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Chapter 10

Localizing Proteins by Tissue Printing

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Abstract

The simple technique of making tissue prints on appropriate substrate material has made possible the easy localization of proteins, nucleic acids, carbohydrates, and small molecules in a tissue specific mode. Plant tissues can be used to produce prints revealing a remarkable amount of anatomical detail, even without staining, which might be used to record developmental changes over time. In this chapter we will focus on the protocols for the localization of proteins and glycans using antibodies or lectins, probably the most frequently used application, but the localization of other molecules is reported and the sources indicated.

Key Words: Immunodetection, tissue blotting, protein localization, Western blotting.

1. Introduction

Fifty years ago, the first series of film printing for the localization of several enzymes (protease, amylase, RNase, and DNase) were realized by placing cryostat sections of various organs on gelatin, starch, or gelatin-nucleic acids (1). Those films were then stained for the substrate, giving a negative image. Since both, substrates and enzymes were macromolecules with slow diffusion rates, the images obtained were clear. The availability of several membranes such as nitrocellulose, Nytran, Genescreen and Immobilon (polyvinylidene di-fluoride: PVDF) designed to bind proteins and nucleic acids open a new era in tissue blotting, and a book edited by P. D. Reid et al. (2) included numerous protocols for the visualization of enzyme activities, protein and glycan localization, gene expression, in plants and some animal tissues. Nitrocellulose membrane adsorbs relatively large quantities of proteins that are tightly bound, whereas it usually does not retain salts, and hydrophilic small molecules. This type of membrane has been used for transferring proteins separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis to membranes for immunological detection. The procedure involves the electro elution of the negatively charged proteins from the gel to the membrane, followed by the visualization of the targeted protein with specific antibodies. The basic principle of tissue printing is that much of the contents of the cells at the surface of a freshly cut tissue section can be transferred to an adhesive or absorptive surface with little or no diffusion, by simple contact (3). The obtained imprints reveal anatomical details that are difficult to see without fixing, embedding, and then sectioning; however, the resolution of anatomical prints is inferior to that of classical fixed and stained sections. The procedure is particularly useful with big samples such as sections of fruits, tubers, or stems, revealing the distribution of particular proteins in the whole organ (4, 5).

A digest of different applications of tissue printing for the detection of particular proteins, glycans, mRNA, enzyme activities, and microorganism in plants, is given in **Table 1**. The steps to make a print and to show the localization of proteins using specific antibodies, as well as the necessary precautions to avoid non specific staining in plant tissues, will be described in this chapter.

1. Materials

2.1. Doing a tissue print

1. Whatman No. 1 filter paper (Fisher Scientific Company, Pittsburgh, PA, USA).
2. Blotting membrane: nitrocellulose (type BA-85, Schleicher & Schuell, St. Marcel, France); nylon (Zetaprobe, Bio-Rad, Richmond, CA, USA), Immobilon P, 0.45µm (Millipore Corp, Bedford, MA, USA).
3. Double-edged razor blades, forceps, rubber gloves, paper to protect the membrane, acrylic sheet, marking pen.
4. Hand lens or microscope for viewing the specimen, and the biological material. (**Fig. 1 A**).

2.2 Detection of total proteins in a tissue blot

1. Nitrocellulose (NC) membrane, 0.45-µm pore size (Schleicher & Schuell, St. Marcel, France) (another membrane may be used).
2. Tris buffered saline (TBS) Tween-20 (TBST): 20 mM Tris-HCl (pH 7.5), 0.5 M NaCl, and

0.05% Tween-20.

