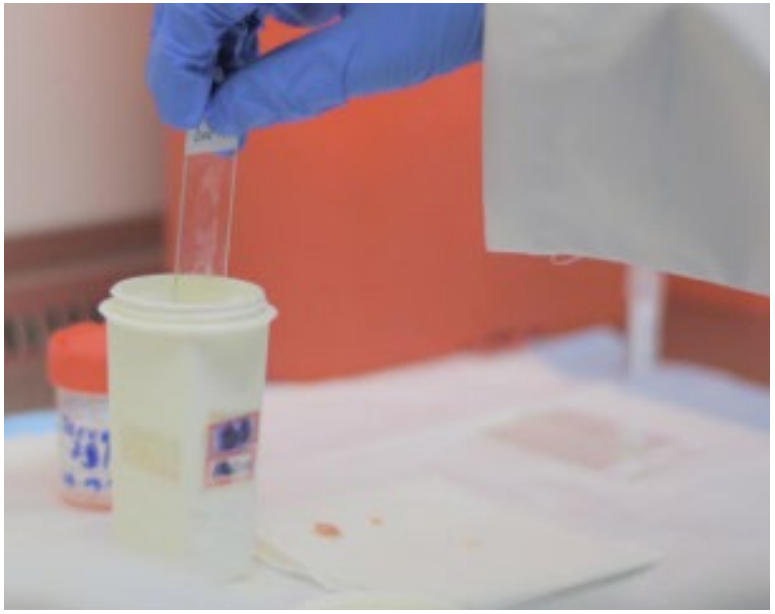
- **Blot the smears** onto paper towels - remove excess of moisture, tissue remains and blood stains

- Allow the smear to air dry for 5-10 min

- Prepare impressions similarly from the **controls**
  - ✓ Negative (healthy brain sample)
  - ✓ Known positive (confirmed rabies sample)

- Confirm the drying of smears

- Place the slides in the slide holder / Coplin jar filled with **chilled acetone**



