Introduction to Fluorescence Microscopy and Immunofluorescence

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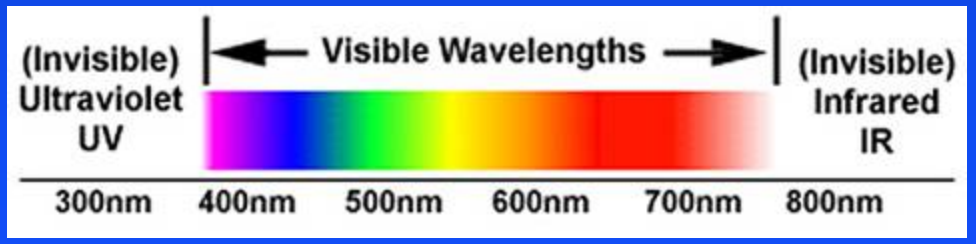
Vocabulary

- Photoluminescence: The ability of living or non-living, organic or inorganic specimens to absorb and subsequently re-radiate light.
- If the light emission persists for up to a few seconds after the excitation light is withdrawn, the phenomenon is known as phosphorescence.
- Fluorescence: light emission occurs only when the excitation light is present.

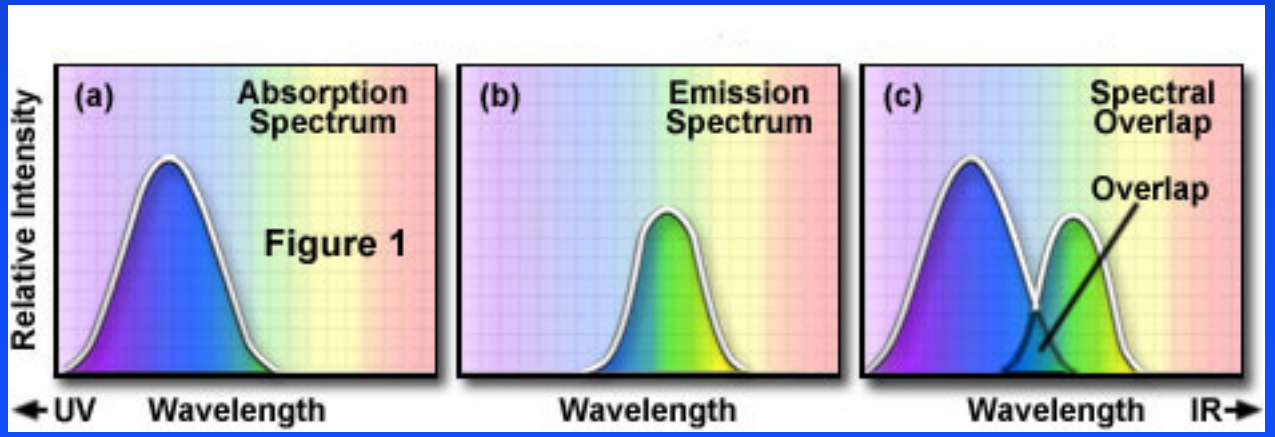
Background

- In the middle of the nineteenth century Stokes observed that the mineral fluorspar fluoresced when ultraviolet light was directed upon it.
- Stokes coined the word "fluorescence".
- Fluorescing light was always of a longer wavelength than the excitation light (Stokes Law).
- FITC
  - Excitation 495 nm
  - Emission: 520 nm

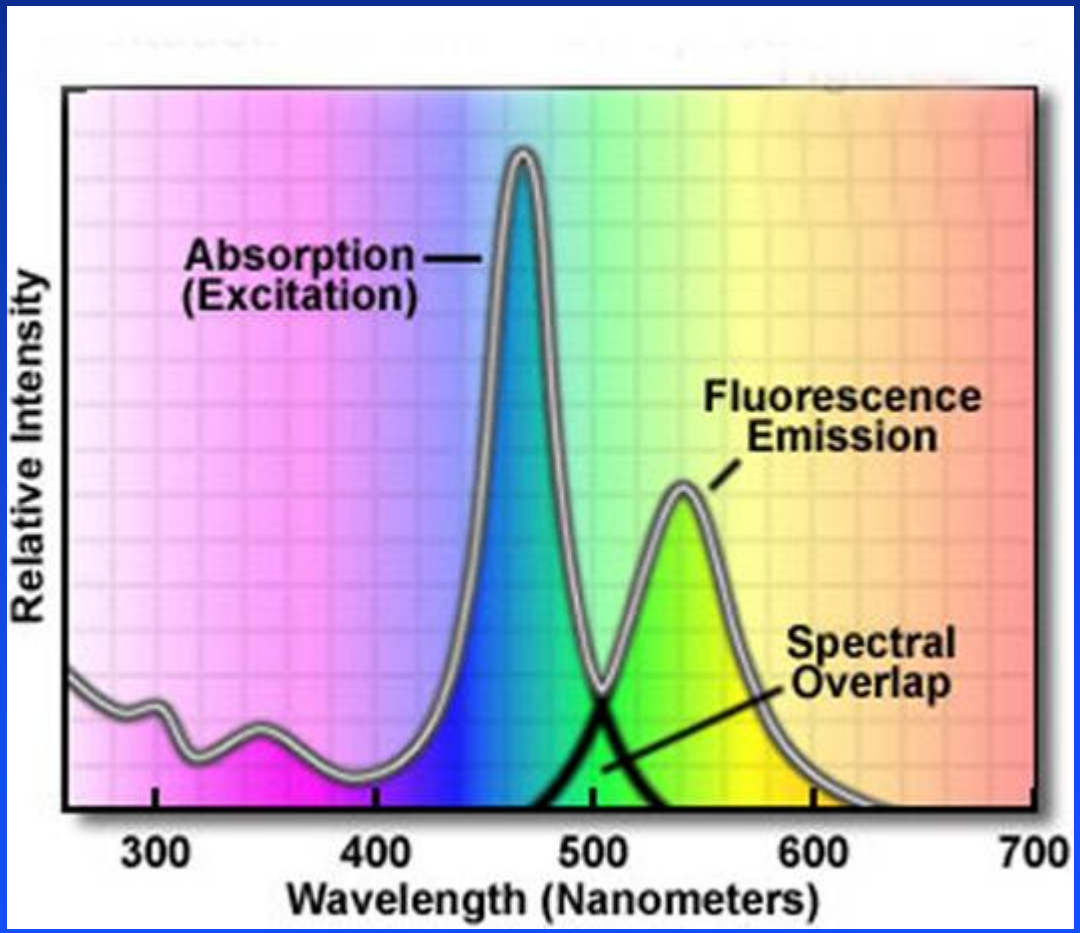


Fluorescence Microscopy

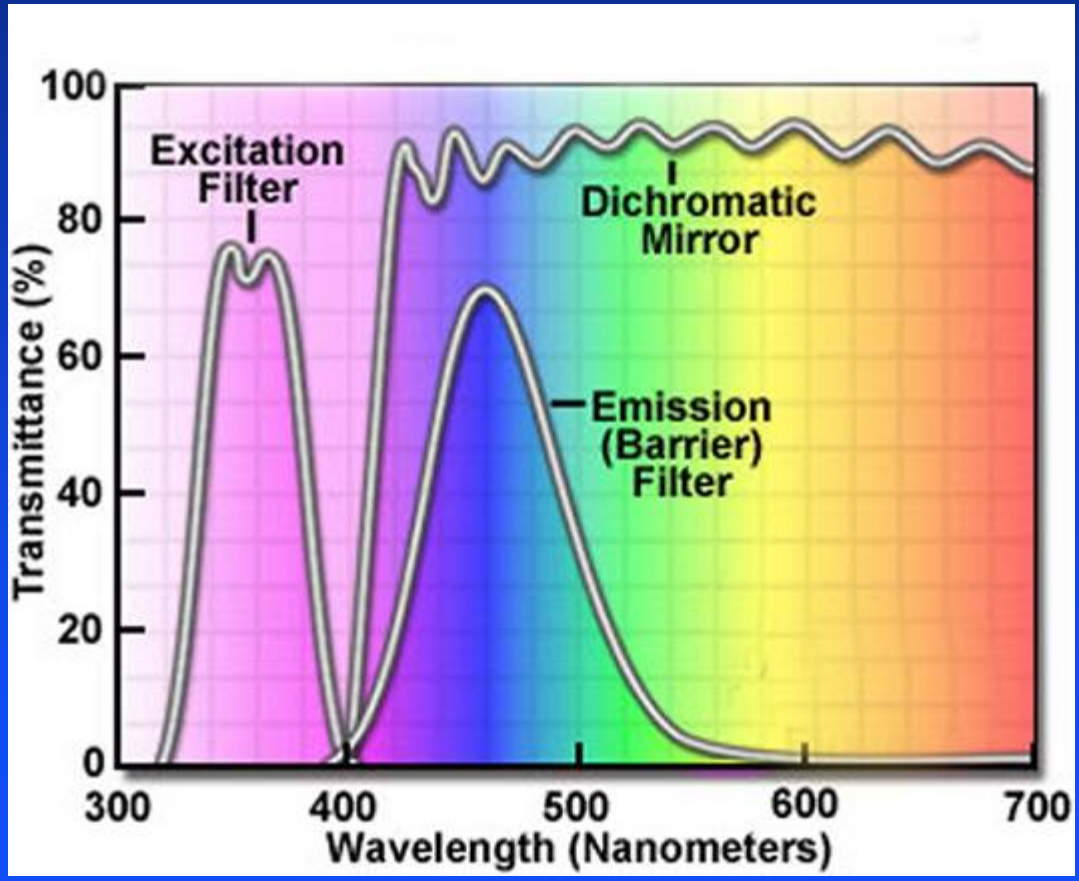
- The intensity of the fluorescence is very weak in comparison with the excitation light (10^{-3} to 10^{-5}).
- The emitted light re-radiates spherically in all directions.
- Dark background is required to enhance resolution.