3. India ink staining: 1 μ l Pelikan India ink/ml of PBS-Tween.

2.3 Revealing proteins with antibodies

1. Nitrocellulose membrane, 0.45 μ m pore size (Schleicher & Schuell, St. Marcel, France) (another membrane may be used)
2. TBST: 20 mM Tris-HCl (pH 7.5), 0.5 M NaCl, and 0.05% Tween-20
3. Blocking buffer: 0.25% gelatin and 0.25% bovine serum albumin (BSA) in TBST.
4. Primary antibody: Antibodies against the targeted protein (potato lectin in this case), in blocking buffer (1:15,000 dilution for the antibody against the deglycosylated lectin)
5. Secondary antibody: Goat anti-rabbit alkaline phosphatase (AP) conjugate F(ab)² fragment (Sigma Chemical Company, MO, USA), 1:20,000 dilution in blocking buffer
6. AP buffer: 0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl, and 5 mM MgCl₂
7. AP substrates: nitro blue tetrazolium (NBT) (Promega, Fitchburg, WI, USA), 50 mg/ml in 70% methanol diluted 33:10,000 in AP buffer, and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Promega, Fitchburg, WI, USA) 50 mg/ml in dimethyl-formamide diluted 66:10,000 in AP buffer.

2.4 Localizing cell wall proteins in plants

1. NC membrane (Schleicher & Schuell type BA-85, St. Marcel, France)
2. 0.2 M CaCl₂·2H₂O.
3. Primary antibody: Specific anti-extensin polyclonal antibodies raised from purified soybean seed coat extensin (**6**) diluted 1:15,000.
4. Secondary antibody: AP conjugated antibody anti-rabbit immunoglobulin IgG (Fc)
5. Tris buffered saline (TBS): 0.9% NaCl in 20 mM Tris-HCl (pH 7.4) plus 0.3% Tween-20 and 0.05% sodium azide (NaN₃).
6. Blocking buffer: 0.25% (w/v) BSA, 0.25% (w/v) gelatin, and 0.3% (v/v) Tween-20 in TBS
7. AP buffer: 50 mM Tris-HCl (pH 9.5) plus 1 mM MgCl₂
8. AP substrate solution: 66 μ l NBT and 33 μ l BCIP (Promega, Fitchburg, WI, USA) in 10 ml AP buffer.
9. 3 MM Whatman filter paper (Fisher Scientific Company, Pittsburgh, PA, USA)

3. Methods

3.1 Doing a tissue print

1. Place several layers of filter paper on a smooth, hard surface, and place a blotting membrane on top. Use a double-edged razor blade to cut a tissue sample (**Fig. 1 B**). (*see Note 1*).
2. Using forceps transfer the tissue section to the membrane. (*see Note 2*). Several successive sections can be printed on the same piece of membrane (**Fig 1 C**).
3. Place a small piece of nonabsorbent paper over the section to protect the membrane from fingerprints (**Fig. 1 D**). (*see Note 3*).
4. Apply the appropriate amount of pressure to the section for the type of print desired. A chemical print requires only light pressure, but a physical print requires several times as much. The proper pressure also varies with the tissue used (**Fig. 1 E**).
5. Gently remove the protective paper and the section with forceps, and air-dry the print with warm air, and observe (**Fig. 1 F**).
6. Prints may be illuminated from the top or from the one side by white or UV light and may also be viewed with transmitted light.

