NONRADIOACTIVE *IN SITU* HYBRIDIZATION ON CRYOSECTIONS

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SLIDE PREPARATION

Wear Gloves! Wear Gloves! Wear Gloves! Wear Gloves! Wear Gloves!

As your hands are a good source of RNase, avoid touching any of the slides or slide boats with bare hands throughout all steps.

Use slide boats and dishes that are designated for *in situs.*

Acid clean the slides.

Be sure to rinse in tap water and running distilled water. Avoid any contamination with general labware.

Subbing

DEPC water is defined as double distilled water mixed vigorously with 0.1% Diethylpyrocarbonate (DEPC) for 2 hours and then autoclaved.

Heat 500 ml. of DEPC water to 50°C. Do not let it boil! Add 5g of gelatin while mixing the water with a stir bar.

Place the flask in a bucket with ice and cool to about 40°C. Add 0.5g of chromium potassium sulfate (chrome alum). Use warm. Avoid creating bubbles when dipping the slides in the subbing solution. Cover and let dry overnight.

Poly-L-lysine Coating

(Remember you are wearing gloves!!)

Dissolve 0.60g of Tris base in 500 mL of DEPC water (to make 10 mM) and adjust the pH of the buffer to 8.0.

Dissolve 25 mg of poly-L-lysine in 500 mL of the 10 mM Tris buffer.

Dip the subbed slides in the poly-L-lysine solution for 10 - 30 minutes. Let them dry overnight in a 37-50°C oven.

When the slides are dry store them in the slide boxes dedicated for *in situ* hybridization.

HYBRIDIZATION PRE-TREATMENT

(The solutions are made according to protocols found in the Genius System User's Guide for Membrane Hybridization, Version 3.0 from Boehringer Mannheim)

All reagents should be stored at 4°C or -20°C.

All glassware and labware is oven baked overnight at 210°C or autoclaved.

Sections are cut on a disposable knife and handled with brush dedicated for *in situ* and gloves are worn. After sectioning the slides are placed in a vacuum desiccator for a minimum of 2 hours or overnight. The slides are then stored with desiccant in the -90°C freezer. When slides are removed for use, care is taken to avoid warming the remaining slides thus preventing the condensation of moisture on them.

Remove formamide aliquot from freezer.

20X Standard Sodium Citrate (SSC)

- Dissolve 87.6g of NaCl in 350 mL of double distilled water.
- Add 44.12g Sodium citrate

Bring to a final volume of 500 mL with double distilled water.

Hydrate the slides in ethanol series, 100% 2X, 1X each 95%, 70%, *50%*, and finally 2X SSC, 1 minute each.

Proteinase K Buffer: 0.1 M Tris pH 8.0 50 mM EDTA

- 25 mL 1M Tris
- 25 mL 0.5M EDTA (pH 8.0)

Bring solution up to 250 mL with DEPC water

Prewarm 250 mL Proteinase K and buffer to 37°C. Add 250 µL of Proteinase K [10 mg/mL] to the buffer just before use.

Incubate the slides in the Proteinase K solution. This is tissue and probe dependent. A time course series should be done to determine optimal signal localization. Too short, detection is sterically inhibited, too long, signal is lost. 5 minutes for 10 µm sections is the maximum.

Rinse briefly in room temperature (RT) DEPC water.

Triethanolamine (TEA) should to be made fresh every time.

- 9.3g in 490 mL DEPC water and pH to 8.0 using NaOH pellets.

Rinse in 0.1 M TEA, pH 8.0, for 3 minutes. Add 650 µL Acetic anhydride to a dry dish and pour in the remaining 250 mL TEA. Incubate the slides in this solution for 10 minutes.

Dehydrate the slides in the reverse order of the steps used in hydration. Drain the slides and let them dry at RT for at least 1 hour.

HYBRIDIZATION

Boil the probe for 10 minutes and then quickly cool on ice; add hybridization solution components to the tube containing the probe.

Tris/EDTA/NaCl (TEN) Buffer:

- 5 mL 1.0M Tris-HCl, pH 7.5
- 30 mL 5M NaCl
- 1 mL 0.5M EDTA

Hybridization Solution:

- 36 µL TEN buffer
- 250 µL 100% D.I. Formamide
- 100 µL 50% Dextran Sulfate (50% in DEPC water)
- 50 µL 10% Blocking soln.
- 2 µg Digoxigenin (DIG) RNA probe (=X µL)
- 64 µL - X µL (vol. of probe) DEPC water

Place enough probe solution on the coverslip to completely cover the sections (approximately 60 µL). Gently lower the slide to the RNase free and siliconized coverslip.

