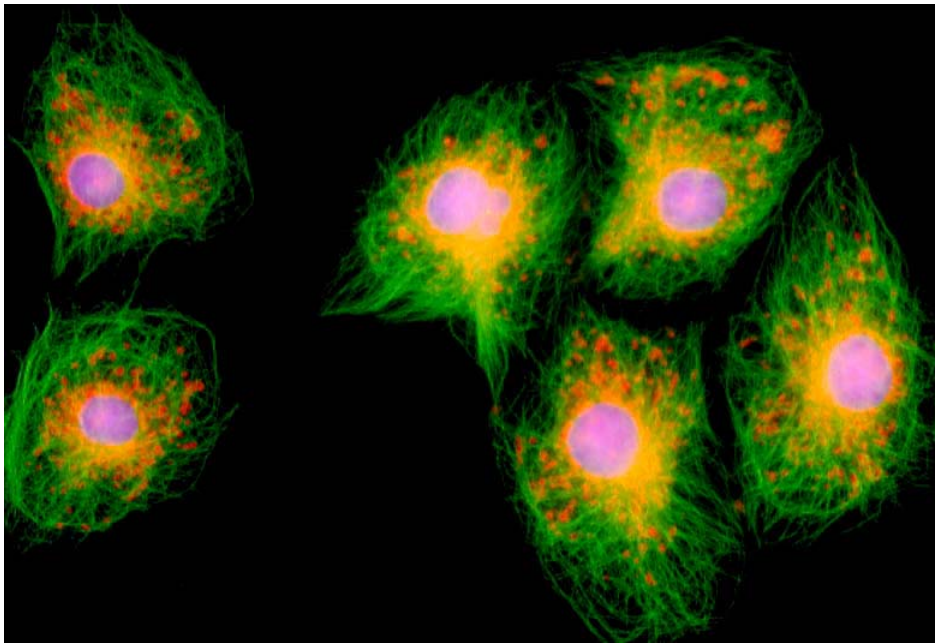


Olympus Protocols
for
Fluorescence Staining
of Cells



OLYMPUS

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Laboratory Apparatus and Supplies for Specimens Preparation

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Centrifuge
Inverted Microscope

Actin Filaments/Rhodamine Phalloidin

(1) Fixation

4% Formaldehyde (Neutral) at Room Temp. for 10-20 mins

(2) Wash

Rinse in 3 changes of D-PBS (-), 5 mins each, at Room Temp.

(3) Permeabilization

0.5% Triton X-100, D-PBS (-), at Room Temp. for 5 mins

(4) Wash

Rinse in 3 changes of D-PBS (-), 5 mins each, at Room Temp.

(5) Stain

5 μ l Rhodamine-phalloidin store soln./70 μ l D-PBS (-); 1 unit dye for 1 coverslip
37 C, for 45 mins

Rhodamine-phalloidin store soln. (Molecular Probes, R-415); 300 units/1.5 ml of
MetOH

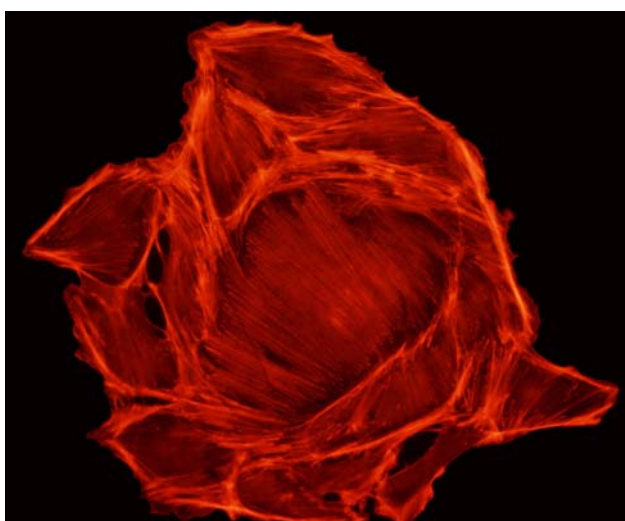
(6) Wash

Rinse in 3 changes of D-PBS (-), 5 mins each, at Room Temp.