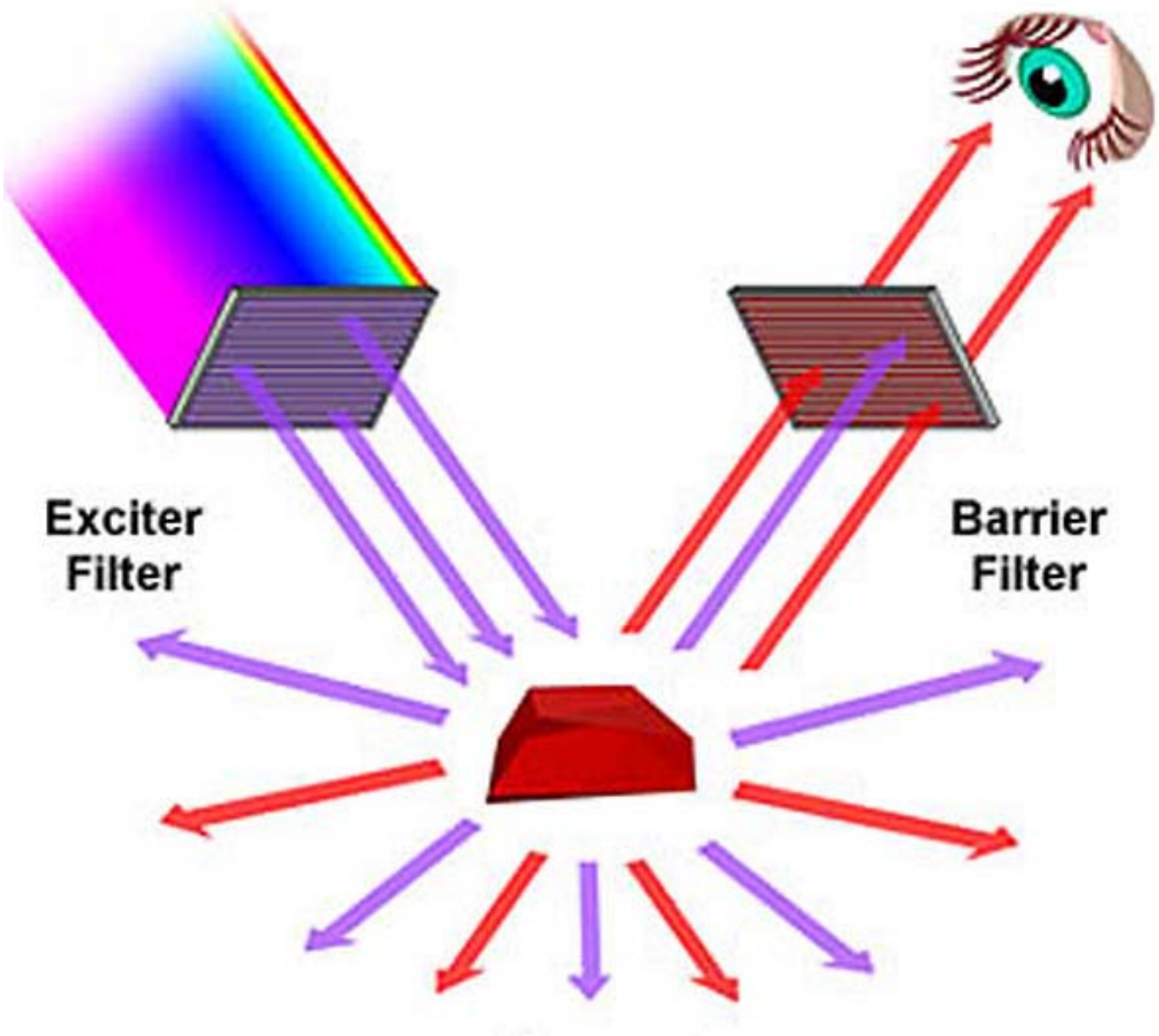


Spectral Overlap



Barrier Filters

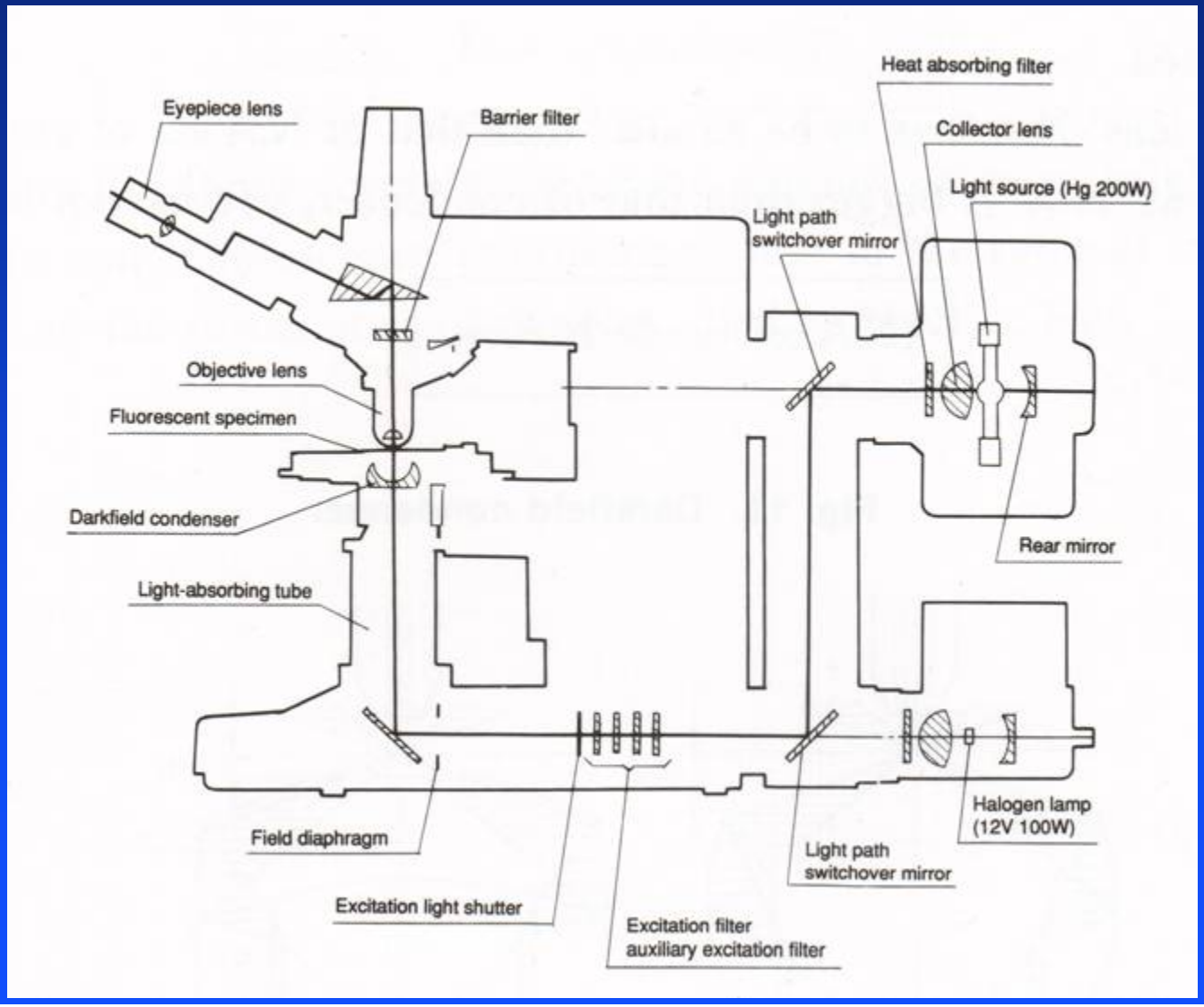


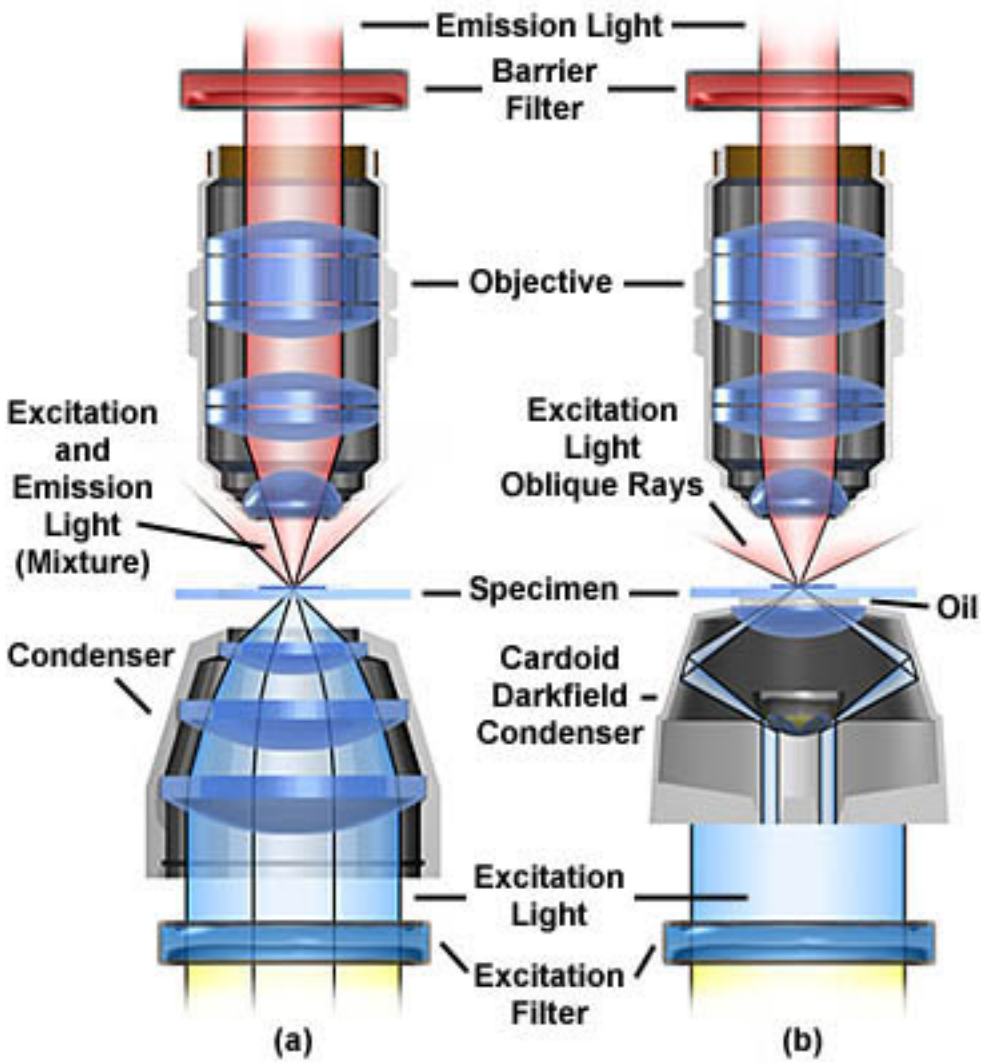

Exciter
Filter
Barrier
Filter

Fluorescence Microscopy

- The basic task of the fluorescence microscope is to permit excitation light to irradiate the specimen and then to separate the much weaker re-radiating fluorescent light from the brighter excitation light so that only the emission light reaches the eye.