- Allow it for

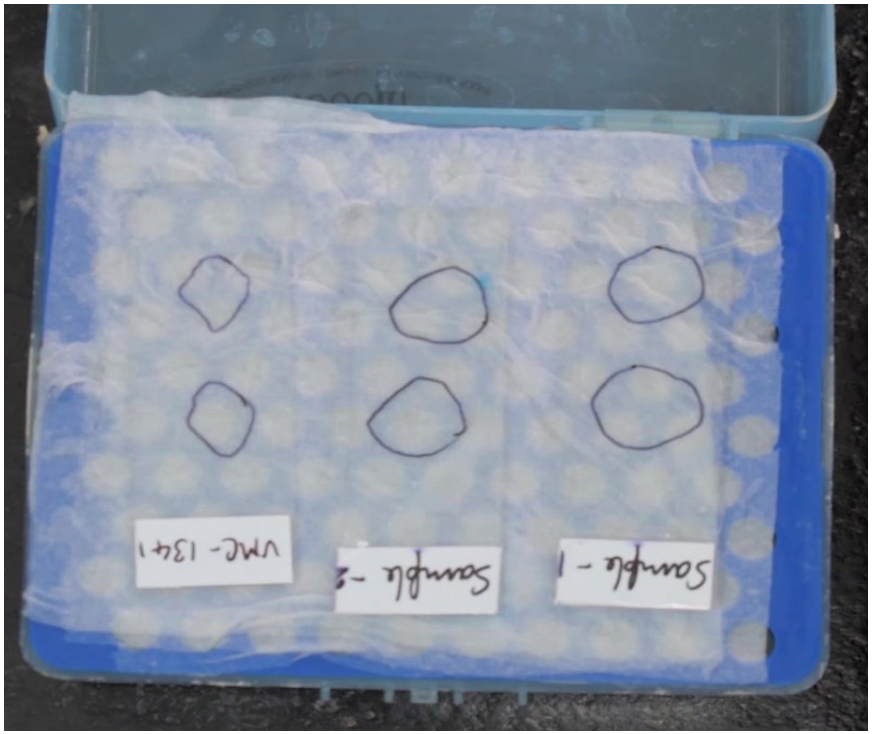
  One hour at -20 ° C / -80 ° C or

  overnight at 4 ° C

2. DFA testing for Rabies virus antigen

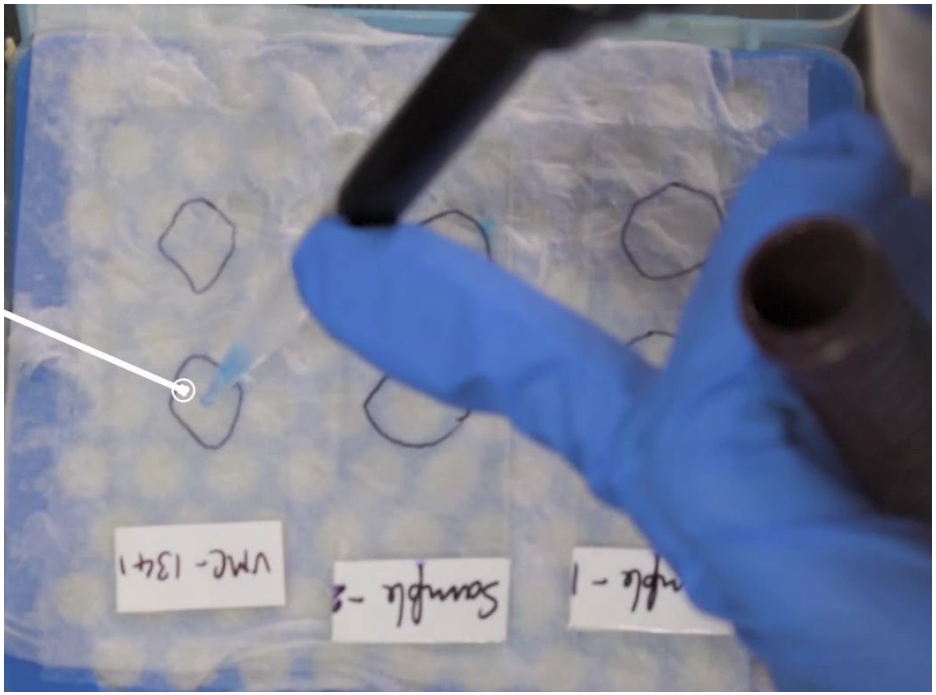
- Remove slides from acetone & air-dry at RT

- Place slides in a **moist / humid chamber**



- Add working dilution of conjugate - to cover the impressions

  (RDL - 1:15 dilution; approx 50 µl / impression)



- Incubate humid chamber at 37 ° C for 30-45 min

- Drain excess conjugate from slides
- Wash with a gentle stream of PBS for 1-2 minutes
- Invert slides on a paper towel - remove excess liquid
- Allow to air dry

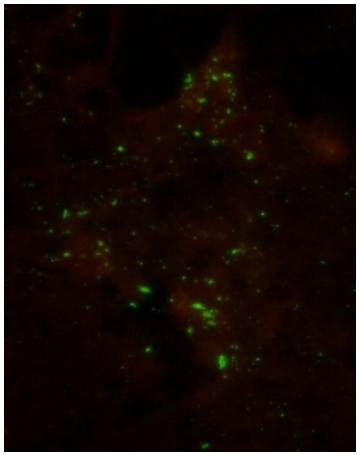
- Mount the slides with a drop of **mountant** (50% Glycerol in PBS)

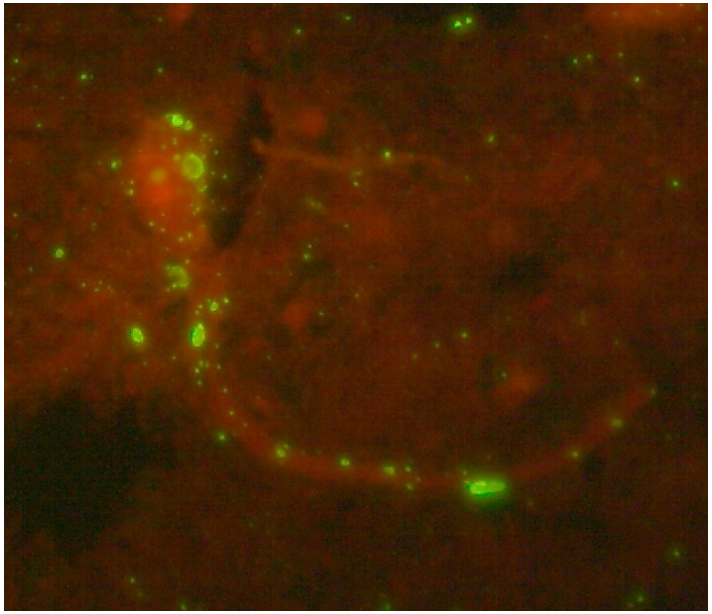
3. Observation and interpretation of results