(7) Mount

Slow Fade-Light antifade Kit (MPI, #S-7461)

On the double staining of microtubules and actin filaments, start at the step (5) after incubating in the secondary antibody for the immunofluorescence staining of microtubules.



PtK2/DP70

Microtubules/anti-beta tubulin/Alexa Fluor 488

(1) Fixation

2% p-formaldehyde, 0.1% glutaraldehyde, 4% sucrose in 0.1M cacodylate buffer (pH7.4), at Room Temp. for 7 mins

(2) Wash

Rinse in 3 changes of 0.1M cacodylate buffer (pH7.4) containing 7% sucrose, 5 mins each at Room Temp.

(3) Permeabilization

0.5% Triton X-100, 4% polyethylenglycol, 1mM EGTA, 0.1M PIPES (pH7.2), at Room Temp. for 5 mins

(4) Wash

Rinse in 3 changes of D-PBS(-), 5 mins each, at Room Temp.

(5) Blocking

1.4% skim-milk in D-PBS(-) (Snow Brand Milk Products Co.,Ltd., Tokyo/Sapporo), at Room Temp. for 10 mins

(6) Wash

Rinse in 3 changes of D-PBS(-), 5 mins each, at Room Temp.

(7) Primary Antibody

2 µg/ml Monoclonal antibody to beta-tubulin (Chemicon International, Inc. MAB3408) in D-PBS(-), 37 C, for 60 mins

(8) Wash

Rinse in 5 changes of D-PBS(-), 5 mins each, at Room Temp.

(9) Secondary Antibody

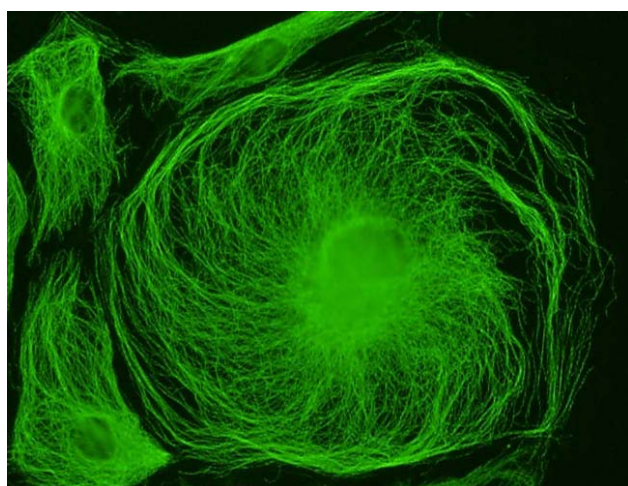
10 µg/ml Alexa Fluor 488-Labeled Antibody to Mouse IgG & M(H+L) (MP, A11001) in D-PBS(-), 37 C, for 60 mins

(10) Wash

Rinse in 5 changes of D-PBS(-), 5 min each, at Room Temp.

(11) Mount

Slow Fade-Light antifade Kit (Molecular Probes, #S-7461)



PtK2/DP70

Endoplasmic Reticulum/DiO C₆(3) <LIVE>

- (1) Culture cells on a glass bottom culture dish.
- (2) Store Soln. of DiO C₆(3) (D-273, MP) : 0.5 mg/ml in Et-OH
- (3) Add the DiO C₆(3) soln. into the culture medium at 0.5 µg/ml (final conc.).
- (4) Incubate at 37 C for 5-10 mins. Only mitochondria are stained by the shortage of the incubation time.
- (5) Rinse in 3 changes of the culture medium without the dye, 5 mins each.

Mitochondria/Rhodamine 123 <LIVE>

- (1) Culture cells on a glass bottom culture dish.
- (2) Store Soln. of Rhodamine 123 (R-302, MP): 1 mg/ml of DDW
- (3) Change the culture medium to the serum free one. Add the Rhodamine 123 into the medium at 10 µg/ml (final conc.).
- (4) Incubate at 37 C for 10 mins.
- (5) Rinse in 3 changes of the culture medium without the dye, 5 mins each.