- The resulting fluorescing areas shine against a dark background with sufficient contrast to permit detection.
- The darker the background of the non-fluorescing material, the more efficient the instrument.

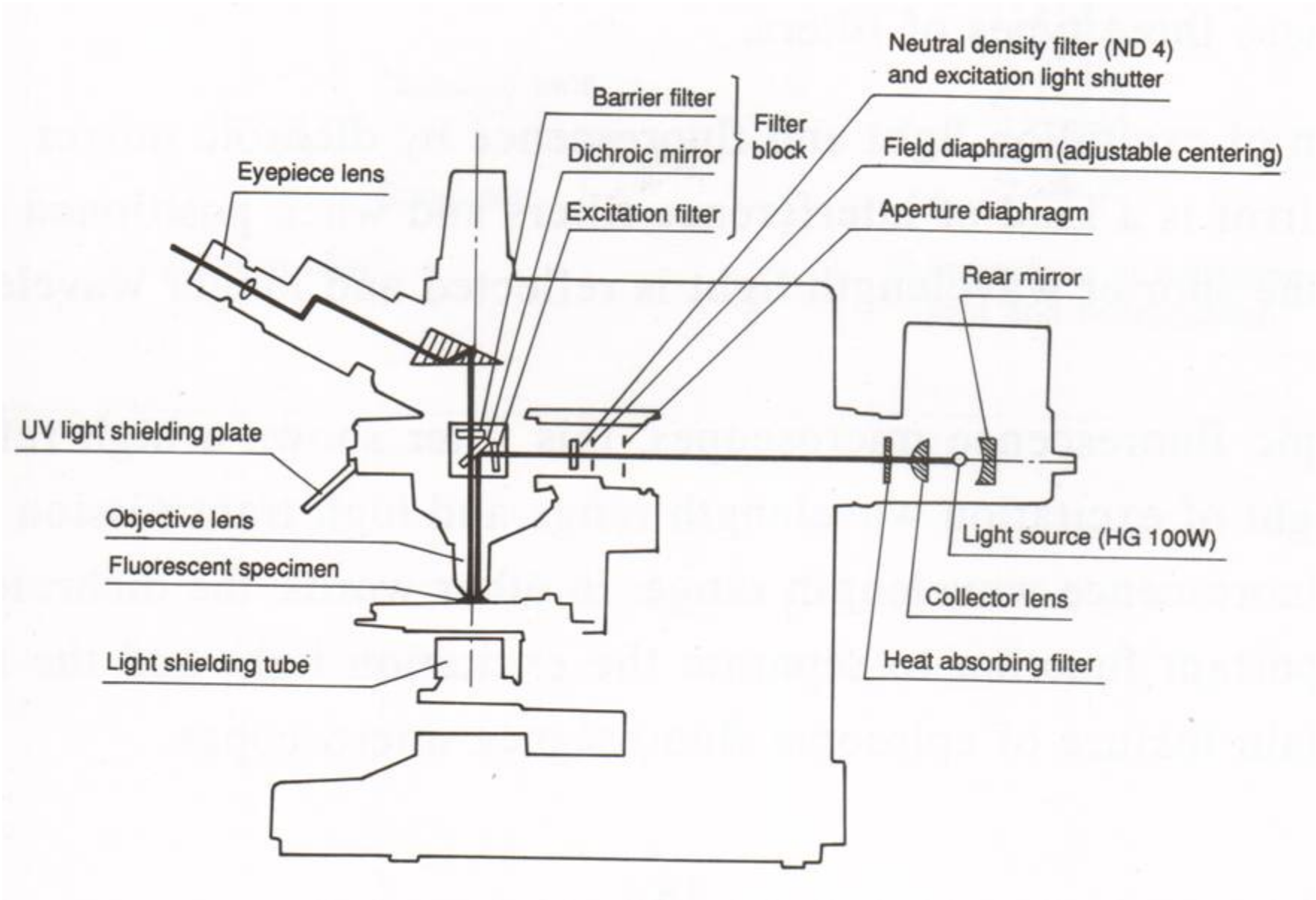
Transmitted Light Microscopy



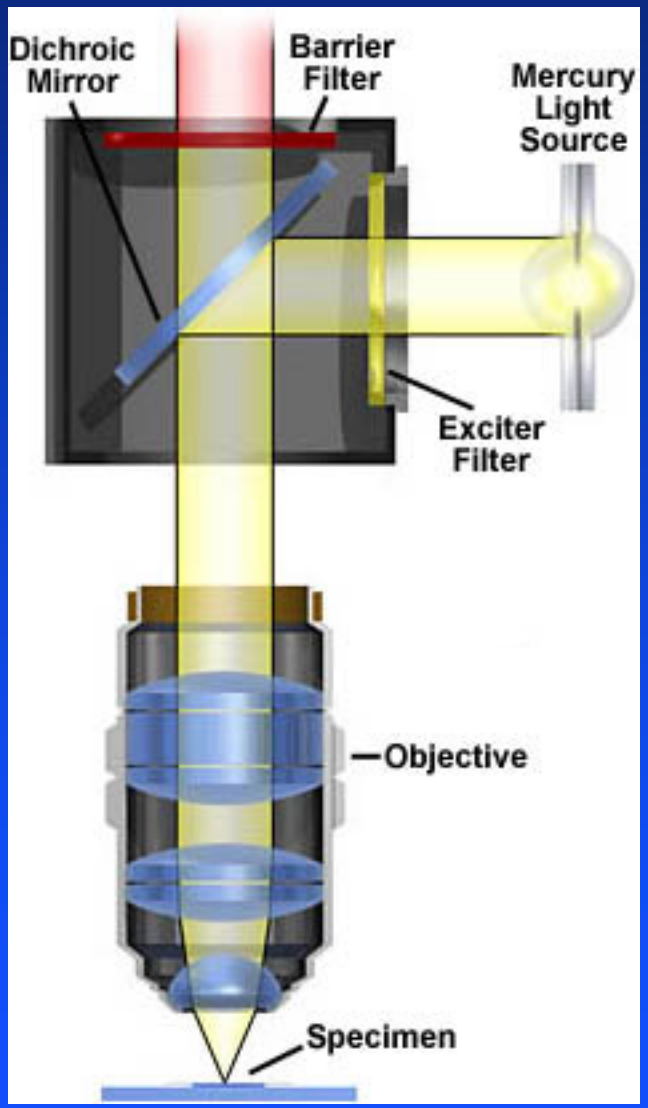

Emission Light
Barrier Filter
Objective
Excitation and Emission Light (Mixture)
Excitation Light Oblique Rays
Specimen
Oil
Condenser
Cardoid Darkfield Condenser
Excitation Light
Excitation Filter
(a)
(b)

