A General Protocol for Yeast *in situ* Hybridization

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[Abstract] This protocol describes the procedure to label selected mRNA species in yeast cells with fluorescence probes and prepare slides for visualization under a microscope. Probe design/synthesis and microscopy operation are not covered in this protocol.

Materials and Reagents

1. HCl
2. Poly L-lysine
3. Formaldehyde
4. β-mercaptoethanol
5. Oxalyticase
6. SDS
7. 70% ethanol
8. Formamide
9. 20x SSC
10. NaH_2PO_4 buffer
11. Triton X-100
12. 1x PBS-DEPC
13. DAPI
14. 40% formamide/2x SSC (see Recipes)
15. 80% formamide (see Recipes)
16. Hybridization solution (see Recipes)

Equipment

1. Incubator
2. Centrifuges
3. Glass beaker
4. Autoclaved glass cylinder
5. Baked glass pipette

6. Hotplate
7. Fume hood
8. Petri dish
9. Foil
10. 50 ml conical tubes
11. Parafilm
12. Light microscope

Procedure

Duration: DAY 2: ~3-4 h, DAY 3:~2-3 h after OD reached, DAY 4: ~1-2 h, DAY 5: ~1-2 h.

DAY 0: Prepatation of coverslips

1. Add 500 ml sterile distilled water to a 1 L glass beaker (measured in an autoclaved glass cylinder).
2. Add 5 ml 12 N HCl with a baked glass pipette.
3. Add coverslips to solution one at a time. Try not stick several coverslips together.
4. Boil for 30 min on hotplate in fume hood.
5. Wash 10 times with sterile distilled water, swirling.
6. Add 300 ml sterile distilled water. Autoclave. Store at 4 °C (good for up to a month).
7. Coat coverslips with poly L-lysine (which keeps cells at the surface of coverslip)
    a. Use 1 petri dish for each strain used in the *in situ* hybridization.
    b. Pull out a few coverslips from the autoclaved beaker into one dish and pick them from there.
    c. Place 6 coverslips in each petri dish. (6 coverslips per strain).
    d. Add 200 µl 0.01% poly L-lysine/coverslip; wait ~ 2 min.
    e. Aspirate off solution leaving a very thin film of solution.
    f. Allow to air dry (~ 2-3 h).
    g. Wash three times with sterile distilled water (not autoclaved), 30 min per wash.
    h. Transfer coverslips to clean dry dish. Stand on edge to dry overnight.

DAY 1: Grow a 5 ml culture in appropriate media O/N on wheel at 30 °C.

DAY 2: In the morning, dilute cultures 1:10 and let them grow.

Check OD_{600} in the afternoon and dilute the culture acco rdingly into 50 ml of media, so that OD_{600} in the morning = 0.3-0.5 incubate cultures in the shaker at 30 °C O/N.

DAY 3: Fixation of yeast cells (Note: KEEP CELLS ON ICE!)

1. Check that yeast grown O/N is at OD_{600} of 0.2-0.5.
2. Based on OD_{600} place "X" volume of cells in 50 ml conical tubes and immediately add the volume of 32% formaldehyde required to give final concentration 3.2%. Mix by inversion.
3. Turn centrifuge on and cool to 4 °C. Prepare balance tubes.
4. Incubate at RT for 45 min (mix by inversion every 5-10 min).
5. Harvest cells (spin at 3,700 rpm/5 min/4 °C).
6. Pour off supernatant (formaldehyde waste).
7. Resuspend cells in 5-8 ml ice cold buffer B (keep tubes on ice).
8. Respin. Pour off supernatant.
9. Repeat wash with buffer B twice (total = 3 washes).
10. Last wash: label oxalyticase tubes.
11. Spheroplasting the cells:
    a. Resuspend cell pellet in 1 ml buffer B+2 µl β-mercaptoethanol (operate in hood).
    b. Transfer resuspended cells to Eppendorf tube containing 0.1 mg oxalyticase.
    c. Keep on ice until all cells transferred.
    d. Incubate at 30 °C for 8 min. Mix by inversion every 2 min.
12. Spin at 3,500 rpm/4 min/4 °C (keep tubes on ice until the end).
13. Label plates.
14. Aspirate off supernatant and wash cells with 1 ml ice cold buffer B.
15. Respin at 3,500 rpm/5 min/4 °C. Remove supernatant.
16. Resuspend cells in 700 µl ice cold buffer B.
17. Spot 100µl of cells per poly-L-lysine coated coverslip in one petri-dish.
18. Incubate at 4 °C for 30 min.
19. Wash coverslips with 25-30 ml ice cold buffer B (2 plates out of fridge at a time).
20. Check for spheroplast--mix 20 µl 1% SDS + 2 µl cells on slide and check under a light microscope.
21. Aspirate off Buffer B and add 25-30 ml 70% ethanol/-20 °C.
22. Store coverslips in 70% ethanol/-20 °C until ready for *in situ* (usually good for a few weeks or more if ethanol does not evaporate off).

