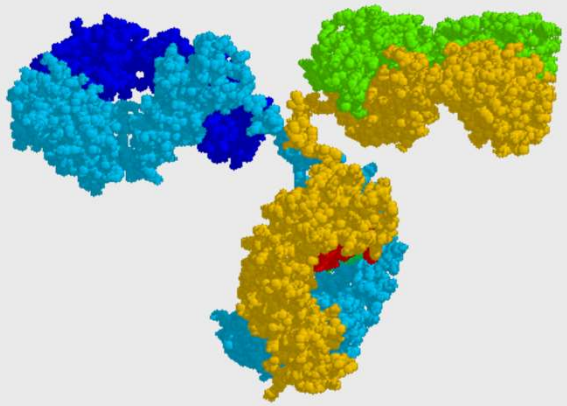
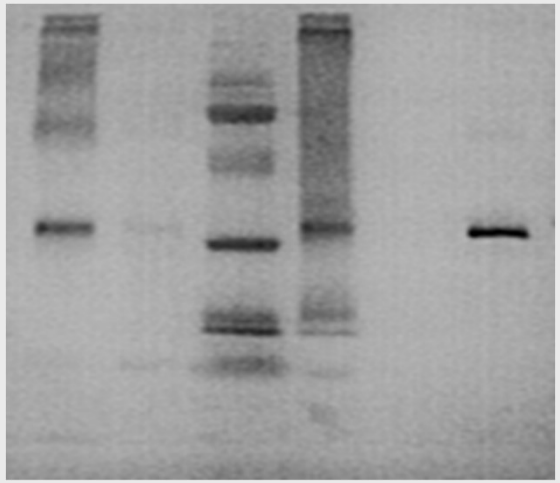
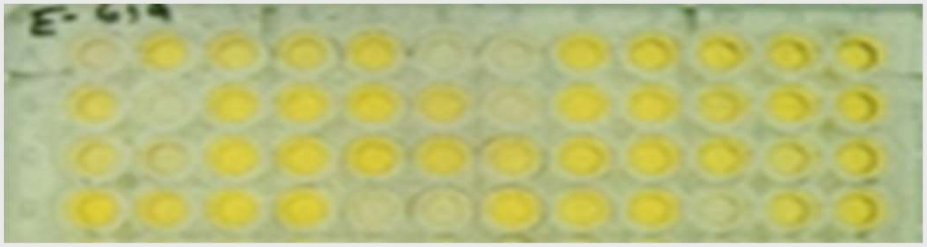
Assays Based on Detection of Viral Proteins







Objectives:

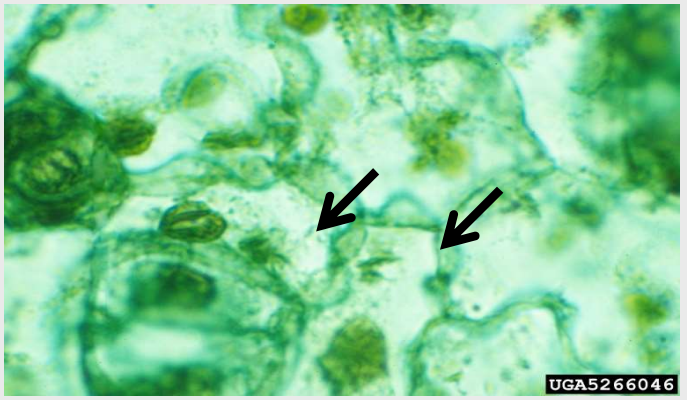
- Understand the mechanics of the different protein-based assays used for plant virus detection
- Understand the uses of, as well as advantages and disadvantages of, different protein-based assays used for plant virus detection

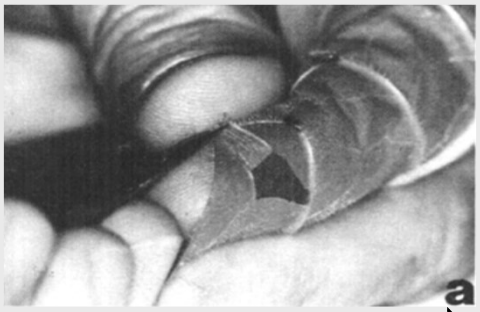
C. Assays Based on Viral Proteins:

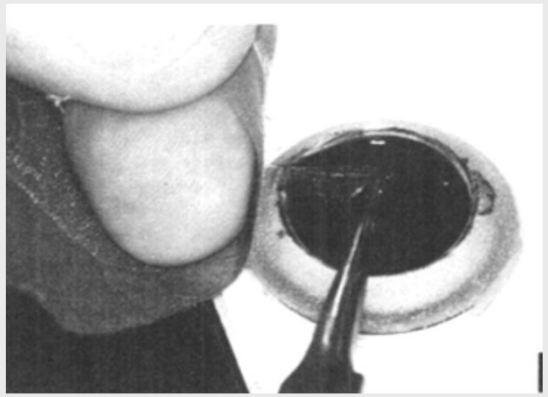
1. Inclusion Body Visualization
2. Visualization of Virions by EM
3. Virus purification
4. Serological Assays (many)
5. Microarrays

1. Inclusion Body Visualization

Leaves,
Petioles,
Flowers



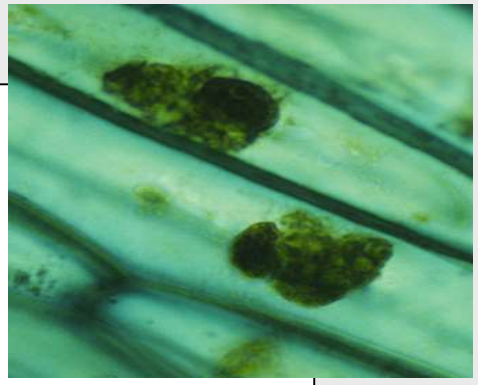




Stain with orange/green to visualize inclusions composed of viral proteins

Christie and Edwardson 1986 Plant Dis. 70:273-279

1. Inclusion Body Visualization:



Strengths:

- Low tech, inexpensive reagents
- Relatively fast
- Relatively easy for some viruses
- Identifies new viruses faster than most techniques

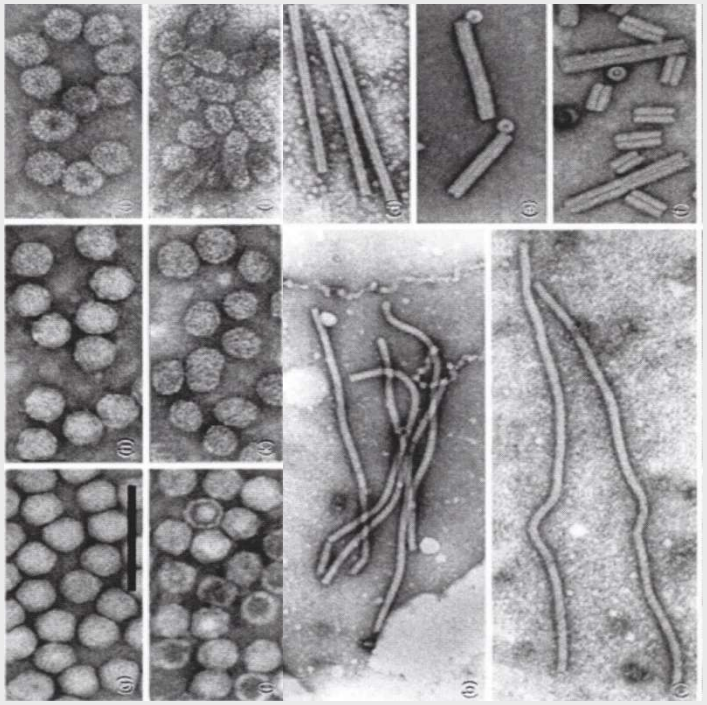
Limitations:

- Too slow for large numbers of samples
- Not as portable as other techniques
- Inclusions not readily visualized for some viruses
- Requires experience to interpret results
- No standards available as positive controls

2. Visualization of Virions: Electron Microscopy of Virions

Various techniques:

- Negative staining of partially or fully purified preparations from infected tissue
- "Leaf dip" negative staining
- Embedding and sectioning of plant tissue



2. Electron Microscopy of Virions

- **Strengths:**
  - Broad spectrum assay – does not require virus-specific reagents
  - Virus particle size and shape gives a diagnosis to genus level
  - May be combined with serological techniques (ISEM) to
    to identify at the species level, for detection of viruses not resolved by leaf dip assay
  - Essential in the identification of a new virus
  - Leaf dip (neg.staining) technique is fast, direct, and simple
- **Limitations:**
  - Difficult to handle large number of samples
  - Requires very expensive equipment
  - Embedding, sectioning requires specialized expertise, tedious
  - Cannot resolve isometric virions in leaf dips or viruses in low concentrations

3. Basic Serological Assays:

- Capture of viral antigens:
  - **ELISA**
  - **Lateral flow assays**
  - **Immunosorbent microscopy (ISEM)**

- Immobilized viral antigens:
  - **ELISA**
  - **Western Blots**
  - **Dot Blots**
  - **Tissue Blots**
  - **Direct Tissue Blots**
  - **Microarrays**
  - **Fluorescent microscopy**
  - **Immunosorbent microscopy (ISEM)**

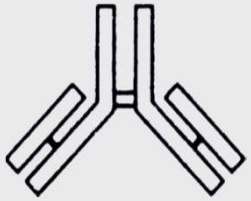
3. Basics Of Serological Tests For Detection Of Plant Viruses

Required:

- An antiserum with specificity for the test antigens (virus)
- A positive control consisting of a reactive source of the virus antigen used in preparing the antiserum
- Samples from non-infected plants to serve as a negative control.
- Sample(s) to be assayed.