Transmitted Light Microscopy

- Because a darkfield condenser is used, the excitation energy reaching the specimen is relatively low and the fluorescence intensity is lowered.
- Darkfield condenser limits the NA of the objectives and reduces image brightness.
- For maximum intensity, both the condenser and the objective must be oiled.
- Darkfield condensers require accurate centering.
- Thick specimens (and slides) are not satisfactory.
- At low power, only the center of field of view is lighted.


Neutral density filter (ND 4)
and excitation light shutter
Barrier filter
Filter
block
Dichroic mirror
Field diaphragm (adjustable centering)
Eyepiece lens
Excitation filter
Aperture diaphragm
Rear mirror
UV light shielding plate
Objective lens
Light source (HG 100W)
Fluorescent specimen
Collector lens
Light shielding tube
Heat absorbing filter

Epifluorescence



Epifluorescence

- Darkfield condenser is not required; the objective acts as a condenser.
- Centering, focusing, and oiling the condenser is not needed.
- Objectives with the highest NA should be used to maximize brightness.
- Specimen thickness does not interfere with flourescence intensity.
- Fading only occurs in the field of view.

Light Sources

- Ultrahigh pressure mercury lamps
  - 50 W, 100 W, 200 W
- Xenon high-pressure lamps
  - 75 W, 150 W, 450 W
- Low-voltage lamps
  - 12V 50W, 12V 100 W tungsten halogen lamps

Mercury and Xenon Bulbs

- Arc lamp glass envelopes are filled with mercury or xenon gas at moderately high pressure
- Never handle these lamps when they are hot
- Avoid applying a mechanical force that might cause the lamp to explode.
- Avoid touching the new lamp with fingers
  - Oils from the hands are acidic and may etch the quartz envelope enough to weaken it.
  - Residue from fingerprints can become fused to the exterior of the bulb when it becomes hot.
- If a bulb does explode, use mercury cleanup and decontamination procedures.

Bulb Alignment

- After installing a new bulb in a mercury or xenon arc lamphouse, the arc must be carefully aligned and focused to achieve a homogeneous field of illumination.
- The arc itself is very small (about 1 or 2 millimeters in length), and the image of the arc must be positioned along the optical axis of the microscope, at the center of the condenser aperture in the vertical illuminator, to ensure even illumination.
- Poorly aligned light paths can cause false-negative results in FA testing.

http://www.microscopyu.com/tutorials/java/arclamp/index.html

Fading

- Bleaching
  - Irreversible decomposition of the fluorescent molecules because of light intensity in the presence of molecular O_2.
- Quenching
  - Caused by free-radical oxidation, oxidizing agents or the presence of salts of heavy metals or halogen compounds.
- Fluorescent-Resonance Energy Transfer (FRET)
  - The transfer of energy to other so-called acceptor molecules physically close to the excited fluorophores.

Antifade Reagent	Comments	Reference
p-phenylene-diamine	The most effective reagent for FITC. Also effective for Rhodamine. Should be adjusted to 0.1% p-phenylenediamine in glycerol/PBS for use. Reagent blackens when subjected to light exposure so it should be stored in a dark place. Skin contact is extremely dangerous.	G. D. Johnson & G. M. Araujo (1981) **J. Immunol. Methods**, **43**: 349-350
DABCO (1,4-diazabi-cyclo-2,2,2-octane)	Highly effective for FITC. Although its effect is slightly lower than p-phenylenediamine, it is more resistant to light and features a higher level of safety.	G. D. Johnson ***et. al.***, (1982) **J. Immunol. Methods**, **55**: 231-242
n-propylgallate	The most effective reagent for Rhodamine, also effective for FITC. Should be adjusted to 1% propylgallate in glycerol/PBS for use.	H. Giloh & J. W. Sedat (1982), **Science**, **217**: 1252-1255
2-mercapto-ethylamine	Used to observe chromosome and DNA specimens stained with propidium iodide, acridine orange, or Chromomysin A3. Should be adjusted to 0.1mM 2-mercaptotheylamine in Tris-EDTA	S. Fujita & T. Minamikawa (1990), **Experimental Medicine**, **8**: 75-82

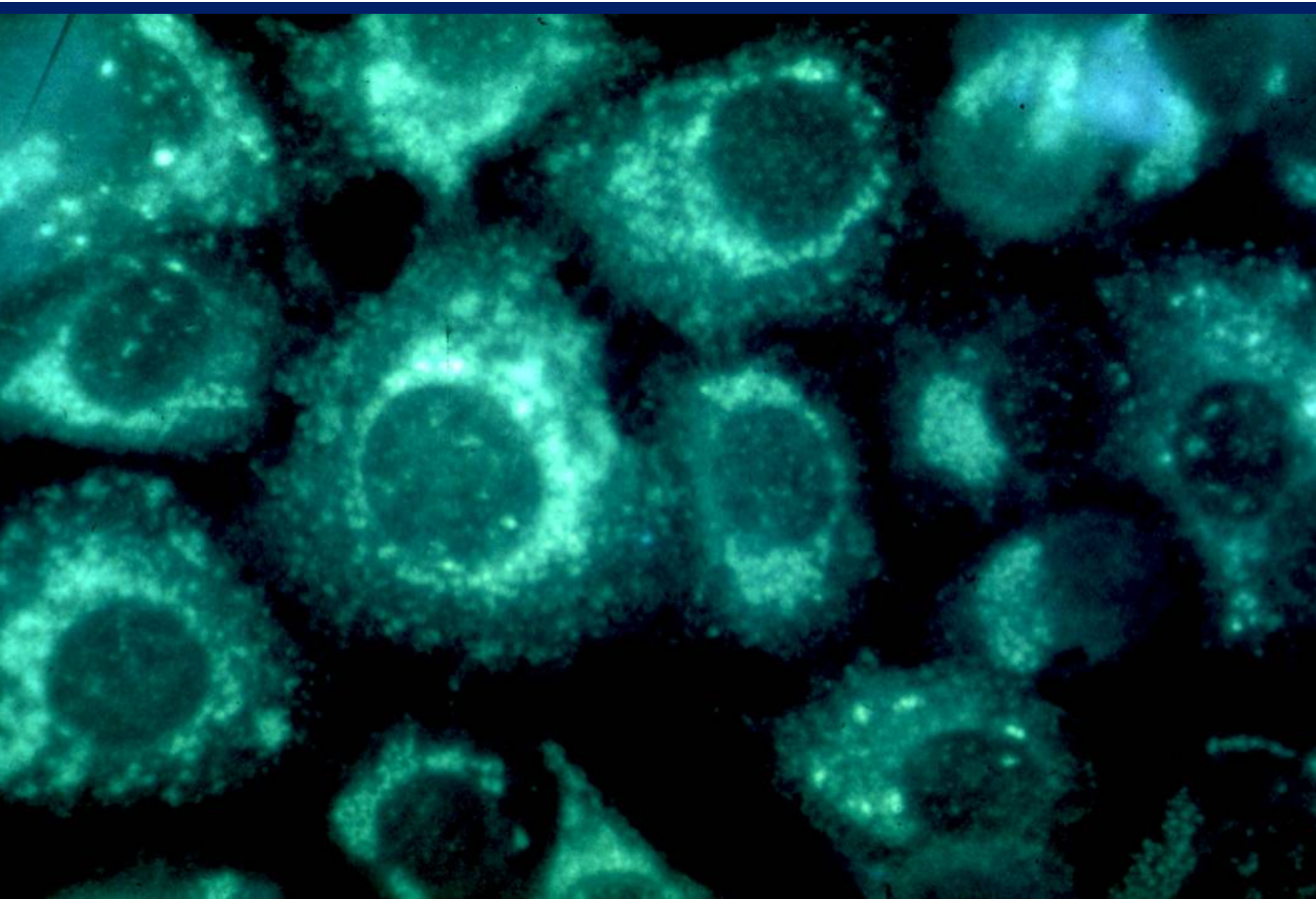
Historical Perspective

- Early investigations showed that many substances autofluoresce when irradiated with ultraviolet light.
- In the 1930's Haitinger and others developed the technique of secondary fluorescence--employing fluorochrome stains to stain specific tissue components, bacteria, or other pathogens which do not autofluoresce. [acridine orange]
- In the 1950's Coons and Kaplan used fluorescein-tagged antibodies to localize antigens in tissues. [Immunofluorescence]