DAY 4: *In situ* hybridization (Note: Wear Gloves)

Preparation of probes:

1. Dry down probe in one tube per coverslip (*note: In general, hybridize 2 coverslips of each strain desired and protect from light!*). Wrap in foil, use bench drawer.
2. For each coverslip, add in one eppendorf tube (a master mix can be done, followed by aliquoting 14 µl/tube):

a. 10 µl fluorescently labeled probe (1 ng/µl stock) (ex: ASH_1 or lacZ)+
b. 4 µl SSSDNA combined with tRNA (5 mg/ml stock)

3. Place open tubes in speed vac and dry probe for 30 min (cover centrifuge with foil).
4. Store at -20 °C until ready to use (may be stored O/N).

Hybridization:

1. Prepare the following reagents from stock solution (coplin jars hold 10 ml/4 coverslips each): 40% formamide/2x SSC, 80% formamide, hybridization solution. They need to be prepared fresh, and discarded after one use.
2. Pour 10 ml 2x SSC (DEPC treated) into each coplin jar.
3. Remove covers lips from ethanol in petri dishes using tweezers. Blot edge of coverslip on kimwipe.
4. Place 4 coverslips in each coplin jar (note which side has cells).
5. Incubate coverslips in coplin jars at room temperature for 3-5 min.
6. Pour off 2x SSC by placing tweezers over coverslips and inverting coplin jars.
7. Incubate coverslips in 40% formamide/2x SSC at RT for 3-5 min.
8. Prepare a glass plate covered with parafilm.
9. Dissolve probe in 12 µl 80% formamide/NaH_2PO_4 buffer.
10. Heat at 90 °C for a few minutes. Protect from light and do not vortex.
11. Add 12 µl hybridization solutions to probe. Mix (stir) with a pipette tip.
12. Spot 21 µl onto glass plate covered with parafilm.
13. Remove coverslips from 40% formamide/2x SSC solution. Dab edge of coverslip on kimwipe.
14. Let coverslips fall face down (where cells are!!!) onto hybridization solution.
    Note: Start from one edge and let fall slowly, practice to avoid bubbles. Do not move coverslips from now on.
15. After all coverslips are on parafilm, place second piece of parafilm over coverslips and seal the edges. Wrap in aluminum foil.
16. Incubate at 37 °C for at least 3 h, preferably over night.

DAY 5: Washing and mounting

Note: For the following steps, wrap coplin jars in aluminum foil to protect from light. Prepare 2x the amount of reagents (2 washes).

1. Pre-warm 40% formamide solution at 37 °C for 30 min before washing.
2. Place coverslips in coplin jar by pulling parafilm with tweezers.
3. Wash coverslips in 40% formamide/2x SSC for 15 min at 37 °C.
4. Repeat #3.

5. Wash coverslip in 2x SSC /0.1% Triton X-100, 15 min at RT.
6. Prepare 10 ml 1x PBS-DEPC + 1 µl DAPI per jar.
7. Wash coverslips in 1x SSC for 15 min at RT.
8. Repeat #7, prepare slides (labeling).
9. Wash coverslips in 1x PBS/DAPI.
10. To mount coverslips on slides, take coverslips out of coplin jars and place face up on kimwipe to blot (mount only 4 at a time and keep the rest protected from light).
11. Spot 12 µl mounting media onto each slide and return media immediately to -20 °C.
12. Place coverslips face down on mounting media. Blot twice gently with a kimwipe to remove excess mounting media.
13. Seal all edges with clear fingernail polish. Store slides at -20 °C.

Notes

1. Wear gloves all times!!!
2. Use plastic pipetes.
3. Use glass cylinders.

Recipes

1. 40% formamide/2x SSC (pour into an autoclaved glass cylinder)

#of jars	2	3	4	5	6	7	8
Formamide	8 ml	12 ml	16 ml	20 ml	24 ml	28 ml	32 ml
20x SSC	2 ml	3 ml	4 ml	5 ml	6 ml	7 ml	8 ml
DEPC-H_2O	10 ml	15 ml	20 ml	25 ml	30 ml	35 ml	40 ml

2. 80% formamide (12 µl for each tube of probe)

# of coverslips	8	10	12	24
Formamide	80 µl	100 µl	120 µl	240 µl
1 M $NaHPO_4$ (pH 7.0)	1 µl	1.25 µl	1.5 µl	3 µl
DEPC-H_2O	19 µl	23.75 µl	28.5 µl	57 µl

3. Hybridization solution

# of coverslips	8	10	12	24
DEPC treated water	60 µl	75 µl	90 µl	180 µl
20x SSC (DEPC)	20 µl	25 µl	30 µl	60 µl
BSA(20 mg/ml)	20 µl	25 µl	30 µl	60 µl

References

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