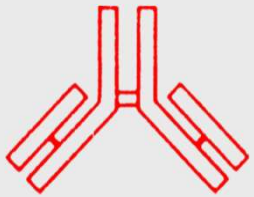
Negative controls are essential!
plant proteins also are antigenic and they can react with antisera that contain antibodies to the host proteins.

Uses of Antibodies and Terminology

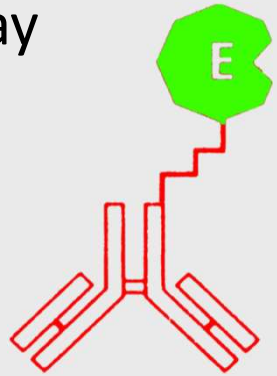


1° Antibody – first antibody in the assay, binds to the antigen

2° Antibody – second antibody used in the assay



- May or may not bind to antigen
- May be non-modified OR
- May be conjugated to a reporter
- Referred to as the “conjugate” when a reporter is attached



Enzyme-linked immunosorbent assay (ELISA)

Most widely used test for the routine detection of plant viruses. The results can easily be quantified (spectrophotometry) and the whole testing procedure can be automated.

Different Strategies:

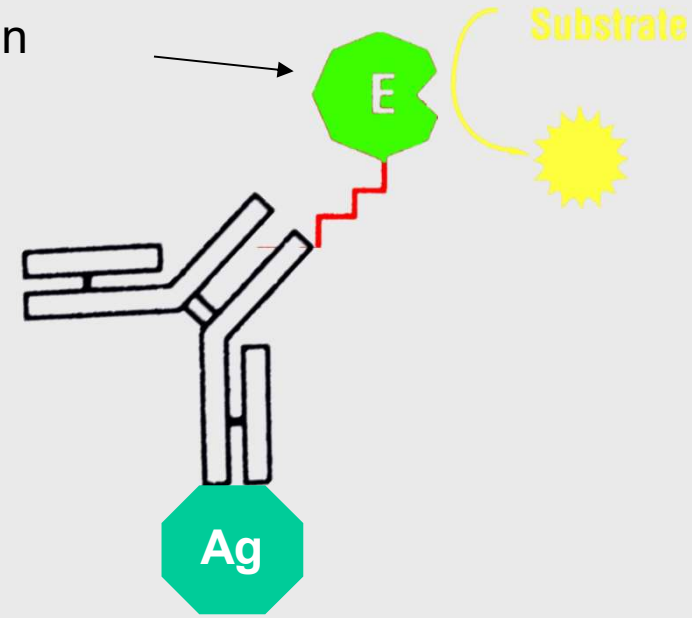
- Direct **or** Indirect
- Antigen-labeled **or** Antibody-Labeled

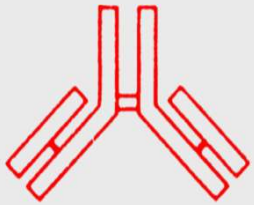
Direct Versus Indirect Detection Methods

Direct Detection –

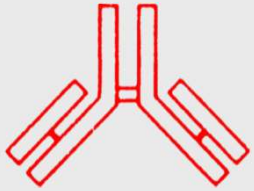
Assay in which the primary antibody is labeled with a reporter

The reporter is an Enzyme

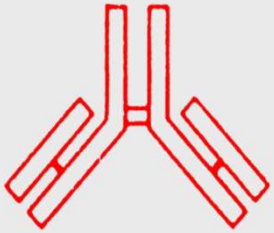




When virions are injected into a rabbit -→ the rabbit makes antibodies against the virus:
Referred to as “rabbit antivirus antibodies”



Antibodies are proteins – so they can be used to produce antibodies



A rabbit antibody when injected into goat ----→ results in goat antibodies which bind to rabbit antibodies:
Referred to as “goat anti-rabbit antibody “

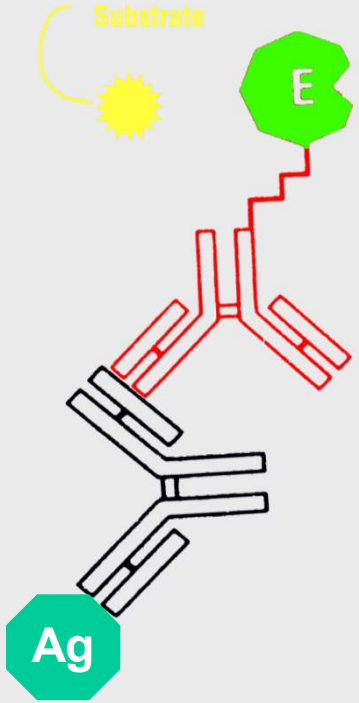
This gives researchers great flexibility to design serological tests

Direct Versus Indirect Detection Methods

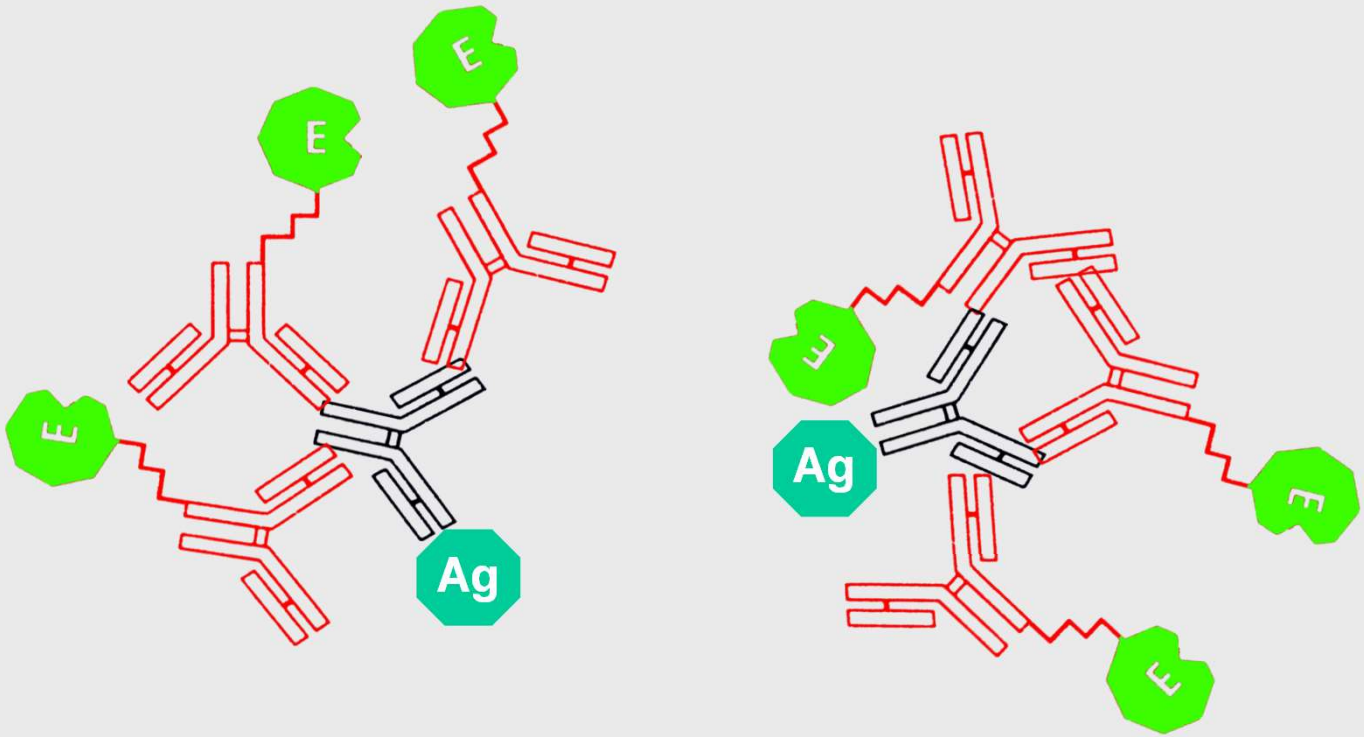
Indirect detection – describes methods in which unlabeled primary antibodies are followed by labeled secondary antibodies. The secondary Ab bind to epitopes on the primary antibodies.