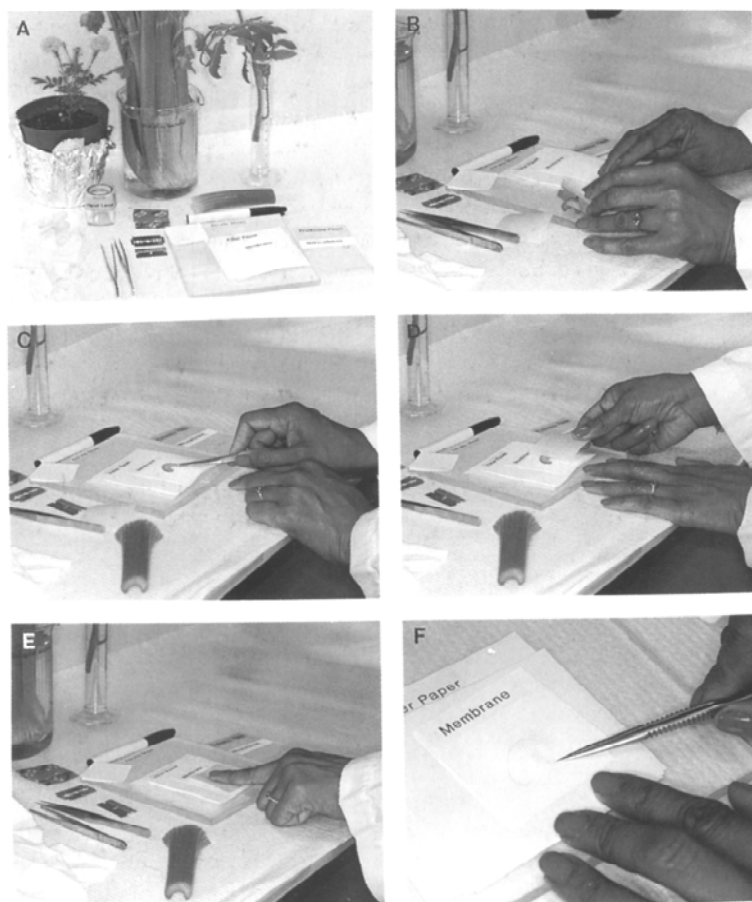


Figure 1

Detailed description of the steps involved in tissue printing. A: materials required; B: cutting the tissue; C: placing the section on the membrane; D: protecting the section and the print; E: applying pressure; F: removing the section. (Reprinted from “Tissue Printing”, R. Taylor, Tissue printing demonstration, p. 6, 1992, (2) with permission from Elsevier).

3.2 Detection of total proteins in a tissue blot

It is useful to have an image stained for total proteins in the blot, which will indicate the pattern of protein distribution in the tissue, as well as some indications on the different tissues present in the section. We have used India ink staining (7) for total protein on nitrocellulose membranes since Coomassie Blue stain gives a strong background. The same tissue slice can be printed several times, showing the tissues with higher amount of proteins (**Fig 2**).

1. Wash the dry nitrocellulose filter 2X with TBST for 5 min with constant shaking.
2. Incubate the NC membrane with India ink staining mix until the image appears clearly (*see Note 4*).
3. Wash the print with water 5 min, and dry it.



Figure 2

Tissue print of a cross section from a celery stalk stained for total proteins with India ink. The same section was printed a first time (upper image) and a second time (lower image). Most of the proteins from the cut cells were transferred on the first print; the second one shows mainly the apoplastic proteins abundant in some tissues.

3.3 Revealing proteins with antibodies

Chemical tissue prints result from the molecules that transfer from the freshly cut cells of a tissue section to the surface of a synthetic membrane, where they are retained and immobilized. The principal steps for tissue printing are: 1) the release of the protein of interest from the tissue; 2) the contact –diffusion transfer of the protein to the

recipient membrane; and 3) the retention and binding of the protein into the synthetic matrix. The protein print is a mirror image of the tissue and can be used to detect and localize specific proteins. Retention and binding of a protein into the recipient matrix depend on the chemistry of the membrane and its ability to interact electrostatically and hydrophobically with the protein of interest. NC membranes have a high binding capacity for proteins and should be tried first for printing a new type of protein molecule. Adding methanol to the transfer buffer increases the capacity and affinity on NC for proteins, presumably by promoting hydrophobic interactions (*see Note 5*).

Methods for detecting the protein of interest bound to the imprinted membranes must be specific and sensitive. Best results are achieved by using antibodies raised against the targeted protein. An enzyme-conjugated secondary antibody raised against the primary antibody is commonly used to visualize the binding between the primary antibody and the antigen. (*see Note 6*). To reduce background from non specific cross reactions, the primary antibody is combined either with 1% (v/v) normal serum from the species in which the secondary antibody was raised or with a low concentration of sodium dodecyl sulfate or Tween-20.

