N-SIM

Specifications:

• Resolution: approx. 100nm in XY and 300nm in Z
• 3D Axial Range: up to 20µm
• Speed: up to 0.6 sec/frame (2D-SIM/TIRF-SIM) or up to 1sec/frame (3D-SIM)
• Light sources:
    Lasers for N-SIM: 488 and 561nm
    Intensilight Hg lamp for standard fluorescence
    Halogen lamp for brightfield
• Camera: Andor Technology iXon3 897 EMCCD camera
• Objective: 100X (N.A. 1.49) oil
• Imaging modes: 2D-SIM, 3D-SIM and TIRF-SIM

Sample preparation:

• Optimise your fixation method to get a good signal to noise ratio.

• Choose a primary antibody that localises strongly to the relevant structure and has low background fluorescence.

• Use Alexa Fluor dye conjugated secondary antibodies wherever possible. These are very bright and photostable. Use fluorophores compatible with the N-SIM lasers: 488 and 561nm.

• Coverslips must be #1.5 or 0.17mm thick (e.g. cover glasses, high performance, D=0.17mm, box with 1000 pc. – Zeiss, cat nr: 474030-9000-000)

• Slides and coverslips must be scrupulously clean (no dust, residual oil, salt from IF washes, etc.). Suggest using 100% EtOH to clean before use.

• Mounting medium should be chosen to match the index of refraction of the objective. Anti-fade agents should also be added to the mounting medium. We suggest 90% glycerol as a good starting point. Avoid mounting media that solidify as these tend to change the light path (e.g. Vectashield). Do not use mounting media that contain DAPI. If you wish to use DAPI, stain then wash before applying mounting media.

Examples (see Appendix 1 for more details):

- ProLong® Gold Antifade Reagent – Invitrogen, cat nr: P36934 or P36930
- Fluoromount/Plus™ – DBS, DiagnosticBioSystems, cat nr: K 048
- PPD (P-phenylenediamine) Antifade Mounting Media
- Murray Antifade Mounting Media
- nPG (n-propyl gallate) Antifade Mounting Media
- Antifade 1: 1,4-phenylene-diamine
- DABCO (1,4 diazobizyclo[2,2,2]octane) Antifade

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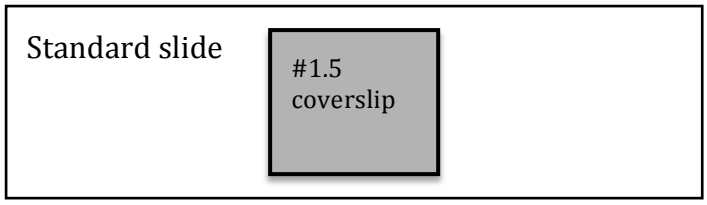
• Coverslips must be in the center of the slide (the distance from the center of the coverslip to both short edges of the slide must be the same, see diagram below. The frosted part of a slide counts as part of the overall length). Only one coverslip may be mounted per slide. This is due to the limited range of motion of the stage and can't be changed!



• Coverslips must be sealed on all sides (no leakage) with clear nail polish or another solid sealing agent. Samples can be stored at 4^{o}C.

• Bring freshly prepared samples and avoid freeze-thaw cycles.

• Immersion oil provided: Type NF (nd=1.515)

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Appendix 1 - Antifade Recipes

PPD (P-phenylenediamine) Antifade Mounting Media (Recipe #1)

P-phenylenediamine (EMD Chemicals Inc. Cat# PX0730)

1. Wrap a glass scintillation vial with foil and drop in a small stir bar. (PPD is light-sensitive.)
2. With a 10 ml pipet add 9 mls of glycerol to the vial.
3. With the 1000 µl Pipetman add 1ml of 1X PBS.
4. Place on stirrer and begin mixing.
5. Weigh out 10 mg of p-phenylenediamine on the Mettler balance. *PPD is toxic. Wear gloves and don't inhale it.*
6. Add the PPD to the vial and stir untill it is all in solution (1-2 hrs.). The medium should appear almost colorless to a slight tint of yellow. If it is an intense yellow or orange color the PPD is most likely contaminated and will have background staining.
7. pH the mounting medium to a pH of 8.0-9.0 using the Carbonate-Bicarbonate buffer. pH paper of range 6.5-10.0 should be used to check the pH of the medium after addition of 12 drops of the Carb-Bicarb buffer and stirring. Additional drops of buffer are added untill the desired pH is reached.
8. Aliquot the mounting medium and store at -70oC.

