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easyIRF preparation of cell monolayers for in resin fluorescence using progressive lowering of temperature

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We use this protocol and it's working

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Disclaimer

Abstract

This easyIRF protocol details the preparation of cell monolayers for in resin fluorescence using progressive lowering of temperature.

Guidelines

Health & Safety requirements:
Ensure you are familiar with all relevant local Health and Safety documents and procedures (Material Safety Data Sheets, Procedural Risk Assessments, Standard Operating Procedures etc.) before starting.

Procedural Risk Assessments: Handling of formaldehyde fixative, benzoin methyl ether, LR White resin, liquid nitrogen.

Abbreviations:
- IRF: In Resin Fluorescence
- PLT: Progressive Lowering of Temperature
- RT: Room Temperature
- AFS: Automated Freeze Substitution

Materials

Materials

1. Incubator set to 37ºC, 5% CO_2
2. Automated Freeze Substitution unit
3. Fridge at 4ºC
4. Vortex (for mixing solutions)
5. pH meter
6. Gilson P10ML pipette and tips
7. Gilson P1000, 1 ml pipette and tips
8. Plastic Transfer Pipette 3ml **SLS Catalog #**PIP4206
9. 15 mL centrifuge tubes, polypropylene **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**CLS430791
10. 50 mL PP centrifuge tubes, Polypropylene tube **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**CLS430921
11. Magnetic stirrer plate
12. Magnetic stirring bars (for stirring buffers)
13. Measuring cylinders (for HEPES buffer prep)
14. 500 ml and 100 ml glass beakers (for HEPES buffer prep)
15. Nalgene™ Rapid-Flow™ Sterile Disposable Filter Units with Nylon Membrane, 500mL, 0.2μm pore **Thermo Fisher Catalog #**151-4020
16. 20 ml syringes (for prep of small volumes of HEPES buffer)
17. 22 µm filter (for prep of small volumes of HEPES buffer)
18. 15 ml glass scintillation vials with caps (for making up acetone & LR White)
19. Parafilm **agar scientific Catalog #**AGG3398
20. Dumont Tweezers 7 **agar scientific Catalog #**AGT5039
21. Aluminium Dishes **agar scientific Catalog #** AGG3912
22. **Optional:** DISPOSABLE WEIGHING DISH-144 **TAAB Catalog #**W083
23. **Optional:** 35 mm TC-treated Culture Dish **Corning Catalog #**430165
24. Aclar Film **agar scientific Catalog #**AGL4458

Chemicals

1. ddH_2O; double distilled water
2. Liquid Nitrogen
3. HEPES **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**H3375
4. Sodium hydroxide pellets
5. 16% Paraformaldehyde **Electron Microscopy Sciences Catalog #**15710

6. Acetone (>=99.8%) Fisher Scientific 10252232
7. Benzoin methyl ether
8. LR White Resin **agar scientific Catalog #**AGR1280

Reagents

1. HEPES buffer 0.8 M pH 7.4
2. HEPES buffer 0.2 M pH 7.4
3. Double strength fix: 8% formaldehyde in 0.4 M HEPES buffer – made fresh
4. Single strength fix: 4% formaldehyde in 0.2 M HEPES buffer – made fresh
5. Acetone dehydration series: 50%, 70%, 90%, 100% (dry) acetone
6. LR White infiltration series: 25%, 50%, 75%, 100% – made fresh

HEPES buffer

Stock solution: 0.8 M HEPES buffer (pH 7.4)

1. Dissolve 9.54 g of HEPES powder (>99.5%; Sigma-Aldrich H3375) in 40 ml of ddH_2O
2. Adjust the pH to 7.4 with sodium hydroxide (NaOH)
3. Top up to 50 ml with ddH_2O
4. Filter sterilise and store at 4ºC

Working solution: 0.2 M HEPES buffer (pH 7.4)

1. Add 12.5 ml of 0.8 M HEPES to 37.5 ml of ddH_2O
2. Store at 4ºC

Double strength fix, make fresh Safety critical

Working solution: 8% EM grade formaldehyde in ~0.4 M HEPES buffer pH 7.4

1. Add 16% EM grade formaldehyde to 0.8 M HEPES stock solution, 1:1 (v:v).

Formaldehyde	**State:** Liquid	**Hazard Codes:** H226, H301, H311, H314, H317, H330, H335, H341, H350, H370	

Single strength fix, make fresh Safety critical

Working solution: 4% EM grade formaldehyde in ~0.2 M HEPES buffer pH 7.4

1. Add 16% EM grade formaldehyde to 0.8 M HEPES stock solution and top up with ddH_2O according to the final volume required (Table 1).

Table 1. Single strength fixative recipe according to final volume required

	Final volume (ml)			
	4.5	10	18	25
16% FA	1.125	2.50	4.50	6.25
0.8 M HEPES	1.125	2.50	4.50	6.25
dH_2O	2.25	5.00	9.00	12.5

100% dry acetone

1. Transfer molecular sieves to a 500 ml glass bottle, add 99-100% acetone until it's brim full, mix, and leave to settle.