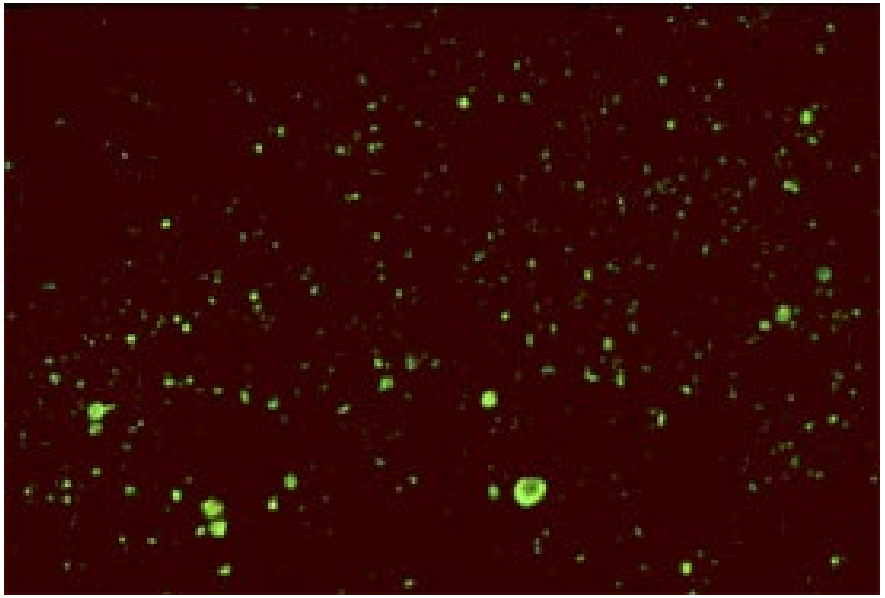
- Observe under a **fluorescent microscope**

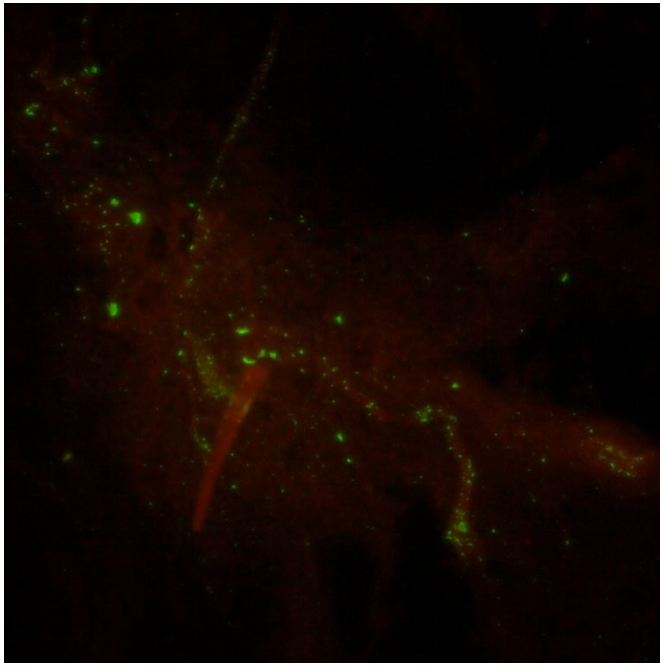
- Scan the impressions for fluorescing inclusions at **200X**