2° Antibody monoclonal or polyclonal anti-mouse), "Goat anti-mouse"

1° Antibody (monoclonal made in mouse) "Mouse anti-virus"

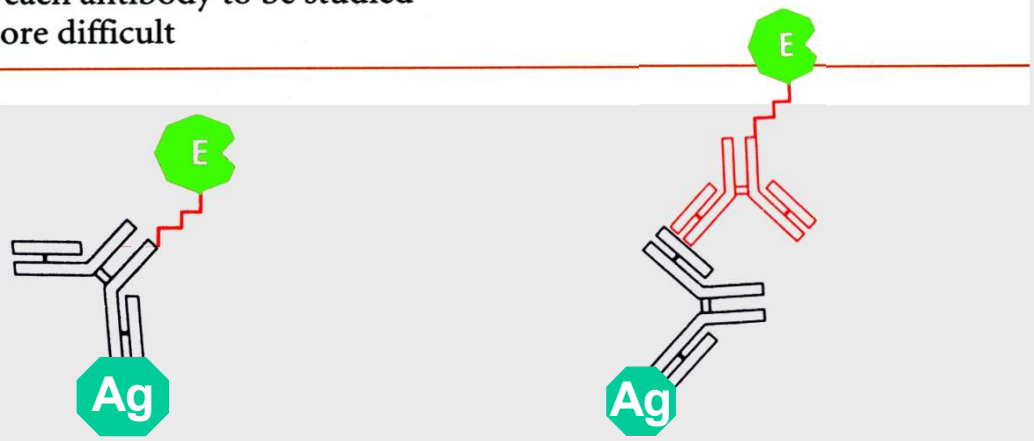


There is an amplification in Indirect Assays
= Increased Sensitivity

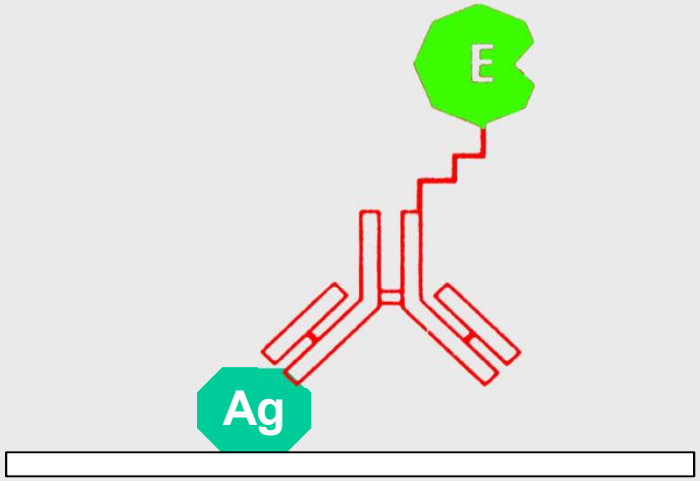


Summary: Comparison of Direct vs Indirect Detection Methods

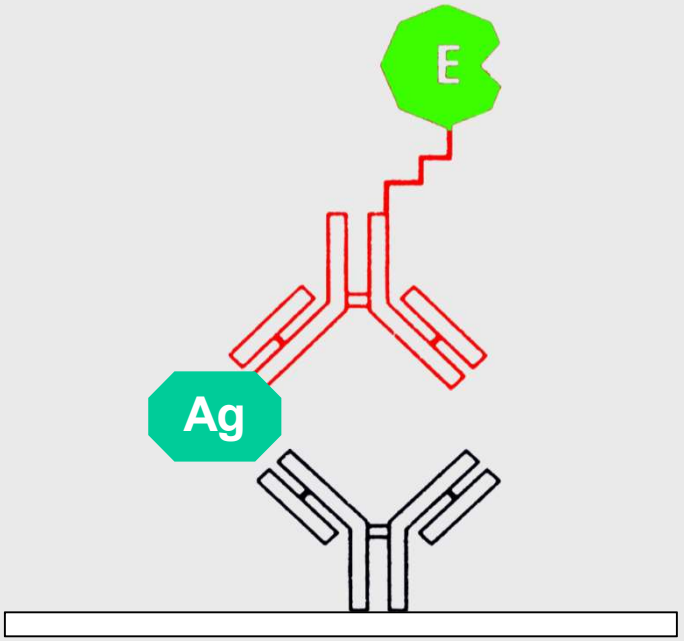
Detection method	Direct detection	Indirect detection
Location of label	Labeled primary antibodies	Labeled secondary antibodies
Advantages	Fewer steps Less prone to background	Stronger signals Widely available labeled reagents Primary antibody is not modified Easier
Disadvantages	Less sensitive New labeling step required for each antibody to be studied More difficult	More prone to bad backgrounds



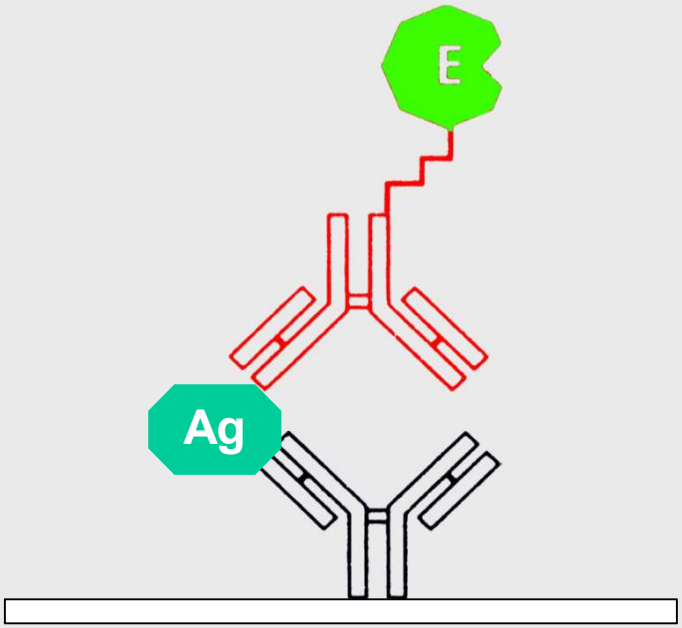
Antigen labeled **vs** Antibody Labeled



Antigen labeled

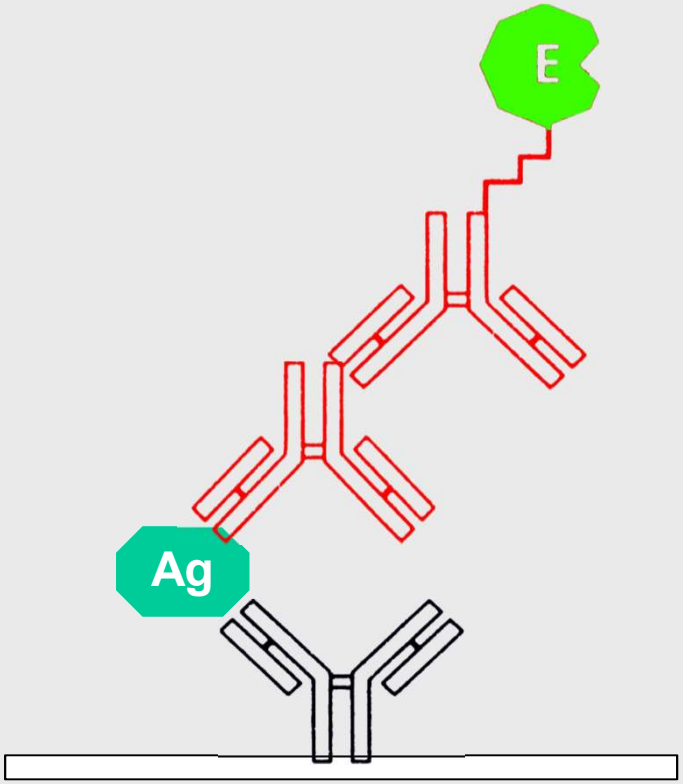


Antibody Labeled
(aka Antibody Trapping)



Antibody Labeled
(aka Antibody Trapping)

