^{35}S Paraffin Section *In Situ (can be on frozen sections too!)*

Day 1.

1) Dewax slides in xylene or toluene, 5 min X 3
2) Rehydrate tissue by quickly putting slides through 100%-100%-95%-70%-50% EtOH.
3) Remove ethanol: 0.15M NaCl (saline), 5 min
   1 X PBS, 5 min (keep these aside)

HERE IS WHERE YOU WOULD START IF YOU HAVE FROZEN SECTIONS

4) Fix in 4% PFA/PBS, 20 min (keep bath)
5) Wash 2 X 5 min with PBS
6) Incubate in 20 µg/ml Proteinase K in TE (50mM Tris•HCl, 5mM EDTA, DEPC water) for 10 min
7) Wash 1 X 5 min with PBS
8) Fix again in 4% PFA/PBS, 5 min
9) Wash 1 X 3 min with PBS
10) During step 10, prepare TEA solution:
    in 500 ml DEPC H_20
    9.3 g triethanolamine
    1.12 ml 10N NaOH
    Quickly add 1.25 ml acetic anhydride (per 500 ml), incubate slides for 5 min, while stirring. Add another 1.25 ml acetic anhydride and incubate another 5 min.
11) Wash 1 X 5 min with PBS
    Wash 1 X 5 min with saline (150 mM)
12) Dehydrate slides by quickly putting slides through 50-70-85-95-100-100% EtOH, but at 70% wait 5 min.
13) Air dry, use the same day for hybridization.

RNA Transcription (Stratagene Kit).

I USUALLY DO THIS THE DAY BEFORE SO THAT I KNOW I HAVE A USEABLE PROBE BEFORE STARTING THE PROCEDURE

First, you must start with 0.5 µg/µl linearized DNA and know which direction to use for probe synthesis. For one half reaction, on ice:

1)

DNA, 0.5 µg	0.5 µl
5 X buffer	2.0 µl
10 mM ATP	0.5 µl

10 mM CTP	0.5 µl
10 mM GTP	0.5 µl
0.1 M DTT	0.5 µl
RNAsin	0.5 µl
35S-UTP	5.0 µl
T3/T7/Sp6 poly	0.5 µl = 5 units

37°C, 1 hour

2) To each reaction, add

DNase	0.5 µl
10 mg/ml yeast tRNA	0.5 µl
RNAsin	0.25 µl

37°C, 10 min.

3) Add

DEPC H_20	30 µl
3M NaOAc	5 µl
1M DTT	0.5 µl

4) The total volume should now be ~50 µl. Now do a 1:1 phenol/chloroform extraction (50 µl). Transfer top layer to new tube. Precipitate RNA with 1 µl yeast tRNA, 25 µl NH_4Ac, 125 µl EtOH. Place on dry ice, 5 min. Spin 5 min. Resuspend pellet in 25 µl 10 mM DTT.

5) Radioactivity Count. 1 µl RNA probe + 5 ml scintillation fluid (Brown lab). Good counts are 5.0×10^5 - 2.0×10^6 cpm/µl. Calculate probe dilution in hyb buffer:

(cpm/µl count)(# µl RNA probe)/(desired probe count)

Desired probe count is between 1-2 x 10^4 cpm/µl.

Hybridization.

50 ml Hyb solution:

5M NaCl (DEPC)	3 ml
1M Tris•Cl (pH 8.0)	1 ml
0.5M EDTA (DEPC)	0.5 ml
1M $NaPO_4$, (pH 8.0, DEPC)	0.5 ml
50% Dextran sulfate	10 ml (start early to dissolve)
50X Denhardt's	1 ml
Yeast tRNA (from tubes)	2.5 ml
1M DTT	0.5 ml
Formamide	25 ml
DEPC H2O	6 ml

Add DTT last.

1) Denature the probe @ 80°C for 2 min, spin. Dilute in hybridization solution as previously described.
2) Put the probe/hyb directly on the dry slides, place coverslip on each. Place slides face up in sealed, humidifed (50% formamide, 5 X SSC, DEPC H_2O) slide boxes.
3) Incubate O/N at 55°C.

Day 2. Washing.

1) Fill 65°C and 37°C water baths ahead of time. Prepare 1L per 40 slides 50% formamide, 2 X SSC, 0.1M DTT.
2) Remove coverslips with 5 X SSC, 10mM DTT.
3) Transfer slides to 50% formamide, 2 X SSC, 0.1M DTT, 30 min, 65°C.
4) Transfer slides to NTE bath, 37°C, 15 min.
   NTE: 0.5M NaCl
   10 mM Tris•Cl
   5 mM EDTA
5) Transfer slides to an NTE bath containing 20μg/ml RNase A for 30 min. Keep glassware in contact with RNase A separate.
6) Wash again in NTE, 15 min, 37°C.
7) Repeat Step 3, 20 min, 65°C.
8) Wash in 2 X SSC, 10 min, 37°C.
9) Wash in 0.1 X SSC, 10 min, 37°C.
10) Rapidly dehydrate in EtOH containing 0.3M NH_4Ac:
    30% EtOH
    60% EtOH + NH_4Ac
    80% EtOH + NH_4Ac
    90% EtOH + NH_4Ac
    100% EtOH

11) Air Dry.

At this step you can expose to film overnight, but signal is generally weak. I usually proceed to dipping the slides in emulsion.

DIPPING SLIDES IN EMULSION:

EMULSION: We use Kodak Autoradiography Emulsion NTB-2. You can buy it from VWR Scientific. The catalog# is IB1654433

DO NOT EXPOSE THE EMULSION TO LIGHT AT ANY POINT!!! ALL THE FOLLOWING STEPS ARE IN THE DARK OR UNDER A DARKROOM RED SAFETYLIGHT.

Warm up emulsion to 48°C.
Dip slides into emulsion. (It is expensive, so we use a slide holder that holds one slide and we dip them individually. You could also use a 50mL conical tube, fill it w/ emulsion and dip the slides individually to coat them. Any emulsion left can be wrapped in foil and stored at 4°C.

Place slides into slide holder vertically and let dry for at **least 3 hours.**

Once dry, place slides into slide box with some dessicant (we use dryrite) and let develop for **7-10 days** (It's a long procedure!). For strongly expressed genes 7 days is sufficient, for weakly expressed genes try 10 days. If you don't know, start w/ 10 days just to be safe.

Next develop slides!