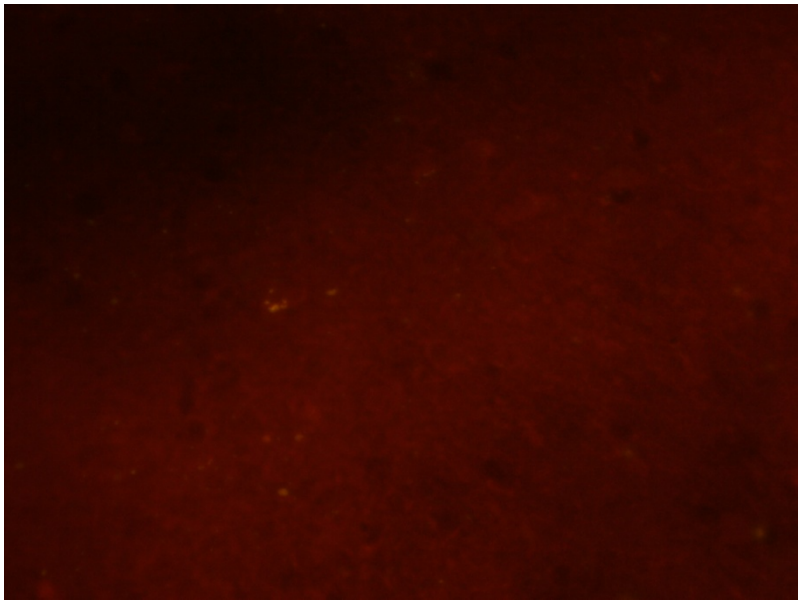
- Rabies virus : **intracytoplasmic inclusions**
  - glaring **apple green areas**
  - Smooth with very bright margins, central area less intensely stained
  - various sizes and shapes
  - **dust-like** particles <1 µm dia, **large round / oval** masses, **strings** 2 to 10 µm