Seal the edges with Permount and incubate in humid chamber at 56°C overnight.

Alternatively: Grace Bio-Labs (Sunriver OR, gracebio@teleport.com) manufactures RNase free siliconized HybridSlips. We use the 22 x 60 mm size (cat. #HS60) with 75 µL of hybridization solution. The coverslips are not sealed and the hybridization is done in a humid chamber.

IN SITU POST HYBRIDIZATION

Formamide solution:

- *50%* molecular grade formamide in 2X SSC.

Do not discard this wash. It is reusable. Preheat the formamide wash to 65°C.

RNase Buffer: 0.5M NaCl, 10 mM Tris, pH 7.5,1 mM EDTA

- 29.23g NaCl
- l0 ml 1M Tris pH 7.5
- 2 mL 0.5M EDTA

Bring up to 1L with DEPC water

Preheat two RNase buffer washes without the RNase, one 200 mL to 37°C, and the second to 65°C.

Pre heat two 2X SSC washes to 37°C

The following washes are at room temperature on the shaker table:

Remove the Permount and soak the slides in 2X SSC for 30 minutes. If the coverslips have not already floated off, leave the slides in the buffer and gently tease the coverslip off.

If the hybridization was done using the HybridSlips, gently slide the coverslips off and wash the slides in 2X SSC for 30 minutes then continue with the 65°C formamide wash.

Wash an additional 30 minutes in fresh 2X SSC.

Rinse in the preheated formamide solution for 1/2 hour. Agitate slightly for the first *5* minutes.

Rinse in 2X SSC at 37°C, two times 10 minutes each.

Add 400 µL RNase A [10 mg/mL] to the 200 mL 37°C RNase buffer and incubate the slides in the solution for 30 minutes.

Wash the slides in the 65°C RNase buffer (without RNase) for 30 minutes.

Immunohistochemical Detection

Wash 2-3 hours in 2X SSC + 0.05% Triton X-100 + 2% Blocking reagent.

5X Maleate Buffer:

- 58g Maleic acid in 850 mL double distilled water. pH to 7.5 using lots of

NaOH pellets.

- Add 43.8g NaCl

Bring up to 1L with double distilled water (dilute to 1X for working concentration).

Wash in Maleate buffer 2X 5 minutes each.

Carefully dry a ring around the sections without allowing the sections to dry and draw around them with a PAT pen.

Incubate slides in humid chamber 3-5 hours or overnight at room temperature with approximately 200-500 µL DIG-Alkaline Phosphatase (AP) antibody, diluted 1:1000 - 1:3000 in Maleate buffer + 1% Blocking reagent + 0.3% Triton. (DIG-AP Ab. is stored in the dark at 4°C.)

Alternatively: Grace Bio-Labs supplies water tight chamber slips, CoverWell, 200 µL vol. (cat. #PC200). The slide is lowered onto the CoverWell containing 205 µL antisera.

Wash in Maleate buffer 2X 10 minutes each.

Wash in Genius kit 3 buffer 3 1X 10 minutes.

Buffer 3:

- Mix 100 mL of 10X Tris/NaCl stock in 800 mL double distilled water. Check the pH at this time, it should be around 9.5.
- While mixing add 100 mL of the 10X $MgCl_2$ stock

Do not adjust the pH at this point

10X Tris/NaCl stock: 1M Tris HCl + 1M NaCl

- In 350 mL double distilled water add: 78.5g TrisHCl, let dissolve then add:
- 29g NaCl. Use lots of NaOH pellets to pH to 9.5, bring to vol. 500 mL, sterile filter using bottle top filter on a very clean bottle.

10X $MgCl_2$ Stock:

- Dissolve 50.8g $MgCl_2$ in 420 mL of double distilled water. Bring up to 500 mL and sterile filter into a very clean bottle.

Incubate the slides in freshly prepared chromogen substrate prepared in buffer 3. Plate 200-500 µL per slide.

Grace Bio-Labs CoverWells can be used for chromogen substrate incubation. After placing the CoverWell on the slide, place them upside down in the humid chamber. If any precipitate should form during the color reaction it will fall on the CoverWell and not on the section.

Chromogen Substrate:

- 45 µL NBT-solution (Genius kit 3, vial 4)
- 35 µL X-phosphate soln (Genius kit 3, vial 5)
- 10 mL buffer 3.

or

- NBT/BCIP ready-to-use tablets from Boehringer Mannheim catalog # 1697-471

Incubations are dependant on the strength of the signal. Increasing the temperature to 37°C only accelerates the reaction. It will not intensify it, and the nonspecific background becomes a problem. Time course for the incubations can run from a couple hours to overnight. Be sure to use a very humid chamber to prevent the slides from drying.