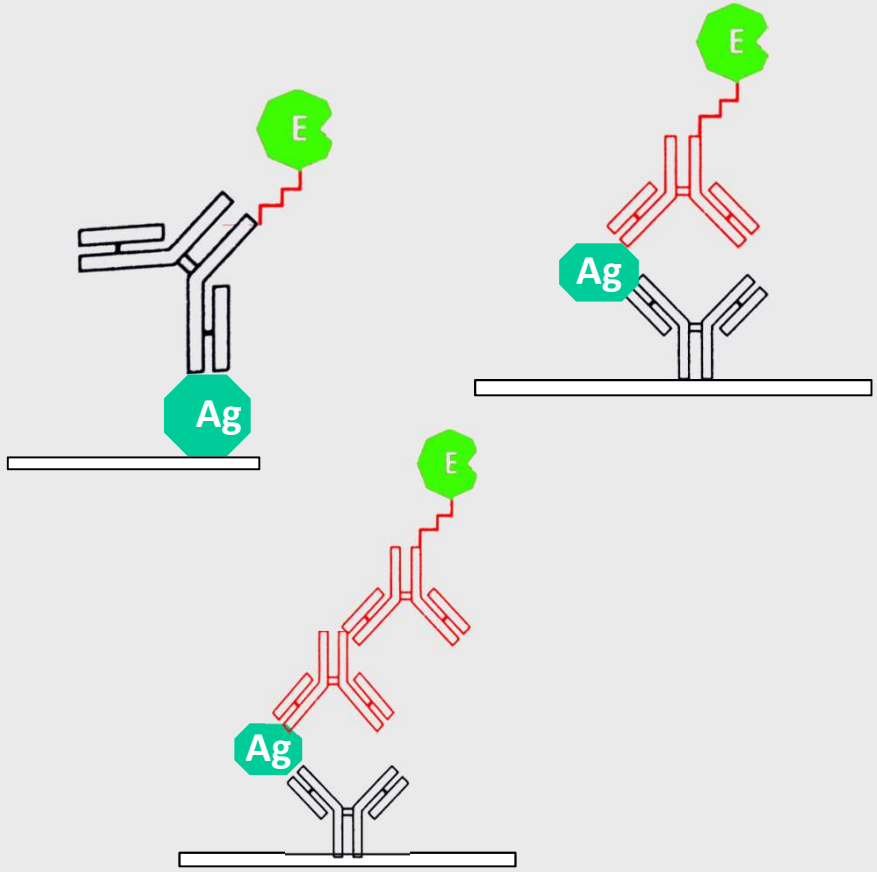
Double Antibody assay



Triple Antibody assay

These “sandwiches” can be used in many assays:

ELISA
Dot or Immunospot
Westerns
EM
Etc.…



WESTERN AND "DOT" BLOTS

Detection of viral antigens immobilized on nitrocellulose membranes or other membranes.

Dot blots - grind tissues in Tris-buffered saline, containing one or more additives, spot onto nitrocellulose

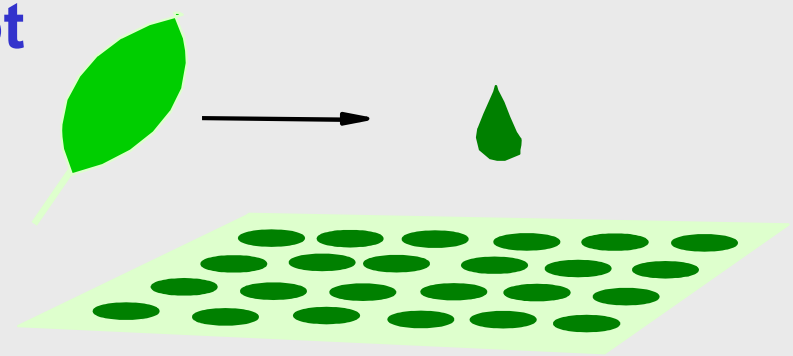
freehand,
blotting manifold,
whole leaves with pressure ("tissue or squash blots")

Western blots – extract total proteins, separate in a gel matrix,
transfer contents of gel to nitrocellulose

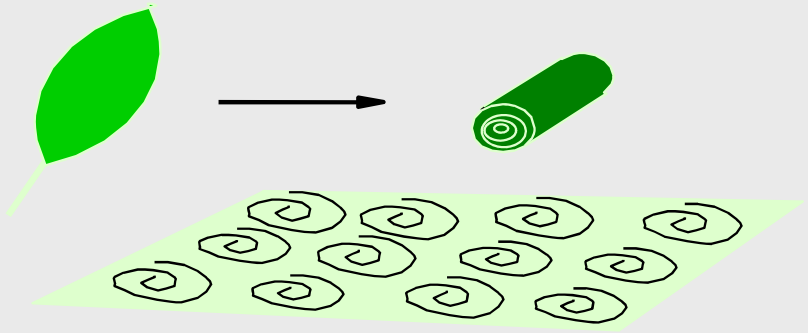
A pre-wetted membrane may be placed in a plastic, 96-well vacuum manifold, and the samples are applied to the membrane. This is followed by procedures similar to those in Western blots.

Blots

Dot Spot



Squash Blot



- Cut tissue with razor blade, Transfer antigens to the membranes by pressing the cut tissues lightly against nitrocellulose membrane.

- Various procedures for detection of possible antigens are similar to those used for the other immuno techniques.

- Various plant parts can be tested: rolled-up leaves, bulbs, stems, petioles.