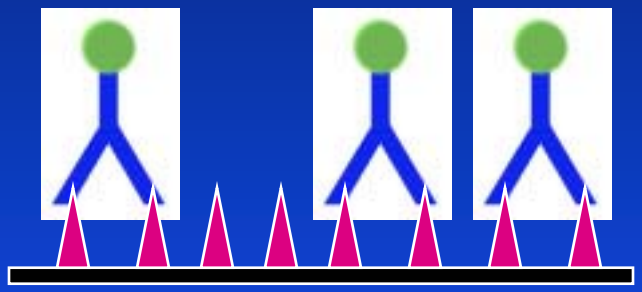
Today

Immunofluorescence is used to:

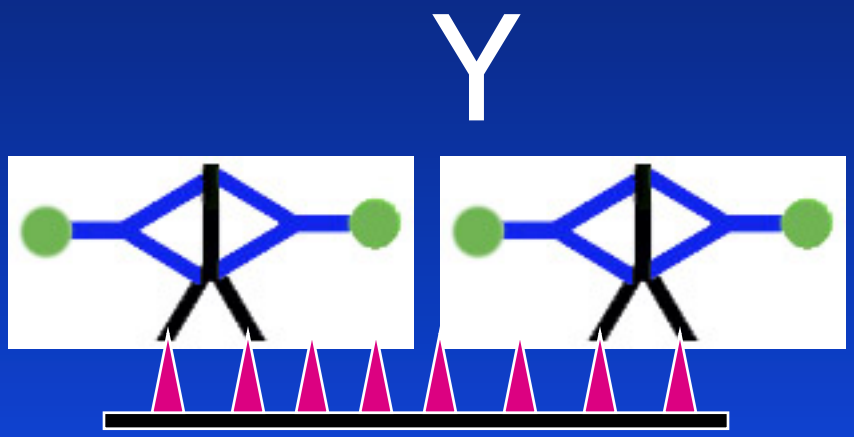
- Identify infectious agents in clinical specimens
- Serotype and identify viruses from cultures
- Quantify antibody levels
- Localize antigenic determinants on cells
- Identify different cell types



Fluorescent Antibody Staining



Direct Fluorescent Antibody (DFA)

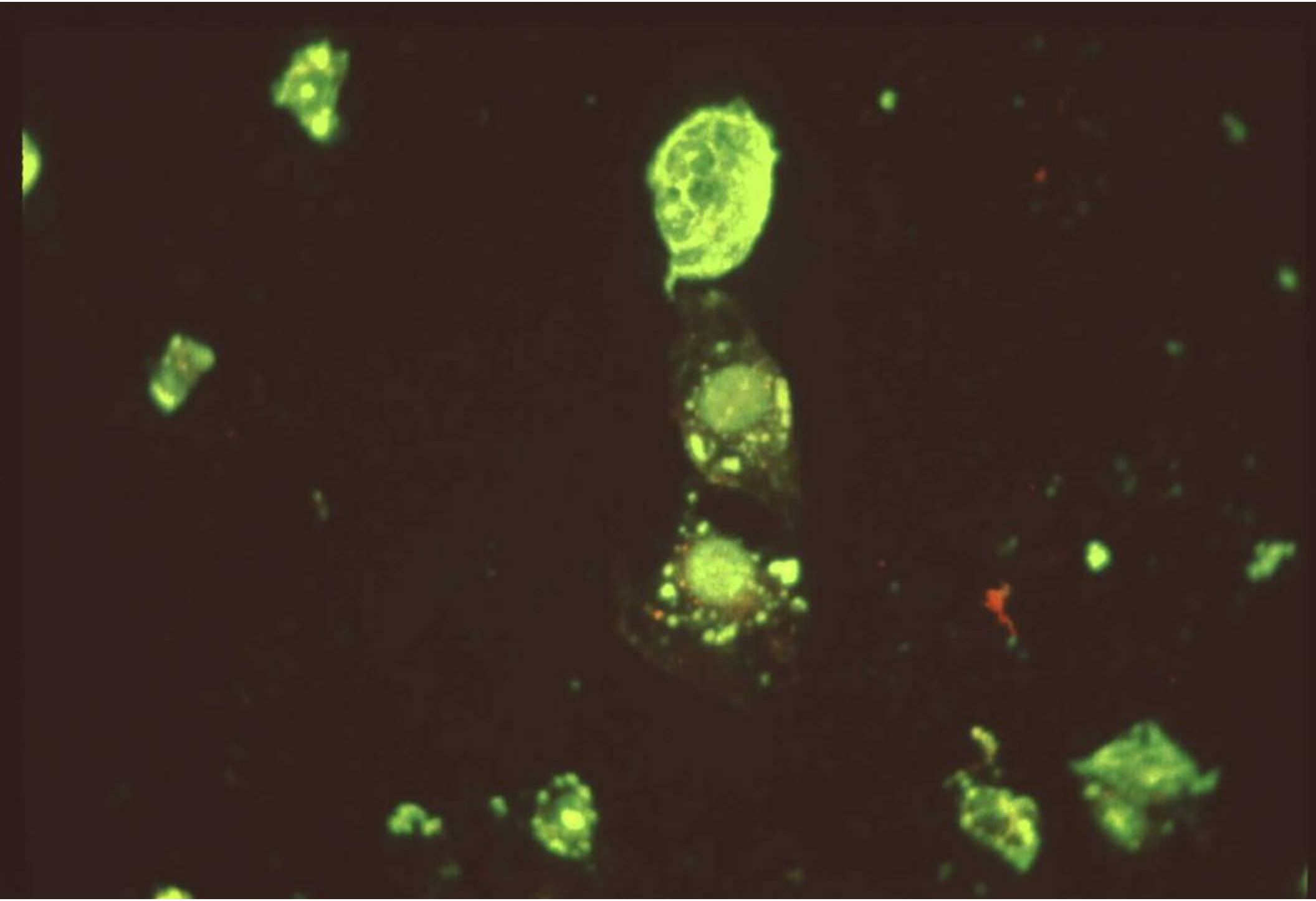


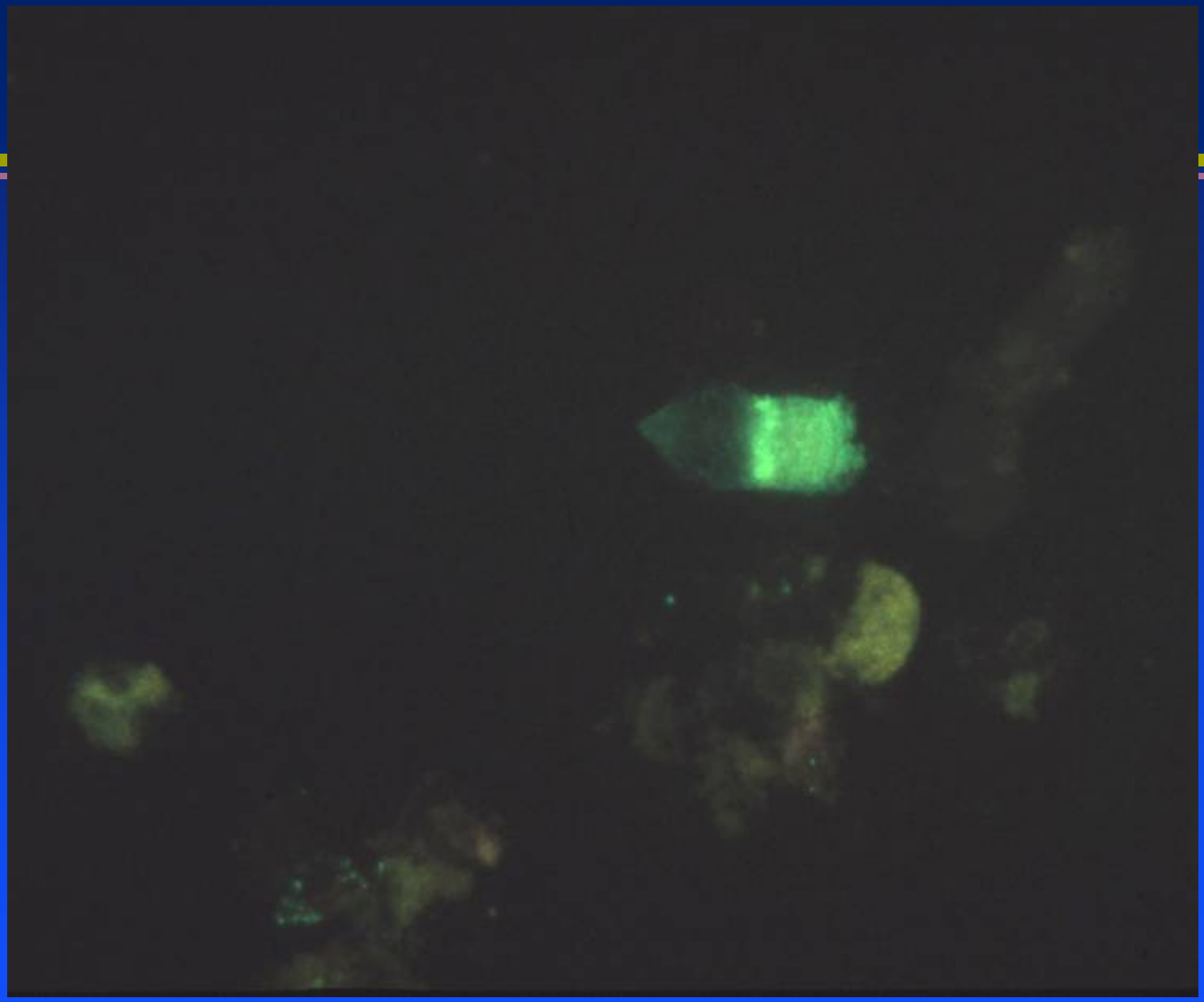
Indirect Fluorescent Antibody (IFA)