Acetone	State: Liquid	Hazard Codes: H225, H319, H336	

Photo-activated LR White Resin, make fresh Safety critical

1. Add 15 ml of catalysed LR White into a 15 ml glass vial
2. Add 0.5-1% benzoin methyl ether (0.75 % = 0.1125g) to LR White and add a cap to the vial
3. Cover with foil and place on the rotator
4. Once dissolved, place in the fridge until use

LR White resin	State: Liquid	Hazard codes: H315, H319, H317, H335	

Benzoin methyl ether	State: Solid	Hazard codes: H301	

Safety warnings

Safety critical: All procedures designated safety critical must be performed in a fume hood (where appropriate) and the following personal protective equipment (PPE) must be worn - lab coat, gloves, and eye protection.

Procedure: Processing samples

1

Note

- This protocol was developed for cell monolayers grown on glass coverslips in 35 mm dishes. Appropriate solution volumes are indicated.
- The protocol can be adapted for use on tissues by extending dehydration and infiltration times.
- For dehydration and resin infiltration steps in metal dishes, fill the metal dish at least half full so that the cells will not dry out, which would destroy the ultrastructure.

Day 1

1d 0h 15m

2 Prepare fresh fixative solutions before starting with sample preparation **Safety critical**

- Pre-warm double strength fix to 37 °C (or the temperature at which the cells were grown)

3 Add an equal volume of pre-warmed double strength fix to the cells in medium and fix for 00:15:00 at Room temperature **Safety critical** 15m

4 Replace double strength fix with 2 mL of single strength fix and leave for 24:00:00 at 4 °C **Safety critical** 1d

Day 2

15m

5 Prepare the Automated Freeze Substitution (AFS) unit **Safety critical**

- Set the AFS unit to 4 °C and fill with liquid nitrogen

Day 2

15m

6 **Prepare solutions fresh before resuming the protocol Safety critical**

6.1 Prepare graded acetone series and pre-cool in the fridge at 4 °C

6.2 Prepare graded photo-activated LR white series and pre-cool in the fridge at 4 °C

Day 2

15m

7 Remove fixative solution from the 35 mm dishes containing the coverslips and replace with 2 mL of [M] 0.2 Mass Percent HEPES, On ice **Safety critical**

8 **Wash the coverslips in:**

8.1 2 mL of [M] 0.2 Mass Percent HEPES, for 00:05:00 , On ice (1/3) 5m

8.2 2 mL of [M] 0.2 Mass Percent HEPES, for 00:05:00 , On ice (2/3) 5m

8.3 2 mL of [M] 0.2 Mass Percent HEPES, for 00:05:00 , On ice (3/3) 5m

9

Note

Dehydration and infiltration notes:

- Transfer pre-cooled 70% and 90% acetone to the AFS chamber. Also add an empty 15 ml glass bottle for waste.
- After each solution change, remove the waste, and transfer the next solution from the fridge to the AFS unit so that it can pre-cool to the appropriate temperature.
- Use pre-cooled metal forceps (orange insulated tweezers) to manipulate samples within the AFS unit.
- Ensure there is enough acetone in the dishes - do not allow the cells to dry out as this will destroy the ultrastructure.

10 Transfer coverslips to metal dishes filled with pre-cooled 50% acetone On ice , and transfer to the AFS unit

11 Reduce the temperature in the AFS over 00:20:00 from 4 °C to -20 °C 20m

12 **Dehydrate cells stepwise at -20 °C in:**

12.1 Pre-cooled 70% acetone for 00:10:00 10m

12.2 Pre-cooled 90% acetone for 00:10:00 10m

12.3 Pre-cooled anhydrous acetone for 00:10:00 10m

12.4 Pre-cooled anhydrous acetone for 00:10:00 10m

13 **Infiltrate cells with a graded photo-activated LR White series at -20 °C in the dark**
Safety critical

Note

We suggest to cover the AFS lid with a box during infiltration to exclude light.

13.1 Pre-cooled 25% LR White for 00:10:00 . 10m

13.2 Pre-cooled 50% LR White for 00:10:00 . 10m

13.3 Pre-cooled 75% LR White for 00:10:00 . 10m

13.4 Pre-cooled 100% LR White Overnight .

Day 3

2d 18h 40m

14 Make fresh photo-activated 100% LR White, pre-cool at 4 °C for 00:20:00 and then transfer to the AFS unit for at least 00:20:00 before use to cool further to -20 °C **Safety critical** 40m

15 Infiltrate cells with the pre-cooled photo-activated 100% LR White for 02:00:00 at -20 °C in the dark 2h

16 **Polymerise resin:**

16.1 Place an Aclar disc (cut to size from an Aclar sheet) over the dish to exclude oxygen during polymerisation

16.2 If needed, top up the liquid nitrogen in the AFS, being careful not to decrease temperature below -20 °C **Safety critical**

16.3 Attach the UV lid to the AFS unit and turn on the UV power

16.4 Polymerise for 48:00:00 at -20 °C with the UV light on 2d

17 Warm samples to 4 °C over 16:00:00 with the UV light turned off 16h

18 Store resin blocks in the fridge at 4 °C