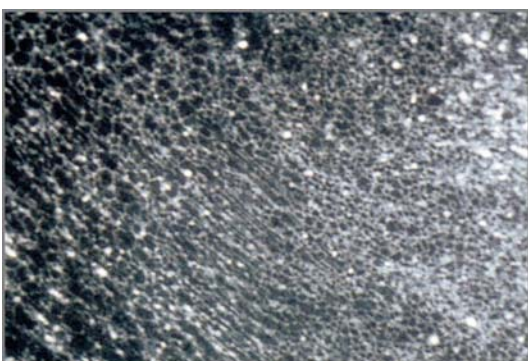
Mitochondria/MitoTracker Red <LIVE>

- (1) Culture cells on a glass bottom culture dish.
- (2) Store Soln. of MitoTrackerRed (M-7512, MP): 1 mM in DMSO
- (3) Change the culture medium to the serum free one. Add the MitoTracker Red into the medium at 250 nM (final conc.).
- (4) Incubate at 37 C for 30 mins.
- (5) Rinse in 3 changes of the culture medium without the dye, 5 mins each.

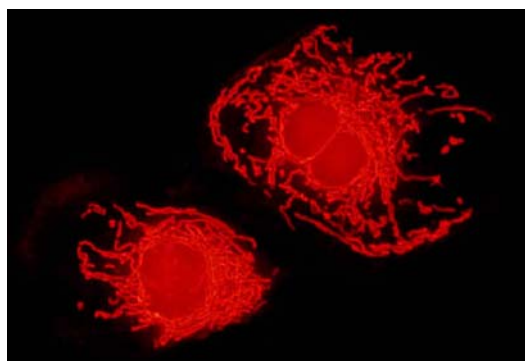
Notes

- Use a micro-CO₂ incubator or a HEPES containing medium on time lapse imaging.
- Add antibiotics into a medium for using the open chamber.

Endoplasmic Reticulum



Mitochondria (MitoTracker)



PtK2/FV1000(ER), DP70(Mt)

Nuclei-1 Acridine Orange

(1) Fixation

70% Et-OH in D-PBS(-), at Room Temp. for 5 mins

(2) Wash

Rinse in 3 changes of D-PBS(-)

(3) Pretreatment

0.1% Triton X-100, 0.08N HCl, 0.15M NaCl, 0.1mM EDTA in DDW, at 4 C, for 5 mins

(4) Wash

Rinse in 3 changes of D-PBS(-)

(5) Staining

6 µg/ml Acridine Orange, 1mM EDTA, 0.15M NaCl in phosphate-citric buffer (pH6.0), at 4 C, overnight~overday (~24 hrs)

Store Solns.

Phosphate-citric acid buffer (pH6.0) [37ml of 0.1M citric acid and 63ml of 0.2M Na₂HPO₄]

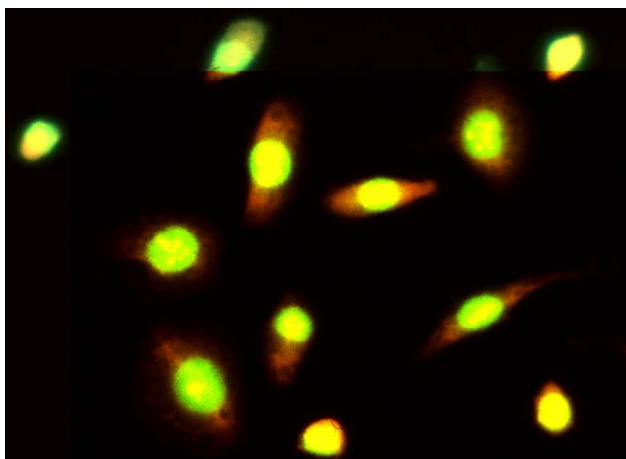
Acridine Orange Soln. [1 mg/ml in DDW]

(6) Mount

Mount in the staining soln. with 10 mM 2-mercaptoethylamin

Store Soln.

2-mercaptoethylamin Soln. [0.1M in DDW]



A549/Film

Nuclei-2 Propidium Iodide

(1) Fixation

70% EtOH in D-PBS(-) at Room Temp. for 5 mins

(2) Wash

Rinse in 3 changes of D-PBS(-)

(3) RNA Digestion

Incubate in RNase Solution at 37 C, for 60 mins

Rinse in 3 changes of D-PBS(-)

RNase Solution/ 100 ml (RNase final conc. 0.1 mg/ml)

0.05 M Tris-HCl pH 7.4

0.05 M NaCl

10 mg RNase (Sigma R-4875)

DNase inactivation: at 100 C, for 60 secs.