DOT BLOTS

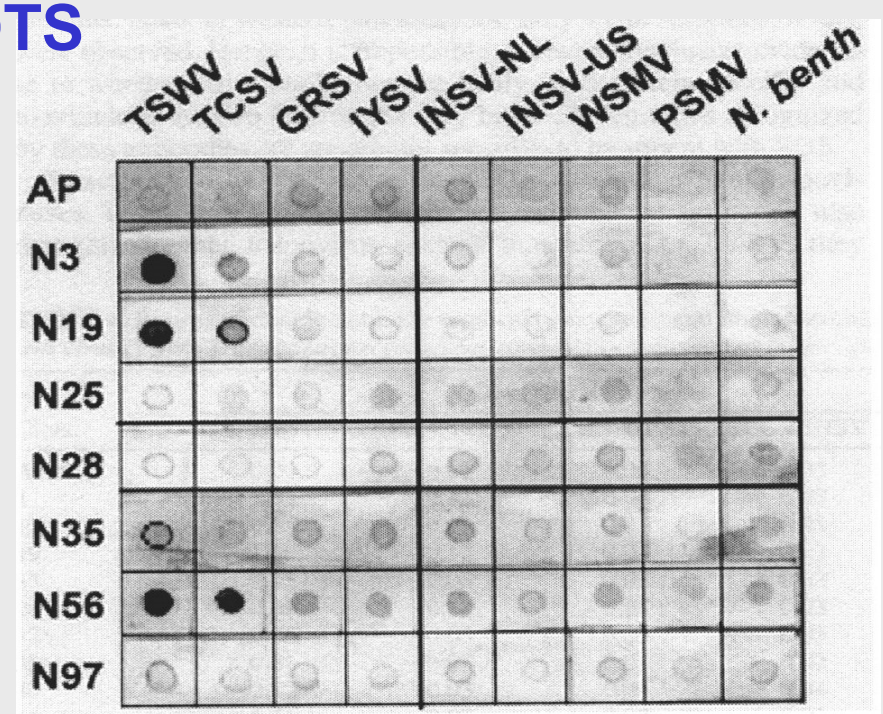


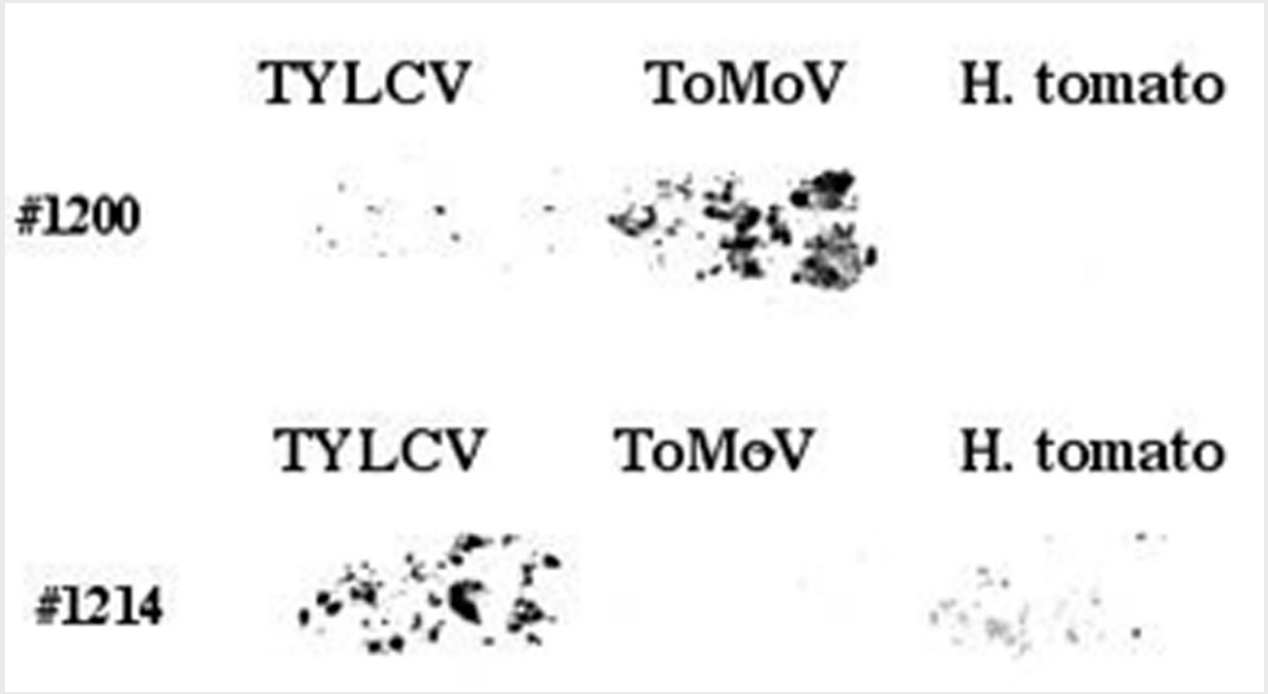
Fig. 4. Reactivity of the *Tomato spotted wilt virus* (TSWV) nucleocapsid-specific single-chain variable fragment alkaline phosphatase fusion proteins in a dot-immunobinding assay to other tospovirus species. TCSV = *Tomato chlorotic spot virus*, GRSV = *Groundnut ringspot virus*, IYSV = *Iris yellow spot virus*, INSV = *Impatiens necrotic spot virus*, WSMV = *Watermelon silverleaf mottle virus*, PSMV = *Physalis severe mottle virus*, and *N. benth* = *Nicotiana benthamiana*.

Griep, R. A et al., 2000, Phytopathology 90, 183-190

Spots can be applied free style or using a manifold (see below)

Tissue Blot Comparison of 2 polyclonal antisera

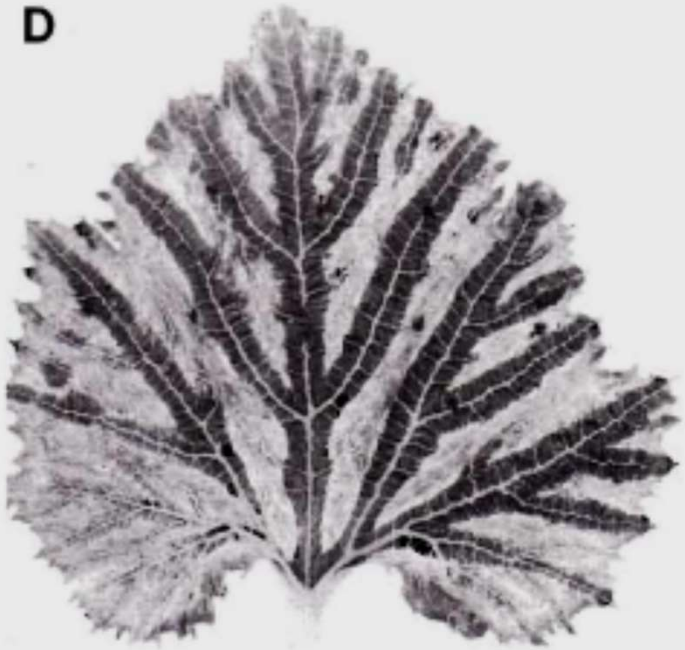
Antisera | Virus-infected Tissue | Healthy Tissue



Tomato yellow leaf curl virus TYLCV; Tomato mottle virus ToMoV

Blots of Entire Leaves

Leaf blotted with a drill press (10K psi) and ZYMV detected with Ab made against ZYMV CP, using the reporter alkaline phosphatase and the substrate NBT/BCIP.

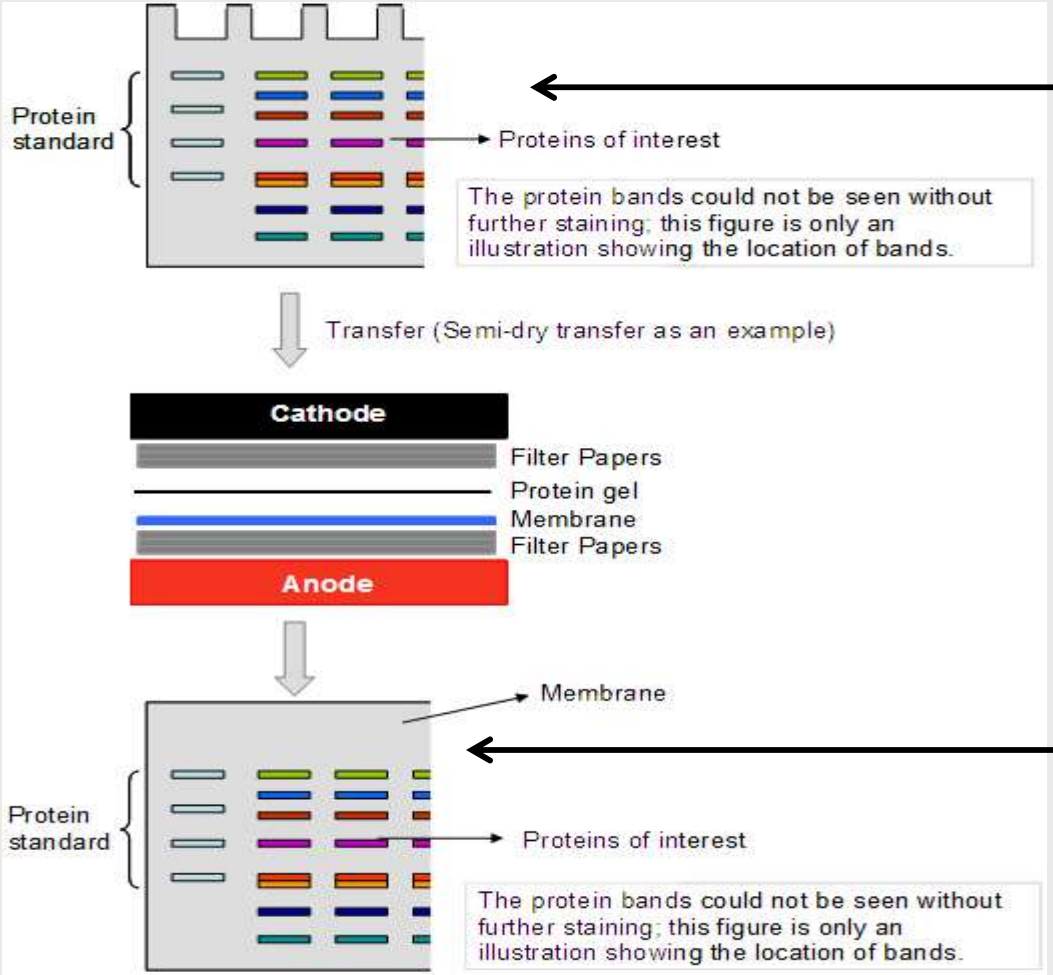


Squash leaf infected with *Zucchini yellow mosaic virus* (ZYMV)

Above: Immunoblot of the same leaf, dark spots indicate presence of ZYMV coat protein