Alkaline Phosphatase Substrate Wash

- Dissolve 1.75g Tris-HCl in 950 mL double distilled water. pH to 7.5, bring up to 1 L.

After color reaction is complete, wash the slides in the Alkaline phosphatase substrate for at least 30 minutes. Sections are mounted under coverslips with glycerol. Seal the edges of the coverslips with Permount.

Things to keep in mind:

The work space must be clean. All glassware is baked at 210°C for at least 24 hours and plasticware is DEPC treated and autoclaved prior to use. After the initial DEPC treatment only autoclave the plasticware. Periodically DEPC treat the plasticware again, especially when there is a problem with the in situs. Gloves are worn at all times during prehybridization steps.

Proteinase K digestion may vary with probes and tissue. It may be wise to do a series of time points to determine the optimal length of digestion. 30 seconds to 5 minutes would be a good start.

Signal detection is dependant on the concentration of probe in the hybridization solution. For most purposes 4 ng/µL should work when 60 µL is plated on the slide, (2 µg probe/500 µL hybridization soln.). Some probes used have given strong signals at 1 ng/µL, others can only be detected at a concentration of at least 4 ng/µL.

The best way to store the probe is to aliquot it into working concentrations in Rnase free and siliconized tubes, and keep frozen at -20°C.

Leftover hybridization solution that contains the probe can be stored at -20°C and used again within 2 weeks or more. It must be boiled again before use.

If you get negative results, first try repeating the immunocytochemistry. Be sure that all your buffers are at the correct pH. The alkaline phosphatase reaction is especially sensitive to the pH of the buffer. ■

References:

L.K. Barthel & P.A. Raymond, (1993), J. Neurosci Methods, 50:145-152

P.A. Raymond *et al.*, (1993) Neuron, 10:1161-1174

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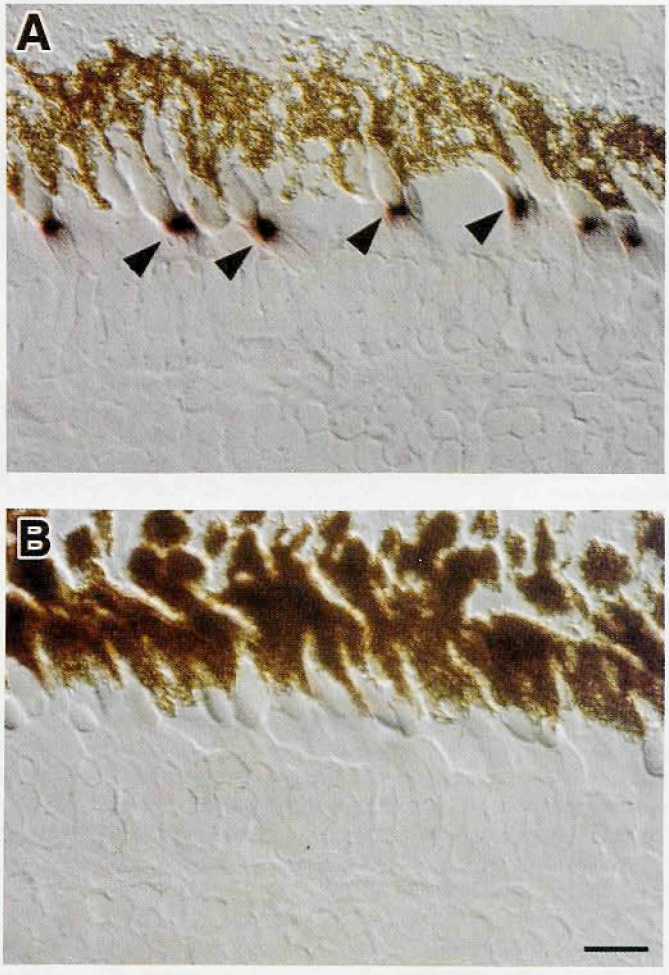

Figure 1: Colorimetric *in situ* hybridization with goldfish green cone opsin riboprobe on goldfish retinal cryosections. RNA probes were labeled with digoxigenin and visualized with an alkaline phosphatase color substrate (NBT/BCIP). A) The goldfish green opsin probe hybridizes to a subset of cone photoreceptors in the goldfish retina. The signal is localized to the myoid (arrowheads) where the mRNA is concentrated. The pigmented epithelium adjacent to the photoreceptors contains brown melanin pigment. B) Sense negative control probe does not label the retina. Bar = 10 µm