(4) Wash

Rinse in 3 changes of D-PBS(-)

(5) Staining

Staining Soln. at Room Temp. for 60 mins

Staining soln.

10 µg/ml PI (1/100 vol. of the store soln.)

10 mM 2-mercaptoethylamine-HCl (Store soln.: 0.1 M in DDW)

Tris Buffer Solution

Tris Buffer Solution

10 mM Tris-HCl, pH 7.4

10 mM EDTA-2Na, pH 8.0

100 mM NaCl

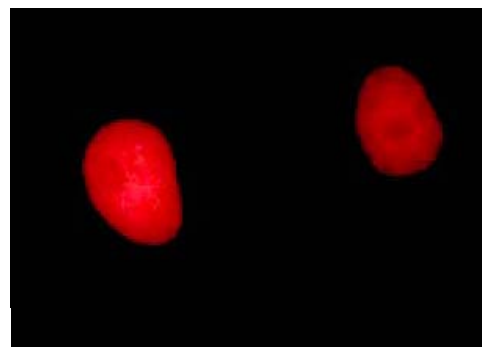
PI Soln.(Store Soln.)

1 mg/ml in 50% Met-OH

(6) Mount

Mount in the staining soln.

On the double staining of microtubules and nuclei, cells are fixed and permeabilized for the immunofluorescence staining, after that RNAs are digested by RNase. Before staining of PI, the primary and secondary antibodies are applied to cells. Use the Slow Fade-Light antifade kit for mounting.



PtK2/Film

Nuclei-3 DAPI

- (1) Fixation
70% EtOH in D-PBS(-), at Room Temp. for 5 mins
- (2) Wash
Rinse in 3 changes of D-PBS(-)
- (3) Staining
Staining Soln. for 60 mins at Room Temp.

Staining Soln.

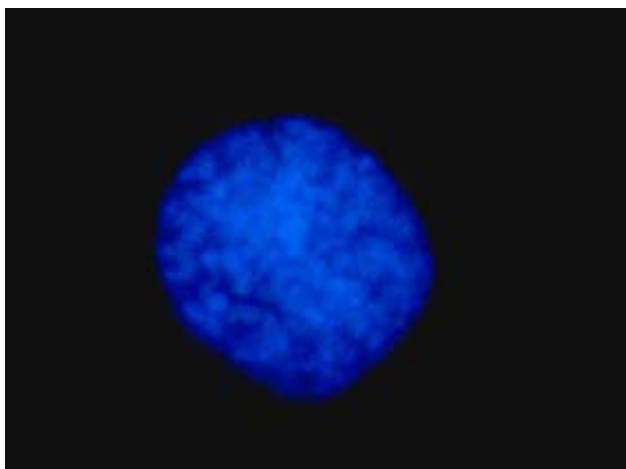
0.5% DAPI Soln. (50 ng/ml)
10 mM 2-mercaptoethylamine-HCl
Tris Buffer Solution

Store Soln.

Tris Buffer Solution [10 mM Tris-HCl (pH 7.4); 10 mM EDTA-2Na (pH 8.0);
100 mM NaCl]
DAPI Soln. [10 µg/ml in DDW]
2-mercaptoethylamine Soln. [0.1 M in DDW]

(4) Mount

Mount in the staining soln.



PtK2/Film

Nuclei-4 YOYO-1

Culture cells on glass coverslips.

(1) Fixation

70% EtOH in D-PBS (-) at Room Temp. for 5 mins

(2) Wash

Rinse in 3 changes of D-PBS(-)

(3) RNase Digestion

Incubate in RNase solution at 37 C, for 60 mins

Rinse in 3 changes of D-PBS(-)

RNase Solution / 100 ml (RNase final conc. 0.1 mg/ml)

0.05 M Tris-HCl pH 7.4

0.05 M NaCl

10 mg RNase (Sigma R-4875)

DNase inactivation : 100 C, 60 sec.

(4) Staining

Stain in the staining solution, at Room Temp. for 60 min.