Polston et al. 1991. Analytical Biochemistry 196:267-270

Western Blotting

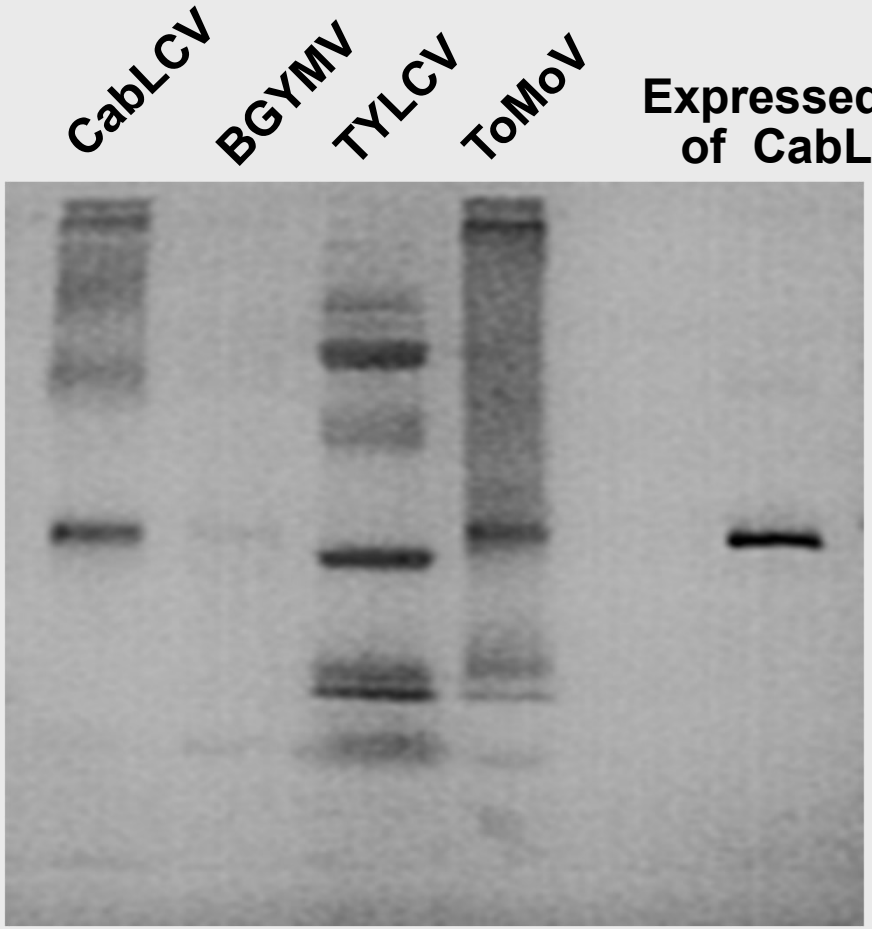


Separate proteins in the extract

Transfer separated proteins from gel to solid membrane

Incubate with an antibody to detect protein of interest

WESTERN BLOTTING



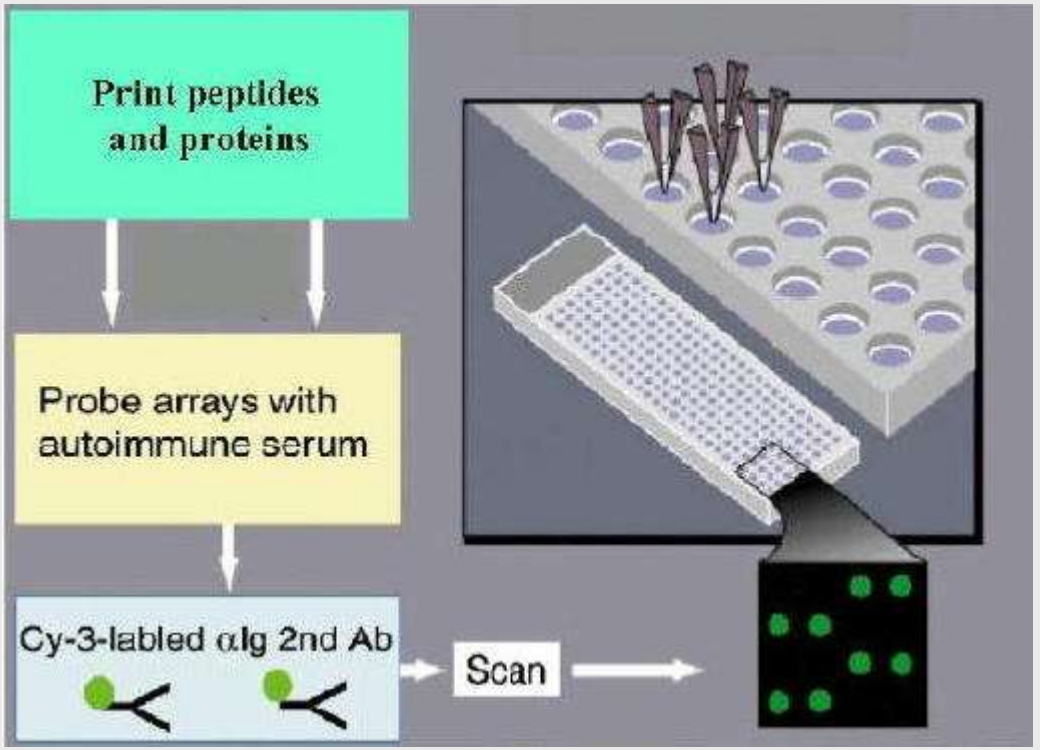
Results using polyclonal antiserum made against the coat protein of *Cabbage leaf curl virus*

← Pure CabLCV coat protein

Microarray:

a 2D array on a solid substrate (glass slide or silicon thin-film cell) that assays large numbers of biological materials using high-throughput highly automated processing and detection methods.

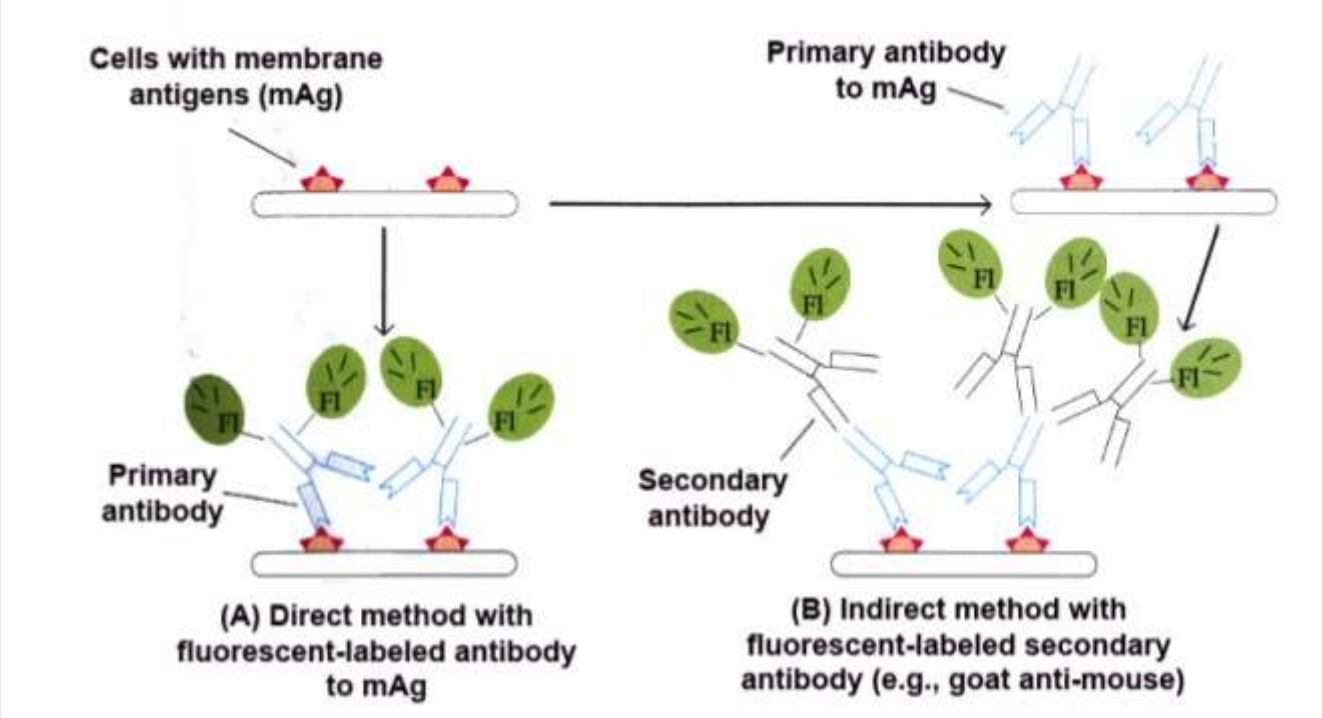
The reporter is a fluorochrome attached to the antibody that allows the detection of the antigen using light or confocal microscopy