The following procedure was used to localize potato lectin by means of polyclonal antibodies against the native and deglycosylated potato tuber lectin (*5*).

1. Cut a section of the tissue about 1 mm thick with a new razor blade, and gently wipe the surface with a filter paper to absorb excess liquid. Put the freshly cut surface on the membrane, and press for 10-15 seconds. (*see Note 7*).
2. Transfer the printed membrane to TBST, and wash away the unbound material two times for 5 min each in a shaker.
3. Block the unoccupied site of the membrane by shaking the membrane in blocking buffer for 30 min at room temperature.
4. Transfer the membrane to the first antibody solution (anti-lectin serum), and incubate overnight in a refrigerator or for 2 h at room temperature. For controls either use preimmune serum at the same dilution or skip the first antibody step. (*see Note 8*).
5. Wash the membrane in TBST three times for 10 min each.
6. Incubate the prints with the secondary antibody (goat anti-rabbit-alkaline phosphatase in this case) for 2 h at room temperature.
7. Wash the membrane several times with TBST (five washes of 5 min each). (*see Note 9*)
8. Incubate the prints in AP substrates at room temperature until the reaction product is observed; treated and control prints should be incubated under the same conditions. (*see Note 10*).

The result of this tissue printing method is shown in **Fig. 3**.

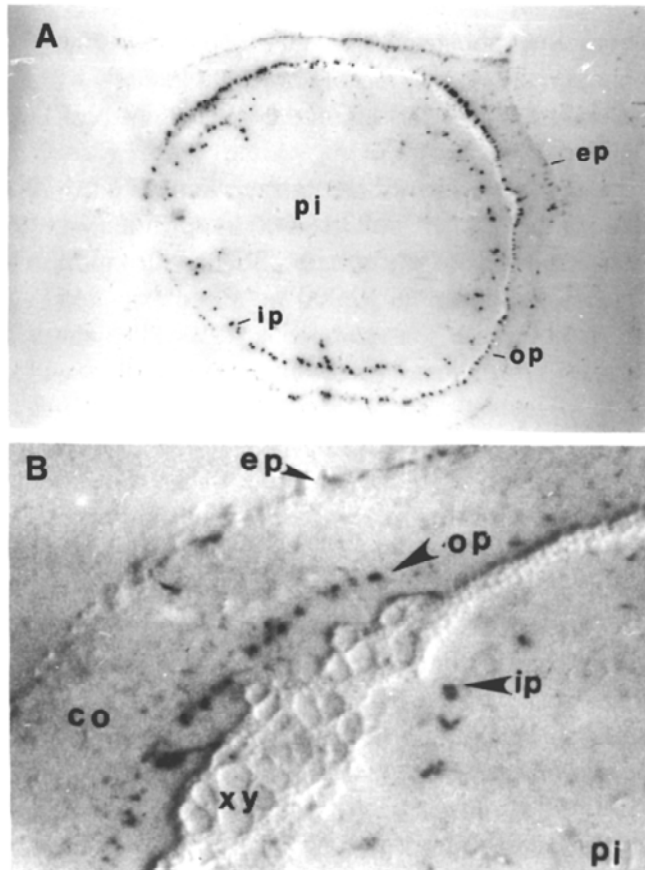


Figure 3

Tissue prints of potato stem incubated with antibodies raised in rabbit against the deglycosylated lectin, followed by goat anti-rabbit AP. A: transverse section. B: magnification of a stem sector with lateral illumination showing greater detail of lectin localization. The lectin is present only in the outer (op) and inner phloem (ip). The stain at the epidermis (ep) is not a positive reaction but a transfer of natural pigments (xy, xylem; co, cortex; pi, pith parenchyma). (Reprinted from "Tissue Printing", R. Pont-Lezica, Lectin and glycans recognition, p. 87, 1992 (54) with permission from

Elsevier).