Staining Solution

0.001-0.01% YOYO-1 solution

10 mM 2-mercaptoethylamine-HCl

Tris Buffer Solution

Tris Buffer Solution

10 mM Tris-HCl, pH 7.4

10 mM EDTA-2Na, pH 8.0

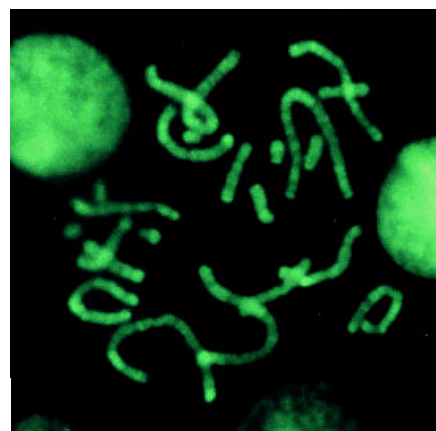
100 mM NaCl

YOYO-1 Solution

1 mM in DMSO (Molecular Probes, Y-3601)

(5) Mount

Mount in the staining solution.



CHV79/Film

Nuclei-5 Hoechst 33342 <LIVE>

(1) Cell preparation

Culture cells on a glass bottom culture dish.

(2) Store Soln. of Hoechst 33342: 1 mM in DDW

(3) Add the Hoechst 33258 soln. into the culture medium at 0.2 μ M (final conc.).

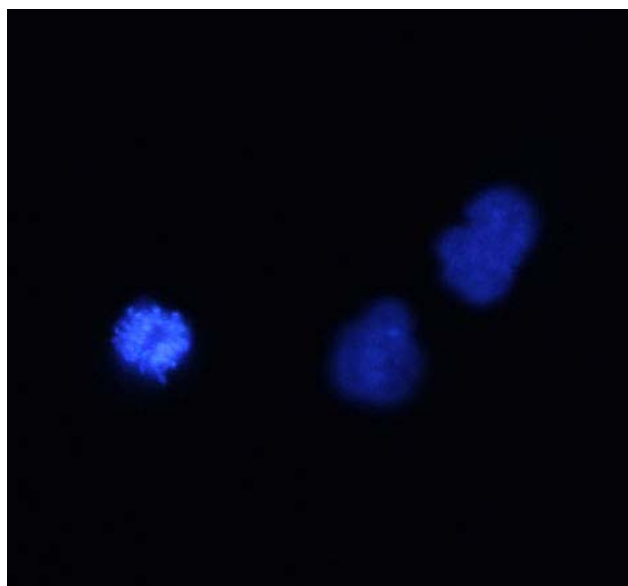
(4) Incubate at 37 C for 30 mins.

(5) Rinse in 3 changes of the culture medium without the dye, 5 mins each.

(6) Change the medium to the Phenol Red free culture medium and incubate for additional 1-2 hours.

Notes

- Use a micro-CO₂ incubator or a HEPES containing medium on time lapse imaging.
- Add antibiotics into a medium for using the open chamber.
- Hoechst 34580 (392/440 nm) is suitable on excitation by the 405 nm Laser Diode.



A549/DP70

Cell Membrane DiO <LIVE>

(1) Cell preparation

Culture cells on a glass bottom culture dish.

(2) Store Soln. of DiO: 10 mg/ml in DMF (Dimethylformamide).

(3) Change the medium into the dye soln. (0.02 mg/ml DiO in 5% sucrose).

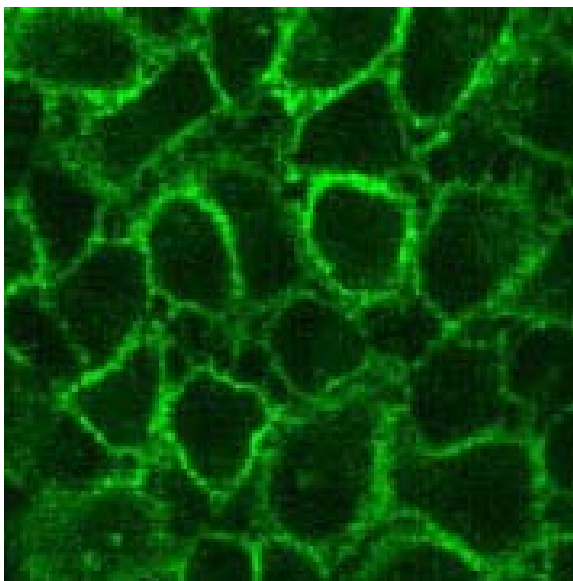
(4) Incubate at room temp. for 30 mins.