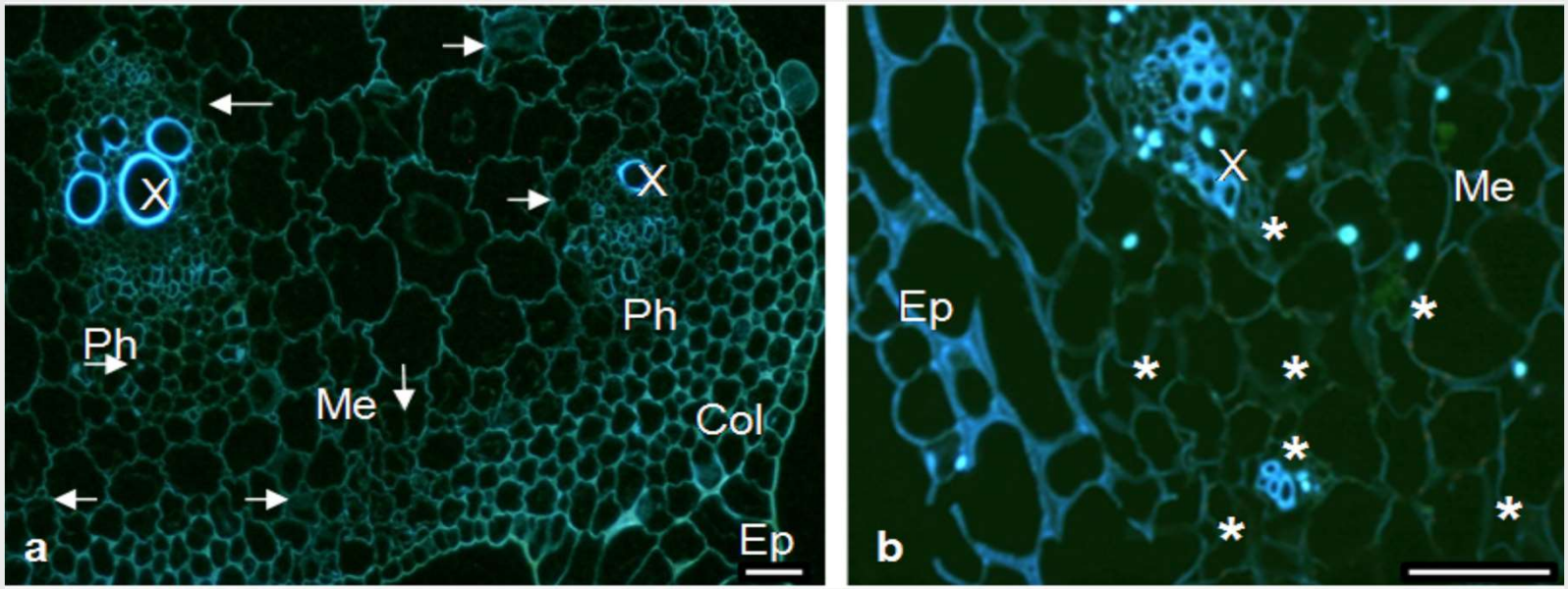


Immunofluorescence Light Microscopy

Same approach -

The reporter is a fluorochrome attached to an antibody to visualize the location of the protein using light or confocal microscopy





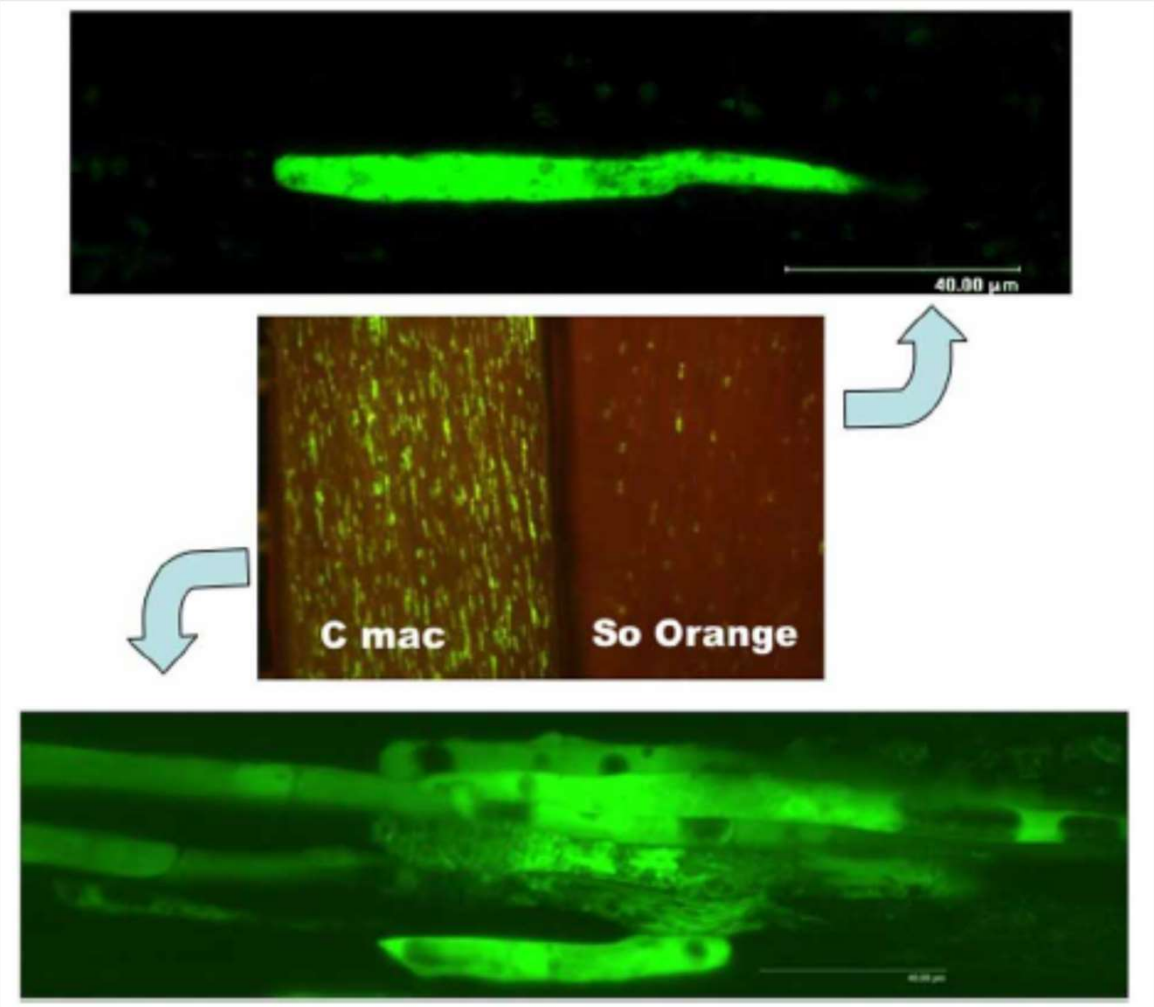
Immunofluorescence detection of *Tobacco rattle virus* (TRV) capsid protein (CP).

a) Green fluorescence (arrows) indicate TRV CP deposition in tobacco leaf petioles. Rabbit polyclonal antibodies directed against TRV CP and secondary anti-rabbit IgG conjugated with FITC were used.
b) Cross section of potato leaf, capsid protein deposition indicated by asterisk. Ep-epidermis, Me-mesophyll, Col - Collenchyma, Ph - phloem, X - xylem. (Bars 200µm)

Common fluorescent reporters:

LOV – fluorescent reporter derived from a plant phototropin
GFP – green fluorescing protein from the jellyfish, *Aequorea victoria*

Tobacco mosaic virus (TMV) modified to express a CP-LOV hybrid protein



Detection of GFP fluorescence in phloem-associated cells of *Citrus macrocarpa* and sour orange
(CTV modified to express a CP-GFP hybrid protein)

Confocal laser scanning microscope (top and bottom)

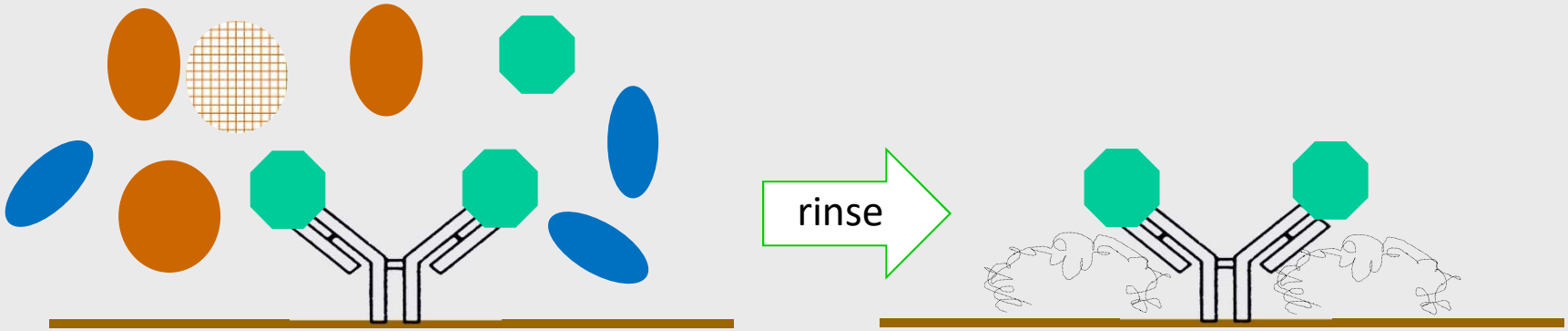
Dissecting microscope (center)

TEM Immunocytochemistry (Immunogold labeling)

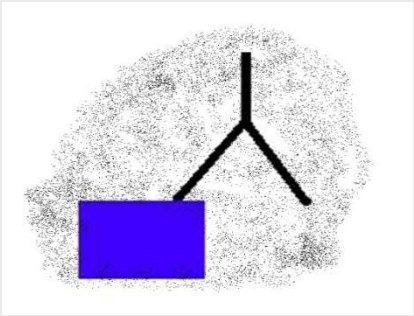
- Utilizes standard immunological reactions.
- Results are visually assessed in the EM.
- Monoclonal or polyclonal antisera can be used.
- Can be performed on particulate samples and thin sections.
- May utilize direct or indirect labeling techniques.