Direct Specimen Detection Using Immunofluorescence

Fluorescent Antibody Detection

- Allows the laboratory to evaluate specimen adequacy
  - Type of cells present
  - Number of cells
- Does not require strict cold-chain transport
- Increased specificity due to pattern and staining evaluations.





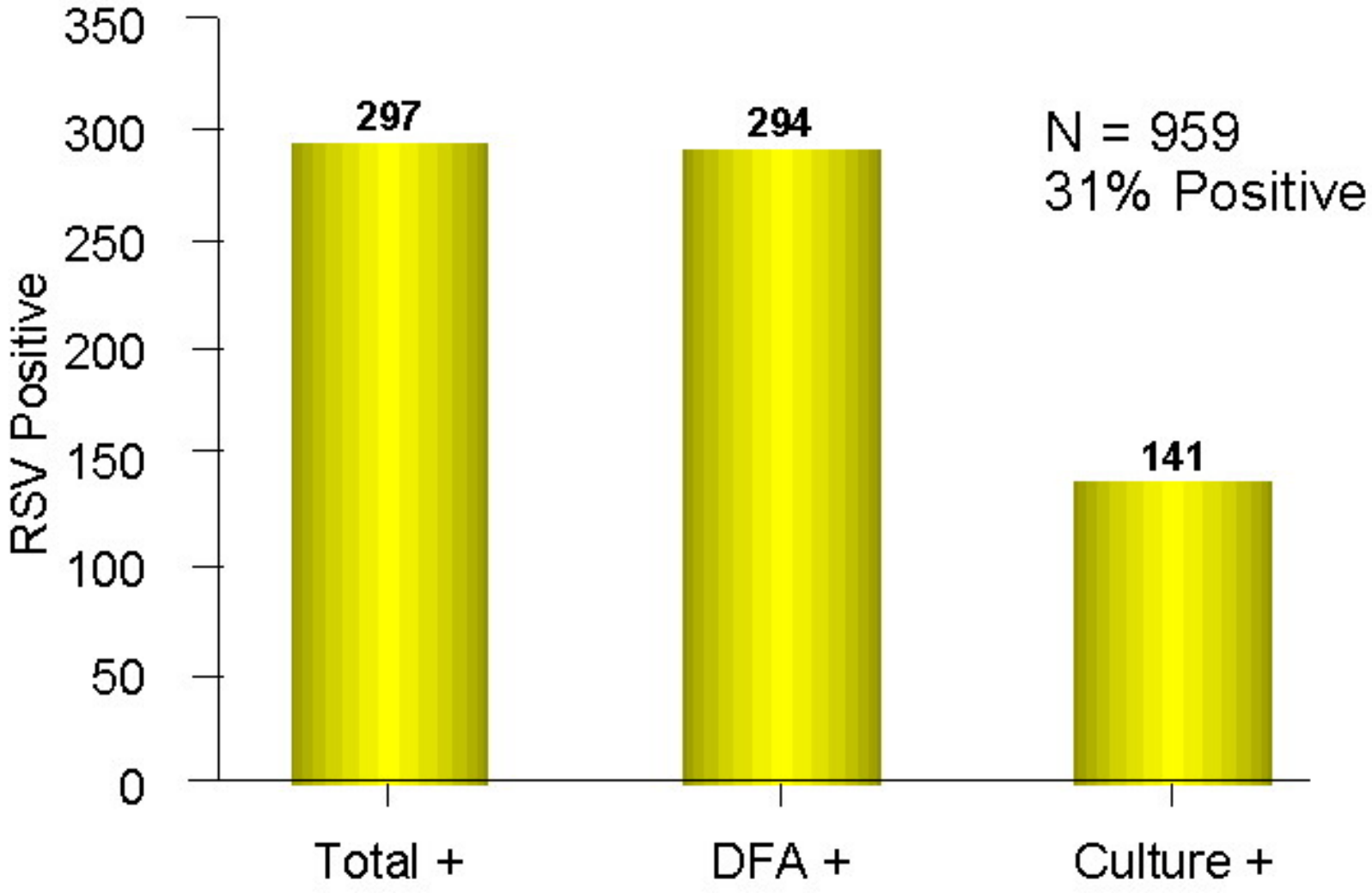
Faster Turnaround Times

- Supports patient cohorting and STAT testing
- Initiate/discontinue antiviral therapies
- Reduced use of antibiotics
- Reduced hospital stays

RSV Testing

- RSV is thermolabile
- DFA methods are significantly more sensitive than culture

RSV Detection



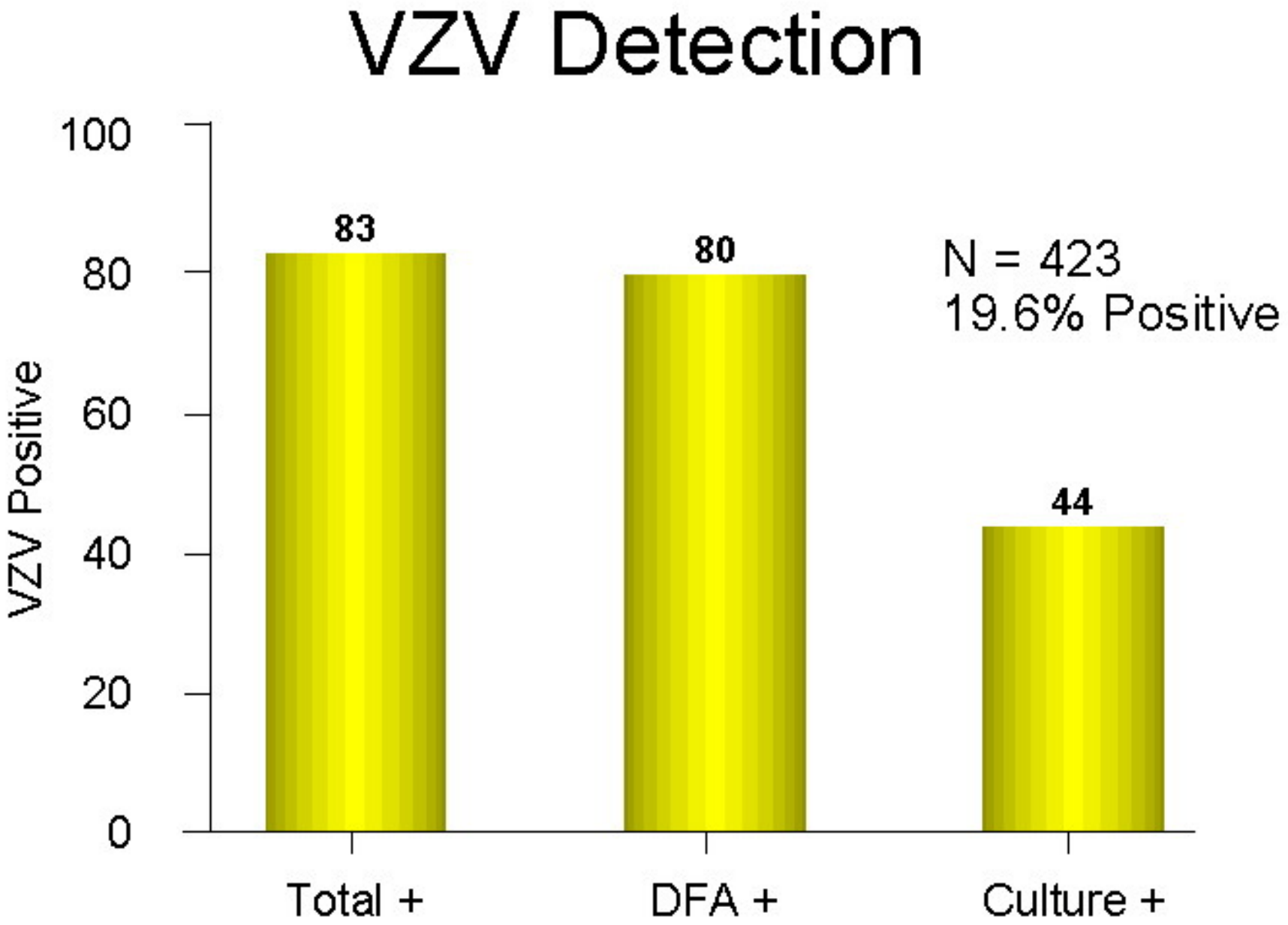
Varicella-Zoster Virus

- Cell culture isolation
- Shell vials
- DFA

Varicella-Zoster Virus

- Tube culture 5 - 7 days
- Shell vials 2 - 4 days
- DFA 1 - 2 hrs

What do we miss?