(5) Rinse in 3 changes of the serum and Phenol Red free culture medium, 5 mins each.

(6) Change the medium to the fresh serum and Phenol Red free culture medium.

Notes

- Use a micro-CO₂ incubator or a HEPES containing medium on time lapse imaging.
- Add antibiotics into a medium for staining in the open chamber.



HeLa/FV1000

Multi Color Staining

For U/B/G/R excitation

U/Nucleus~DAPI

B/Actin Filaments~AlexaFluor 488-Phalloidin

G/Mitochondria~MitoTracker Red

R/Microtubule~Cy5 (Immunofluorescence method with anti-beta tubulin)

SUMMARY OF STAINING

Stain first mitochondria in the living cells with MitoTracker Red, then fix the cells. Stain with the other dyes after fixation.

1. Mitochondria Staining

Preparing MitoTracker (Store solution 1mM in DMSO)

Stain solution

250 nM MitoTracker in Recording Medium

Recording Medium

20 mM Hepes (pH 7.4), 115 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂

0.8 mM MgCl₂, 13.8 mM glucose

(1) Staining solution at 37 C for 30min

(2) Rinse in 3 changes of D-PBS

2. Cell fixation

2% formaldehyde, 0.1% glutaraldehyde, 1.4% sucrose in 0.1M cacodylate buffer(pH7.4) for 7 min. at Room Temp.

After the Mitochondria Staining (See the staining methods on previous pages),

(1) Tubulin Staining [Primary Antibody/Mouse anti-beta tubulin (Chemicon International, Inc.; MAB3408), Secondary Antibody/Goat anti-mouse IgG+IgM Cy5 conjugated (RKL; 610-110-121)]

(2) Actin Filaments Staining [AlexaFluor 488-phalloidin]

(3) Nuclei Staining [DAPI]

(4) Mount in Slowfade-light antifade without rinse



PtK2/FV1000

Ion Concentration <LIVE>

fura-2, indo-1, fluo 3, calcium green-1 etc. for calcium Ion concentration
BCECF, c SNARF-1 etc. for pH

(1) Cell preparation

Culture cells on a glass bottom culture dish.

(2) Dye loading

Use acetoxymethyl ester (AM) dyes

5 μ M Dye/AM and 0.02% Pluronic F-127 (final conc. each) are mixed prior to dilution with the loading medium.

Store solution

1 mM Dye/AM in DMSO

10% Pluronic F-127 in DMSO

Dye Loading and Recording Medium

20 mM HEPES (pH 7.4)

115 mM NaCl

5.4 mM KCl

1.8 mM CaCl_2

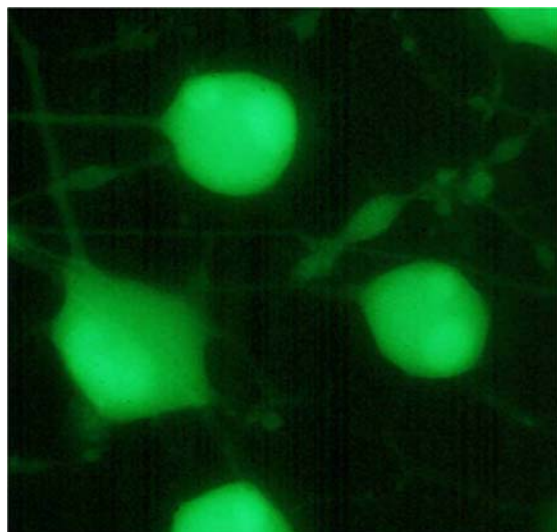
0.8 mM MgCl_2

13.8 mM glucose

Remove a culture medium, and incubate in 200 μ l of the above dye solution for 20 min. at room temperature.

Add 2 ml of the dye-AM free loading medium without washing and incubate cells at room temperature for 30-60 min.