3.4 Localizing cell wall proteins in plants

The presence of a cell wall is one of the outstanding features distinguishing plant cells from those of animals. The cell wall is not an organelle with relatively constant functions; rather, it is subject to continuous developmental processes that govern cell size, division, shape and function. In addition to the well known polysaccharides present in the cell wall, proteins are important modulators of cell wall structure and function (8). One of the best characterized cell wall proteins is extensin, a basic hydroxyproline rich glycoprotein important for cell wall structure. It is a difficult protein to isolate, because high proportion of it becomes insolubilized in the wall, but it can be extracted with a solution with high salt concentration. A modification

of the tissue print method was developed (6) to transfer cell wall proteins to NC, soaking the membrane previously in 0.2 M CaCl₂, and then printing the tissue.

1. Soak the NC membrane in 0.2 M CaCl₂ for 30 min, and dry on 3-MM Whatman paper.
2. Cut fresh tissue into sections of 0.3-3 mm thick with a new razor blade, previously washed in distilled water for 3 sec and dried on Kimwipes. Then transfer each section to the NC membrane as indicated
3. Block the NC with blocking buffer for 1-3 h at room temperature with constant shaking
4. Add the primary antibody to the desired dilution in blocking buffer, and incubate the membrane for 1-3 h at room temperature with shaking
5. Wash the NC three times for 30 min each in TBS with agitation
6. Soak the NC membrane in secondary antibody (AP-conjugated anti IgG) diluted 1:20,000 in blocking buffer for 1-3 h with agitation.
7. Wash the NC membrane with AP buffer, and add the AP substrate solution. Develop the tissue print until a color signal appears, and stop the reaction by washing the membrane in distilled water

4. Notes

1. Use gloves for all the manipulations to avoid finger prints on the membrane. It may be necessary to gently preblot the section on a separate piece of filter paper before printing to remove excess tissue exudates from cut cells and to ensure an accurate print.
2. To avoid double images be careful when blotting and removing the tissue section from the membrane.
3. When printing a thin section (200-300 μm), place a piece of membrane on top of the section to prevent the nonabsorbent paper from marking the membrane under the section.
4. The incubation time for India ink staining can be very variable, according to the amount of protein present in the tissue. For a seed blot from a legume, 15 min incubation will give a very good image. For other tissues such as stem or petiole, it may be necessary to incubate for several hours or even overnight.
5. In some instances NC membranes do not bind the protein of interest at all or else bind it only weakly. Try next a membrane that can react chemically with the protein, and covalently bind it, such as Immunodyne Immunoaffinity membrane (Pall Corp, Cortland, NY, USA). The type of buffer, salt concentration, and pH of the incubation mixture are important for protein binding and must be determined empirically.
6. Detection of the alkaline phosphatase-conjugated second antibody on tissue prints from plant organs was selected over the peroxidase-conjugated second antibody procedure

because the substrates used for detecting the peroxidase, such as *O*-phenylenediamine and hydrogen peroxide will react with endogenous plant peroxidase activity in the tissue sections (9), making the immunoblotting reaction.

7. The same tissue surface can be reprinted several times; the successive images will be weaker, but a good imprint can be found among these when the proteins are very abundant in a particular tissue.
8. To be sure that the antibody used for tissue printing is specific for the targeted protein, it is important to test it against a total protein extract of the tissue previously by SDS-PAGE, followed by a Western blot. Only the targeted protein should be stained, and use the highest dilution to obtain the best results.
9. The last wash is made with TBS without detergent to avoid interference with the alkaline phosphatase reaction.
10. Occasionally some tissues show cross reaction with the goat serum. To avoid such a reaction, two procedures are available: 1) block the prints with blocking buffer containing 1:3000 dilution of normal goat serum, or 2) use a secondary antibody raised in a different animal.

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