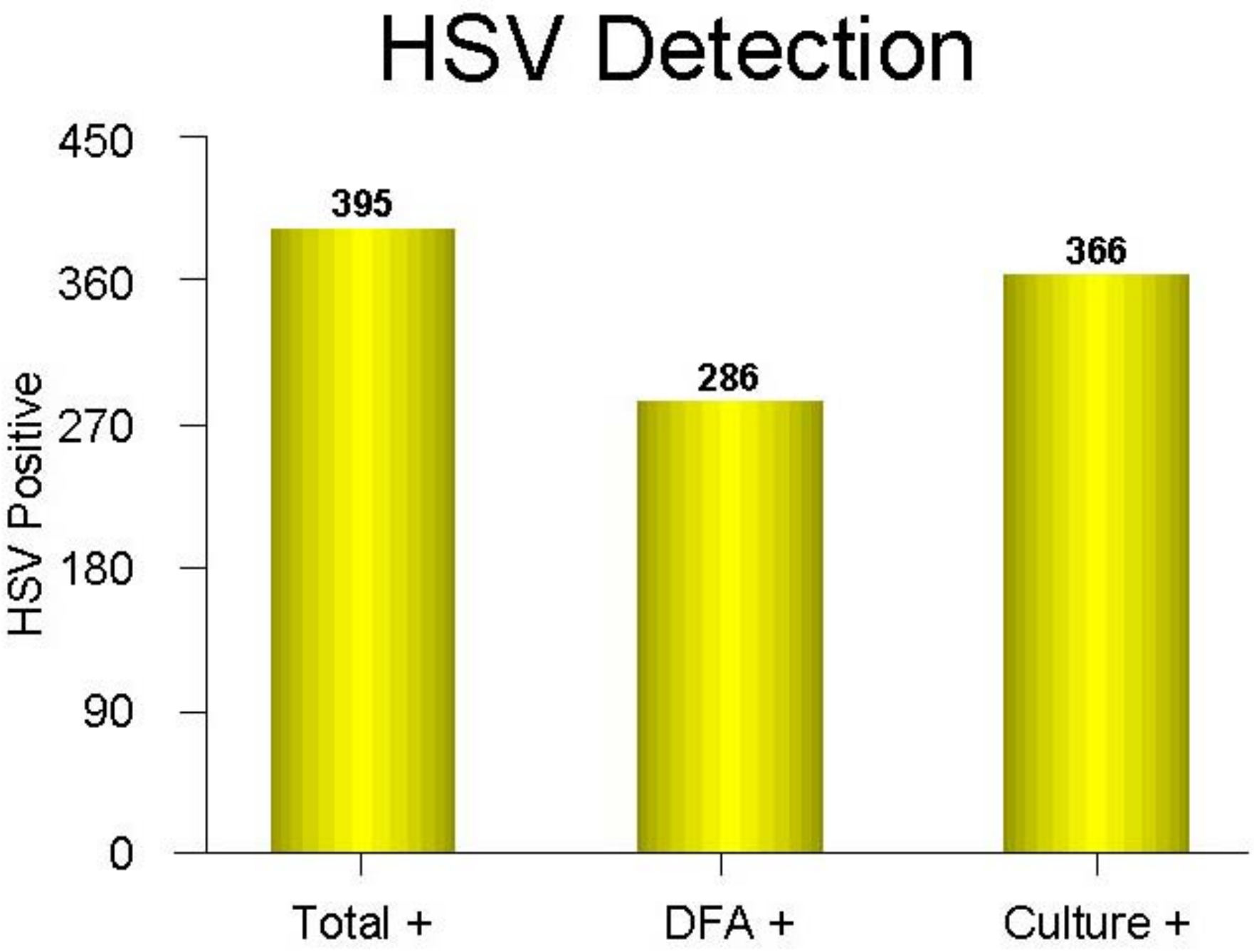

VZV Detection
VZV Positive
100
80
60
40
20
0
83
80
44
N = 423
19.6% Positive
Total +
DFA +
Culture +

Not for Every Specimen

- PCR for CSF and ocular fluids
- Culture other specimens (BAL, tissue)