(3) Rinse cells with the fresh medium before starting the measurement



NG108-15/Film

Anti-Fade Reagents

Mounting Solution (similar to SlowFade)
2.5% DABCO (1,4-diazabicyclo-2,2,2-octane)
0%, 50% or 90% Glycerol
20 mM Tris-HCl (pH 8.0) or D-PBS(-)

Preparation

- (1) Dissolve DABCO in Glycerol warmed at 70 C.
- (2) Adjust pH 8.0 with Tris-Cl buffer

Commercially Available Mountants and Antifades

SlowFade	Invitrogen Molecular Probes	http://probes.invitrogen.com/
ProLong	Invitrogen Molecular Probes	http://probes.invitrogen.com/
FluoroGuard	Bio-Rad	http://www.bio-rad.com/
Vectashield	Vector Laboratories	http://www.vectorlabs.com/
FluorSave	Calbiochem	http://www.merckbiosciences.com/
PermaFluor	Thermo	http://www.thermo.com/com/
Poly-Mount	Polysciences, Inc	http://www.polysciences.com/shop/
Cytoseal 60	VWR	http://www.vwrsp.com/index.cgi
Citifluor	Citifluor Ltd.	http://www.citifluor.co.uk/index.html

Cell Culture

Cells are cultured on glass coverslips or glass bottom culture dishes. The coverslips were soaked in ethanol, rinsed in DDW, and sterilized before use. Seven pieces of round coverslip of 15 mm in diameter can be set in a 60 mm plastic dish. Some cell do not grow well on a glass surface, and when this occur, modifying the glass surface is necessary. Coating the surface with poly-D (or L)-lysine, collagen, fibronectin, *etc.* resolve this problem.

Fixation

Fixatives should preserve cell structure and antigenicity. The most commonly used fixative is 4% formaldehyde.

- (1)Coagulating Fixatives: Ethanol, Methanol, Aceton (organic solvents)
- (2)Crosslinking Fixatives: Formaldehyde, Glutaraldehyde

Chemicals

Cacodylate Buffer

0.2 M Sodium Cacodylate (pH 7.4 /HCl)

Rhodamine Phalloidin

Store Soln.: 300 units/1.5 ml MetOH

Staining Soln.: 35 µl of stock soln. in 500 µl of D-PBS(-)

70 µl (containing 1 unit rhodamine phalloidin) of the staining solution for
1 coverslip

Recording Medium

20 mM Hepes (pH 7.4), 115 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgCl₂,
13.8 mM glucose

Formaldehyde

(1)Use commercially available 10% neutral buffered formalin. ... *or*
(w/wo methanol)

(2)Dissolve p-formaldehyde. ...*or*

To make 20% p-formaldehyde solution

[Stir at below 60 C, add 1N NaOH and stir again. Finally the solution is filtered.]

Dilute to 4% solution with 0.1 M Phosphate buffer (pH 7.4).

(3)Use commercially available 4% p-formaldehyde/phosphate buffer.

Blocking Solution

(1)1.4% skim-milk / D-PDS(-) ...*or*

(2)1% BSA (Bovine Serum Albumin) / D-PBS(-) ...*or*

(3)5% non-immune serum / D-PBS(-)

Website

D-PBS (-)

Dulbecco's Phosphate Buffer, Calcium & Magnesium Free
<http://www.invitrogen.com/>

MP

Invitrogen-Molecular Probes
<http://probes.invitrogen.com/>

RKL

Rockland Immunochemicals Inc.
<http://www.rockland-inc.com/>

Glass Coverslips

15 mm Round No.1 (Matsunami Glass Ind., LTD., JAPAN)
<http://www.matsunami-glass.co.jp/>

Glass Bottom Culture Dish

MatTek
<http://www.glass-bottom-dishes.com/index.html>
Matsunami Glass IND., LTD. (JAPAN)
<http://www.matsunami-glass.co.jp/>
World Precision Instrument
<http://www.physio-tech.co.jp/>

Slides

White Frost, Polished edge No.2 (Matsunami-glass, S2112)
NEO Color Frost, Polished Edge No.2, for Fluorescence (Matsunami-Glass, S0318)
<http://www.matsunami-glass.co.jp/>

Neutral Buffered Formalin

Commercially available 10% neutral buffered formalin (4% formaldehyde) pH 7.4
<http://www.wako-chem.co.jp/index.htm>



Reference

The Handbook; a guide to fluorescent probes and labeling technologies (10th ed.)
<http://probes.invitrogen.com/>