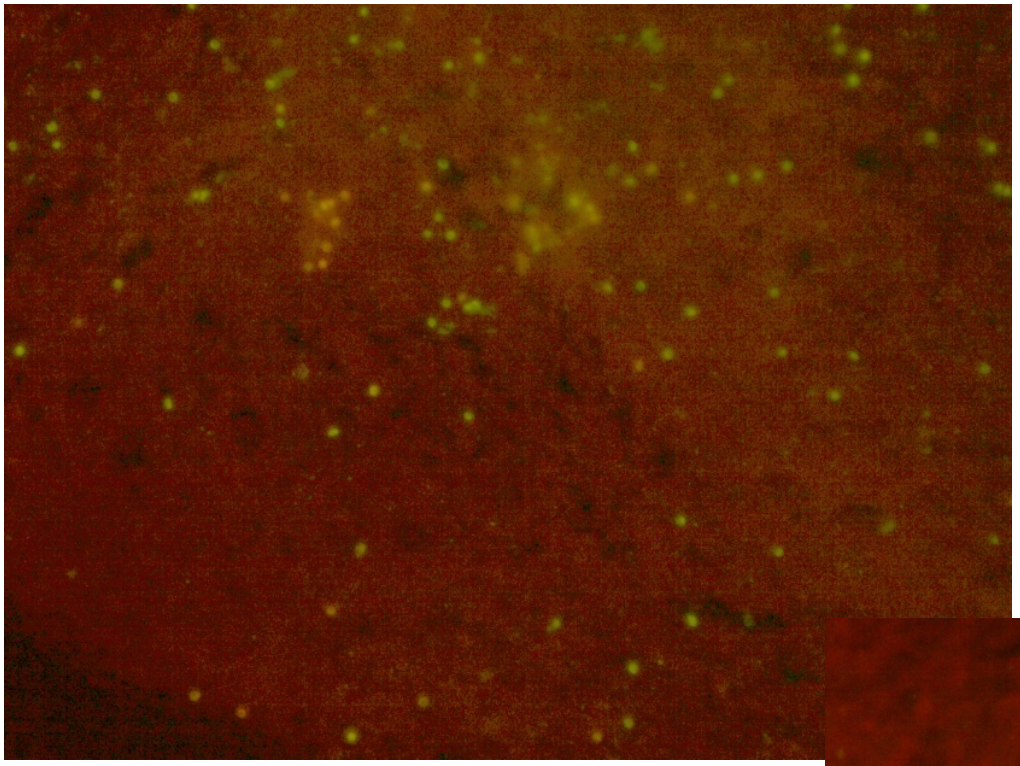


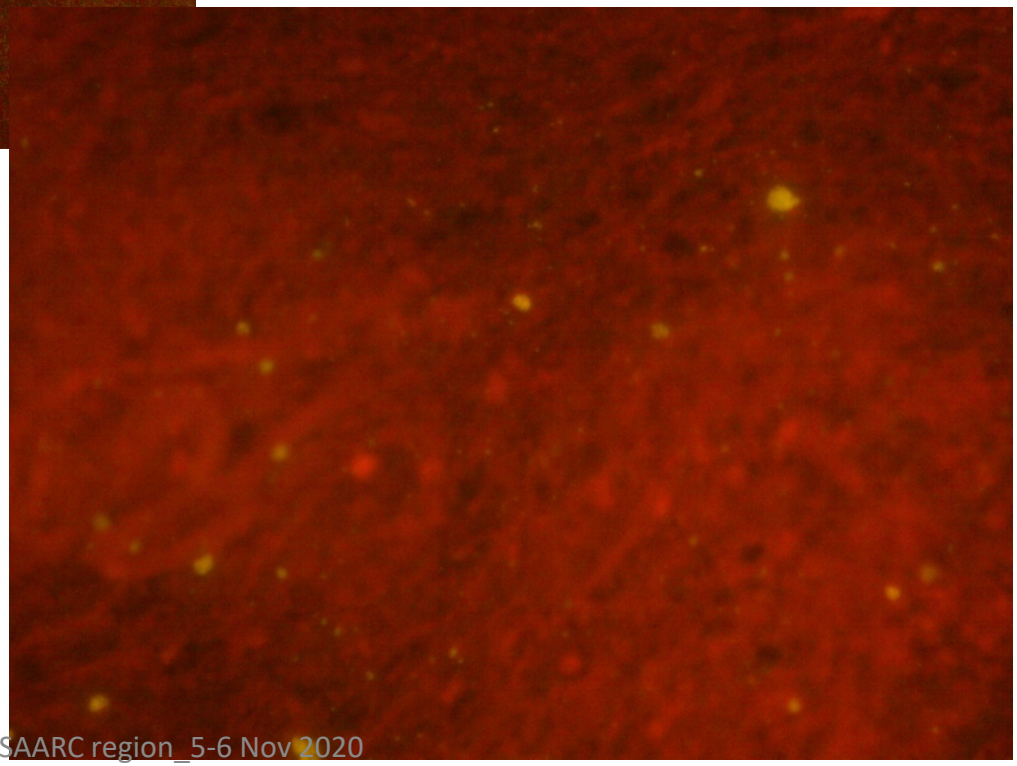




Nonspecific staining

- Presence of dull green yellowish golden yellow areas
- Areas which take **uniform bright green staining**
- Diminished / **weak staining** intensity -
  - ✓ denaturation of rabies virus antigen or
  - ✓ non specific binding of antibody to components of inflamed tissue or artifacts of tissue decomposition.





Samples in glycerol saline

- Wash with Phosphate Buffered Saline (PBS)

Samples in formalin

- Treated with **proteolytic enzyme**
- chemical cross linking of proteins interferes with antigen binding
- DFA on formalin-fixed and digested samples is always **less reliable and more cumbersome** than when performed on fresh tissue.

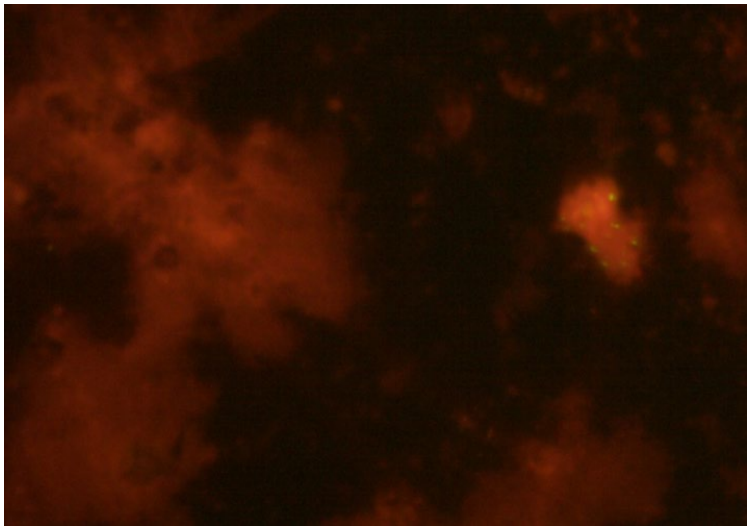
Unsatisfactory samples

- **Deteriorated / decomposed samples**

  lost structural characteristics

  appear slight green, liquified

  Nucleoprotein denatured



Negative results should not be reported on deteriorated samples

Limitations of DFA test

DFA test for rabies antigen detection - the **gold standard for rabies diagnosis**

