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In situ hybridization of yeast cells (RNA and Oligonucleotide probes)

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I. General considerations:

- In situ hybridization of yeast cells is almost identical to mammalian cells, except that the cell wall has to be removed by spheroplasting the cells prior to hybridization. The following protocol is adapted from Long RM, et al. *RNA* 1995 Dec;**1(10)**:1787-1794.

II. Fixation:

- Yeast are grown in 45ml cultures in the appropriate media until they reach early log phase (OD600 between 0.2 and 0.4). Cells are fixed for 10 minutes at room temperature by directly adding to the medium 10ml of 20% formaldehyde, 50% acetic acid. The fixative is removed by three rounds of centrifugation (5 minutes at 3500rpm and at 4C), and resuspension in 10ml of ice-cold buffer B (1.2M sorbitol, 0.1M potassium phosphate, pH 7.5).

  NB: As in mammalian cells, acetic acid improves detection only of nuclear RNA. For oligo probes, it is not necessary, and cells can also be fixed by adding 5ml of 40% formaldehyde to the culture medium, and incubating 40 minutes at room temperature.

III. Spheroplasting:

- Cells are resuspended in 1ml of buffer B containing 20mM Vanadyl-ribonucleoside complex (Gibco-BRL cat#15522-014), 28mM ß-mercaptoethanol, 0.06mg/ml Phenylmethylsulfonyl Fluoride, and transfered to a tube containing 0.1mg of dried oxalyticase. Spheroplasting is done by incubating the cells for 8 minutes at 30C. Cells are then centrifuged 2 minutes at 3500 rpm at 4C, and washed once in ice-cold buffer B. Cells are further resuspended in 650 µl of buffer B, and 100 µl is added to poly-lysine coated coverslips (this is most easily performed in 6 well tissue culture plates, one coverslip per well). Cells are left to adhere to the coverslip by incubating them 30 minutes at 4C. 3ml of buffer B is then carefully added to each well, removed by suction, and replaced by 5ml of 70% ethanol, which is incubated at least overnight at -20C. At this stage, the coverslips can be stored for weeks at -20°C.

IV. Hybridization:

- Cells are rehydrated for 5 minutes at room temperature, in 2x SSC (300 mM NaCl, 30 mM sodium citrate, pH 7.0), 50% formamide. Cells are hybridized overnight at 37C in 40 µl of a mixture containing 10% dextran sulfate, 2 mM vanadyl-ribonucleoside complex, 0.02% RNAse-free BSA, 40 µg *E.coli* tRNA, 2x SSC, 50% formamide, 30 ng of probe.

  NB: The amount of probe can be diminished 5 times without much loss in signal in case background in the cells is high. Often addition of a nonsense probe (N-50, where N is all nucleotides) significantly reduces background.

- For detection of poly(A) RNA using polydT(50), 15% formamide used at 37C. Only 5ng of probe is necessary per sample; hybridization times routinely take an hour, due to the low complexity of the reactions.

V. Washing:

- Cells are washed twice for 30 min at the appropriate stringency: 2x SSC, 50% formamide, 37C for oligonucleotide probes; 0.1x SSC, 50% formamide, 50C for RNA probes.

  NB: Washing conditions may be adjusted for each probe, and can be optimized by Northern blotting. Probes that are labelled to a high specific activity (see below) tend to give higher background, but addition of 0.1% NP40 or 0.1% SDS in the washing buffer can diminish this background.

VI. RNAse treatment (optional):

- RNase treatment can remove background binding of the probes. It is however better to avoid it when possible, since it leads to loss of RNA from the cells. In most cases, it is possible to remove the background by adjusting the stringency of washing and the amount of probe in the hybridization mixture.

VII. Antibody detection (optional):

- Digoxigenin-labeled probes can be detected with sheep anti-digoxigenin antibodies (1/200, Boehringer Mannheim), and then with donkey anti-sheep antibodies conjugated to fluorescein (1/150, Sigma). Slides are incubated for 1 hour at 37C in 2x SSC, 8% formamide, 2 mM vanadyl-ribonucleoside complex, 0.2% RNAse-free BSA, and washed twice for 15 min in 2x SSC, 8% formamide at room temperature.

  NB: Fluorescent antibodies tend to give a high background, which is efficiently removed by the presence of 8% formamide in the incubation buffer.

VIII. Mounting:

- Slides are washed in 1X PBS, and mounted in 90% glycerol, PBS, 1 mg/ml p-phenylendiamine, 0.1 µg/ml DAPI.