* Flakes of PPD are large and should be crushed Carbonate- Bicarbonate Buffer

A. Make up a 0.2M solution of anhydrous sodium carbonate (2.12g/100ml)

B. Make up a0.2M solution of sodium bicarbonate (1.68g/100ml)

Take 4 mls of A + 46 mls of B and bring up to 200 mls with DH2O. The pH will be 9.2.

*Note: If the PPD is contaminated or goes bad (turns a brown color) it will stain DNA, so each preparation should be tested. Check by looking at mitotic cells to be sure that chromosomes are not stained.

Murray Antifade Mounting Media

Preparation:

1. In a suitable bottle, add:
    a. 100 ml glycerol (check for autofluorescence first!)
    b. 5 g (n-propyl gallate)
    c. 0.25 g DABCO (1,4-diazobicyclo-(2,2,2)-octane)
    d. 0.0025 g PPD (para-phenylenediamine)
2. Wrap tube completely in foil to protect from light
3. Mix on stirrer until dissolved (overnight)
4. Store at 4°C.

Use:

1. Do final wash in pH 8 (to pH 8.5) buffer.
2. Drain buffer from slide.
3. Add enough antifade to cover specimen.

4. Let sit 5 minutes, drain off antifade.
5. Mount with fresh antifade. Coverslip.

Note: Try to remove all water from the specimen! Otherwise, effectiveness of the antifade will be greatly decreased.

Thanks to Dr. John Murray at the University of Pennsylvania

nPG (n-propyl gallate) Antifade Mounting Media

Preparation:

1. In a 50 ml falcon tube, add:
    a. 5ml of 0.2M TRIS, pH 8.5
    b. 43 ml glycerol (check for autofluorescence first!)
    c. 2.5g n-propyl gallate
2. Wrap tube completely in foil to protect from light
3. Mix on stirrer until dissolved (overnight)
4. Store at 4°C.

Use:

1. Do final wash in pH 8 (to pH 8.5) buffer.
2. Drain buffer from slide.
3. Add enough antifade to cover specimen.
4. Let sit 5 minutes, drain off antifade.
5. Mount with fresh antifade. Coverslip.

Antifade 1: 1,4-phenylene-diamine (Recipe #2)

<u>1,4-pheylene-diamine</u>

1. Prepare carbonate-bicarbonate buffer (pH 9.0).
2. Dissolve 50 mg 1,4-phenylenediamine in 2 ml 1X PBS.
3. Adjust pH with carbonate-bicarbonate buffer to 8.0.
4. Add 1X PBS to 5 ml.
    Important: Add 2 ml buffer and check pH, then add drop-wise until pH is 7.99-8.00. If pH exceeds 8.0, the procedure must be started over.
5. Mix with 45 ml 86% glycerol. Leave on inverter for at least 1 hr.
6. Aliquot and store at -20°C.

<u>Carbonate-Bicarbonate Buffer (pH 9.0)</u>

Sodium bicarbonate, 0.5 M (pH 8.13) 4 ml
Sodium carbonate, 0.5 M (pH 11.32) 1 ml
Filter sterilize

DABCO (1,4 diazobizyclo[2,2,2]octane) Antifade

DABCO (1,4-diazabicyclo[2.2.2]octane) Sigma, Cat. D2522
Glycerol, 86%
Tris-HCl, l M pH 8.0

Preparation

Components Amount
DABCO 0.233 g
Tris-HCl, 1 M pH 8.0 200 µl
Sterile water 800 µl
Glycerin, 86% 9 ml

Procedure

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1. Combine components.
2. Dissolve by warming to 70°C.
3. Vortex.
4. Aliquot and store at -20°C.