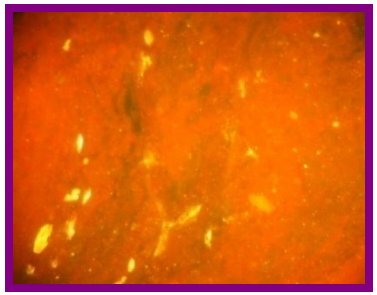
However, several **factors can affect the reliability of test**

- **Sampling of tissues** (brainstem and cerebellum)
- **Sample storage conditions** (extreme heat, storage medium, formalin –fixation, 50% glycerol/saline)
- **Sample condition** (autolysis, desiccation, denaturation of the proteins)

- **Reagents used**
  - Acetone quality (purity, fixation time, temperature)
  - Working dilution of conjugate
  - Mounting medium used
  - Affinity and Avidity of the labeled antibodies used for antigen detection

Non specific reactions

Bright Background staining



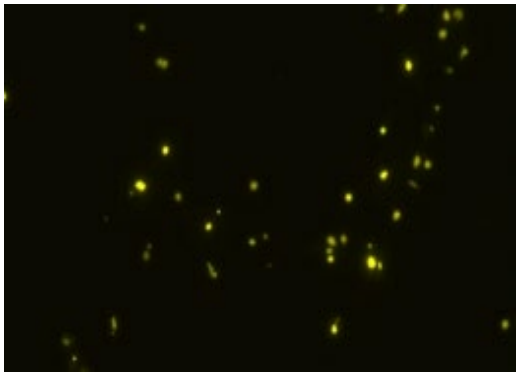
Possible causes

1. Improper working dilution of conjugate
2. Unsatisfactory reagent
3. Tissue related problems (impressions or smears too thick, deteriorated or samples proteins denatured due to heat or chemicals)

Possible solutions

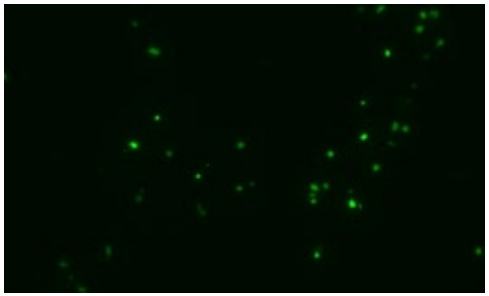
- Check conjugate dilution
- Make thinner impressions
- Use of Evan's blue counterstain – ↓es background staining

Random Crystalline FITC



Possible causes

- Drying of conjugate
- Precipitation of soluble antigen
- Edge effect (fluorescence visible outside of tissue or dried on the edge)



Possible solutions

- Use of moist incubation chamber to prevent drying
- To remove particulates, filter conjugate onto slides
- Prevent freezing and thawing of stock conjugates
- Ensure sufficient washing with PBS to eliminate conjugate

Atypical morphology or sparse distribution outside of tissue

- **Cross reactivity** with a non-rabies conjugate
- Pathogenic organism with mucopolypeptide of **protein A**
- **Group G streptococcus species**
- IgG binding sites in tissues, inflammation & staining of immune cellular infiltrates

Atypical inclusions – tissue autolysis,
denaturation due to freeze & thaw cycles
Thick impression
Inferior grade acetone

Mechanical – Non Adherence of tissue

Possible causes

- Poor fixation
- Dirty slides
- Reagent buffers (pH, salt concentration of PBS)
- Thick impression
- Inferior grade acetone

Weak staining

- Use of wrong working dilution of conjugate
- Reduced epitope recognition of the rabies virus variant by conjugate

To conclude….

Surveillance of rabies in animals based on reliable diagnostics

- basis for control strategies

- DFA for routine diagnosis in animals
- Quality Conjugates
- Fluorescent microscope
- Use of appropriate Controls

Improve surveillance of rabies in animals in SAARC region

Lets join hands

to

Strengthen Animal Rabies Surveillance

to make

Zero Rabies by 2030

– A Reality