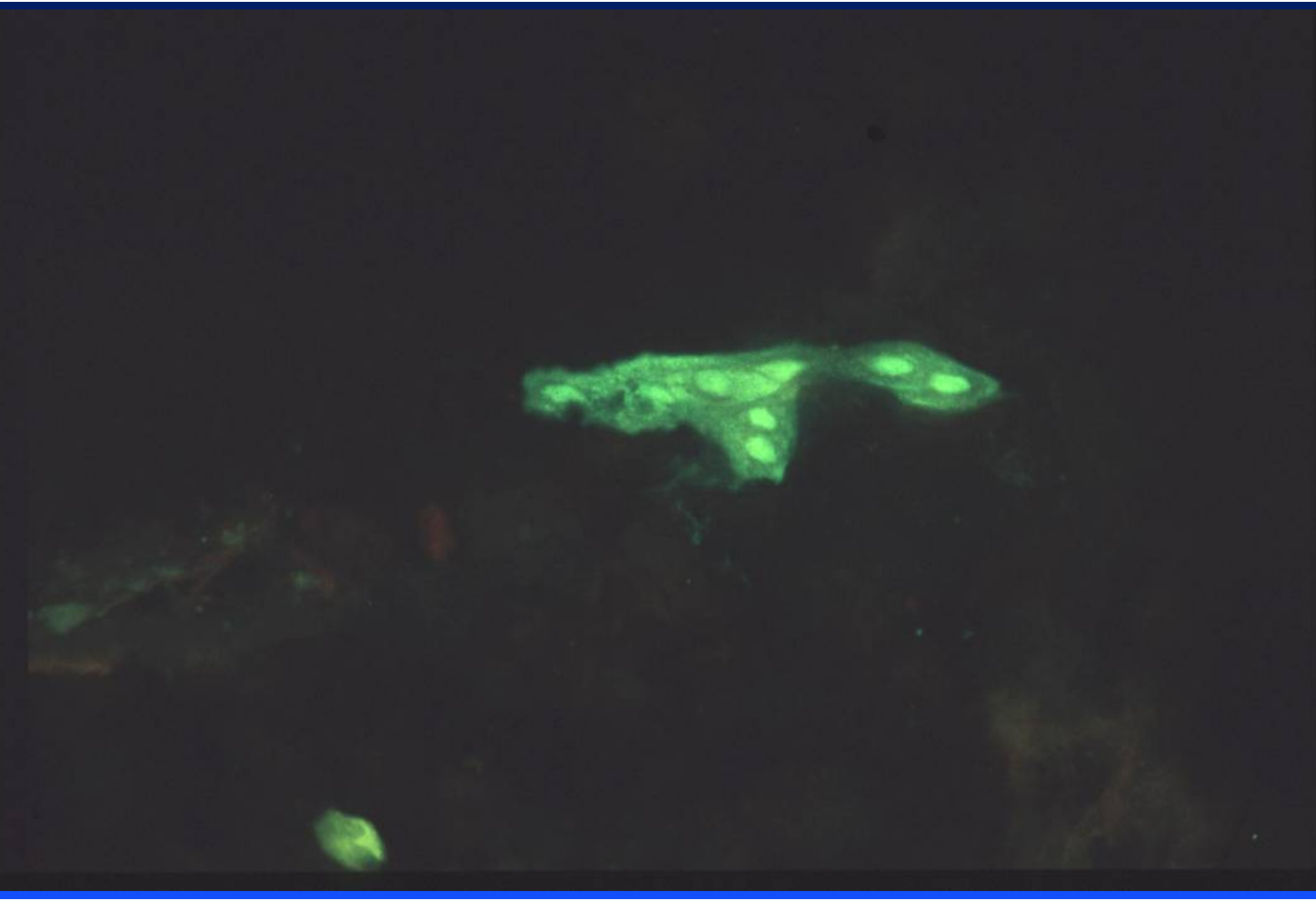
Herpes Simplex Virus

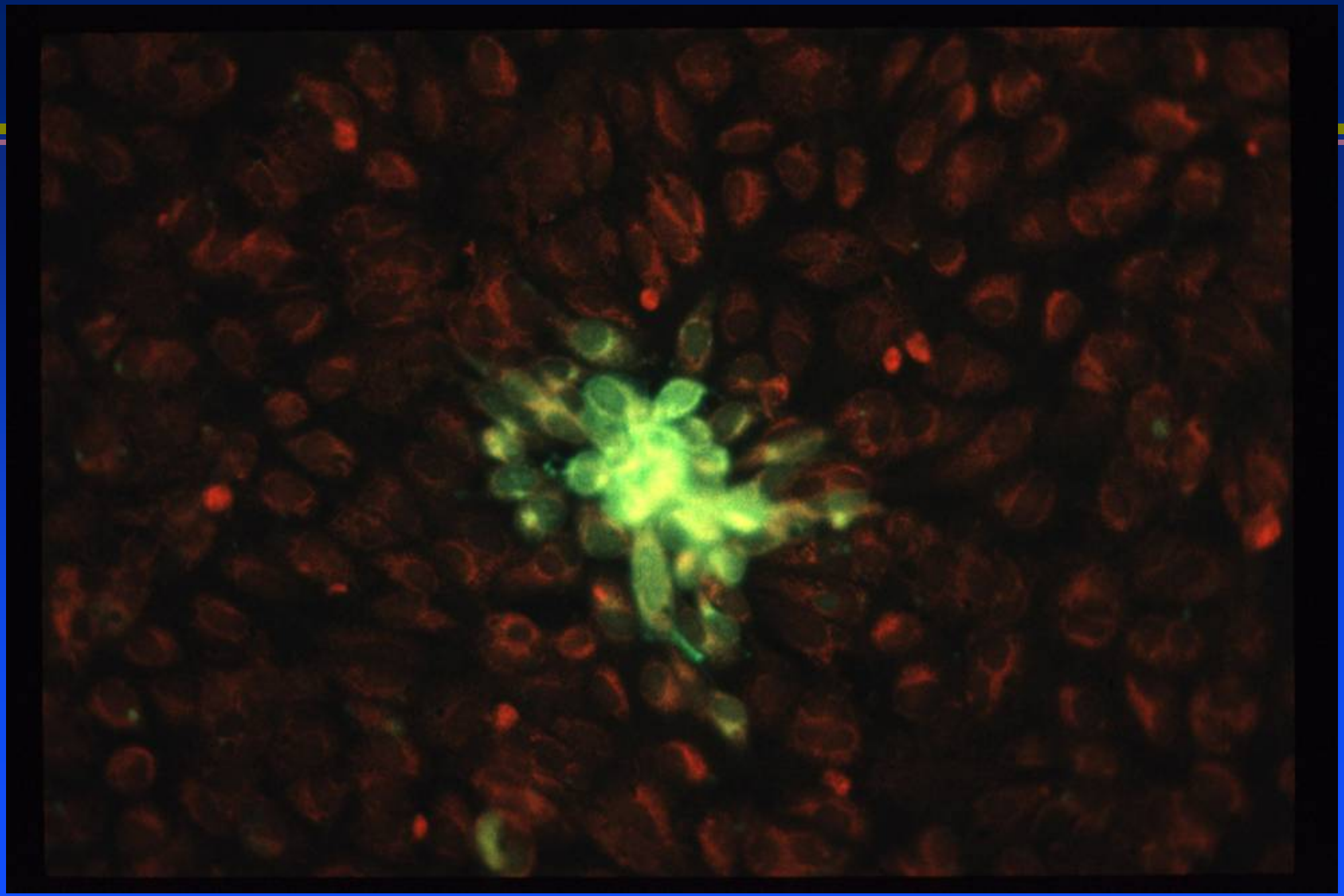
- Culture 1-2 days
- EIA (daily batches)
- DFA 1-2 hrs

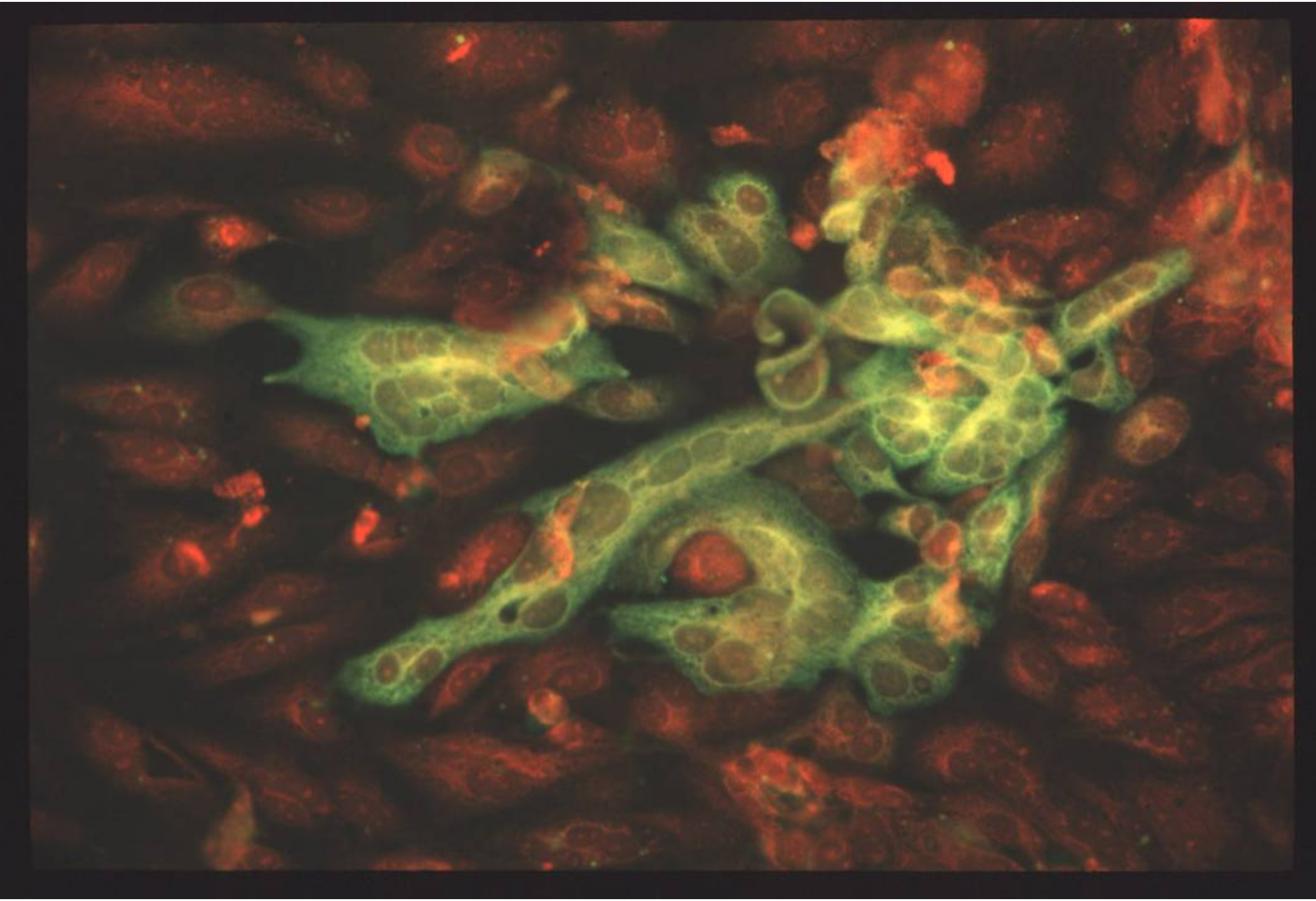

HSV Detection
HSV Positive
450
360
270
180
90
0
395
286
366
Total +
DFA +
Culture +

Bottom Line

- Two-step testing for dermal specimens
  - DFA (detects 72% of positives)
  - Culture DFA-negative specimens
- PCR for CSF and ocular fluids
- Culture other specimens







Respiratory Viruses

RSV	2-5 days
Influenza A/B	2-10 days
Parainfluenza	2-14 days
Adenovirus	2-10 days

Respiratory Virus Testing

- DFA (eliminates >50% of cultures)
- Culture DFA-negative specimens
- EIA only when necessary

Breakthrough Rates

- Overall, 1-10% of DFA-negative specimens were culture-positive

- RSV: << 1%
- Parainfluenza 1,2,3: 1-4%
- Influenza A: 7-10%
- Adenovirus: ~15%