Immunocapture of virions on EM grids

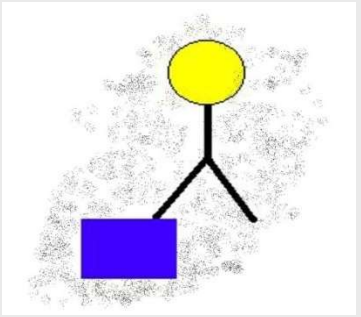
- Coat grids with virus-specific antibody
- Add sample
- Rinse
- Add stain and observe
- Used for viruses which occur in low concentrations



Direct Labeling of Virions in the EM

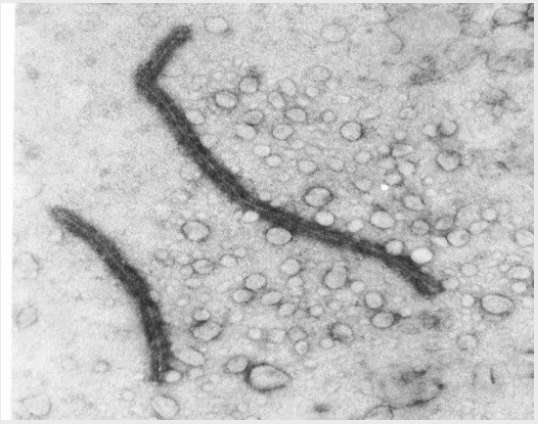


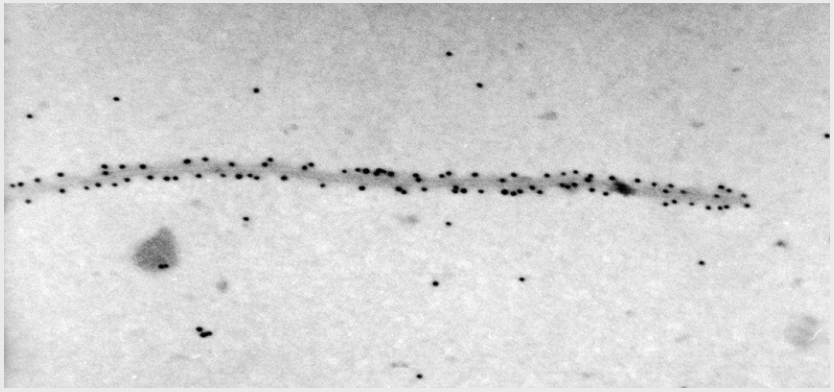
OR

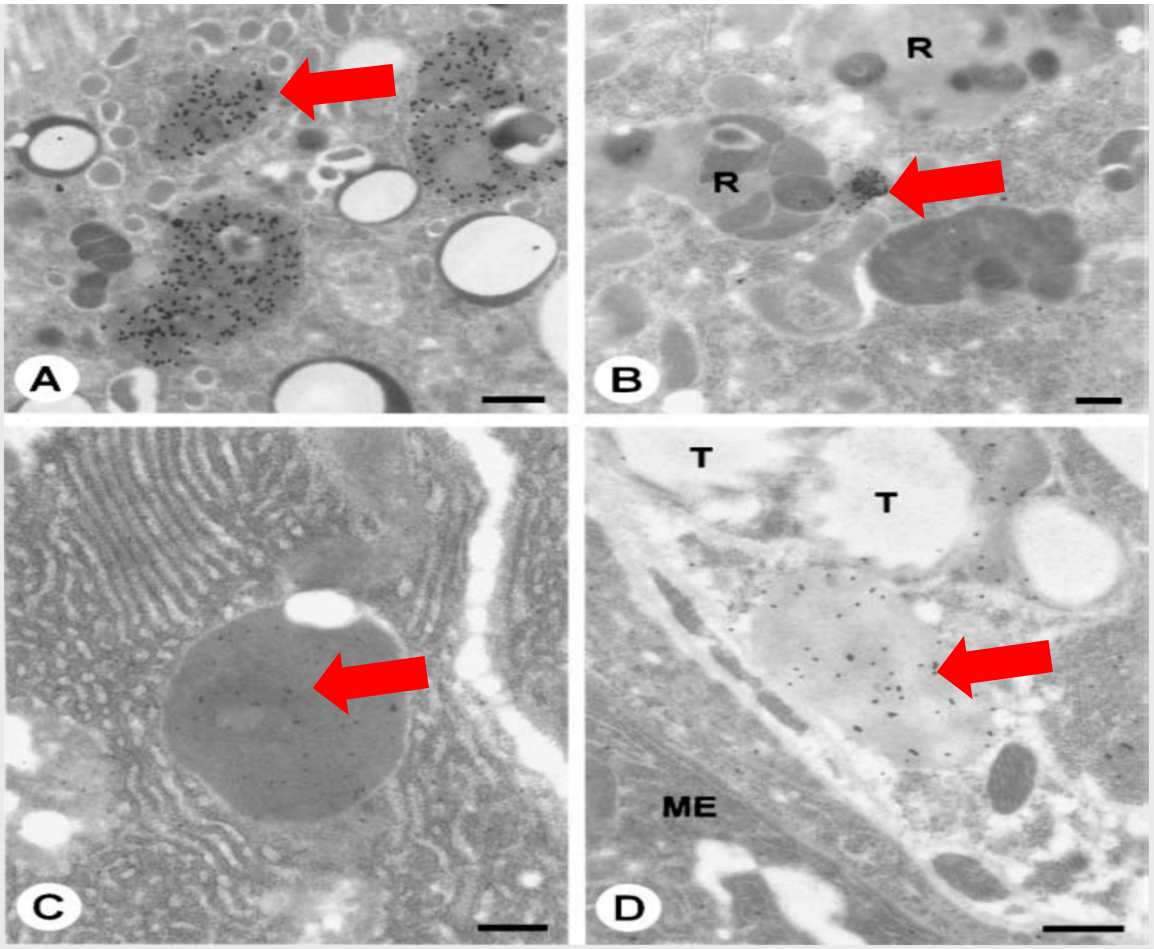


Primary antiserum: Antigen-antibody complex plus an electron dense stain such as uranyl acetate

Primary antiserum conjugated to a gold particle binds to the antigen. Gold particles are electron dense.







Immunogold localization in thrips of 3 viral proteins:
(A) **N protein** and
(B) **NSm protein** in midgut epithelium cells of TSWV-infected L2 thrips larvae 5 days after start of acquisition and of
(C) **NSm protein** production in the salivary gland of adult thrips.
(**D**) Later after acquisition, **NSm** can be found associated with inclusions in muscle cells adjacent to the midgut epithelium.

ME midgut epithelium; *R* residual bodies; *T* trachea. Scale bars represent 200 nm (**A**, **B**, **C**) and 400 nm (**D**) respectively

Summary: Assays Based on Viral Protein – Serological Assays

Strengths:

- Many of the assays are rapid, simple,
- Most don't require expensive equipment or supplies
- Often can be used to identify new strains of known viruses
- Can differentiate between isolates or strains of the same species
- Can be very sensitive - detect ng of virus antigens
- Can be used for diagnosis of a large number of samples

Summary: Assays Based on Viral Protein – Serological Assays

Limitations:

- The techniques depend upon the availability of antibodies, antibodies are not available for all viruses
- Limits detection to known viruses or viruses antigenically closely related to known viruses
- Certain techniques require expensive equipment
- Autofluorescence is sometimes a problem