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Supplementary protocols for 'A simple and fast optical clearing method for whole-mount fluorescence in situ hybridization (FISH) imaging'

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We use this protocol and it's working

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Abstract

We report a single-step optical clearing method that is compatible with RNA fluorescence in situ hybridization (FISH) imaging. We previously demonstrated microscopy imaging with immunohistochemistry and genetic reporters using a technique called lipid-preserving refractive index matching for prolonged imaging depth (LIMPID). Our protocol reliably produces high-resolution 3D images with minimal aberrations using high magnification objectives, captures large field-of-view images of whole-mount tissues, and supports co-labeling with antibody and FISH probes. We also custom-designed FISH probes for quail embryos, demonstrating the ease of fabricating probes for use with less common animal models. Furthermore, we show high-quality 3D images using a conventional fluorescence microscope, without using more advanced depth sectioning instruments such as confocal or light-sheet microscopy. For broader adoption, we simplified and optimized 3D-LIMPID-FISH to minimize the barrier to entry, and we provide a detailed protocol to aid users with navigating the thick and thin of 3D microscopy.

Guidelines

For figures mentioned in the protocol, please refer to the main manuscript.

Protocol materials

- HCR Probe Wash Buffer **Molecular Instruments** Step 61
- Nycodenz AG® **Proteogenix** **Catalog #**1002424 In 2 steps
- 20X SSC **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**S6639-1L Step 15
- Paraformaldehyde, 16% (wt/vol) **Electron Microscopy Sciences** **Catalog #**15710 Step 30
- 10x PBS **Thermo Fisher Scientific** **Catalog #**AM9624 Step 30
- HCR Probe Hybridization Buffer **Molecular Instruments** Step 54
- Methanol **P212121** **Catalog #**PA-33900HPLCCS4L Step 33
- Hydrogen Peroxide, 30% **Fisher Scientific** **Catalog #**H325-500 Step 39
- MilliQ water In 2 steps
- Urea **P212121** In 2 steps
- HCR Amplification Buffer **Molecular Instruments** Step 64
- Formamide (deionized) **Fisher Scientific** **Catalog #**BP228100 Step 39
- 4,6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) **Thermo Fisher Scientific** **Catalog #**D1306 Step 73

Before start

To better preserve mRNA, make sure all equipment and lab surfaces are cleaned properly to prevent RNAse contamination (e.g., RNAse away, Thermo Scientific 21-402-178).

Synthesis of H2O LIMPID

1 Place a 500 ml glass beaker on a large weight balance and tare.

2 Transfer 200 g of deionized MilliQ water **Contributed by users** to the glass beaker. Remove the beaker from the balance.

3 Weigh out the same weight (200 g) of Urea **P212121** powder and gently transfer the urea powder to the glass beaker. This will make a roughly [M] 50 Mass Percent (w/w) urea solution, which will clear the sample (Supplementary Fig. 5 a-b).

4 Add a magnetic stirring bar in the beaker.

5 Wrap the top of the beaker to reduce water evaporation.

6 Heat up the beaker to 60 °C and gently mix the solution with the stirring bar until all powder is dissolved (becomes transparent when fully dissolved).

7 Once all urea powder is dissolved, cool down the solution and transfer 300 g of 50% (w/w) urea solution to an empty 500 ml glass beaker.

7.1 Place the empty glass beaker on the large weight balance and slowly pour 50% (w/w) urea solution until 300 g of solution is transferred.

7.2 Transfer remaining 50% (w/w) urea solution to a glass container with a cap. Close the cap and reduce moisture loss by wrapping the cap with a parafilm.

7.3 Transfer the magnetic stirring bar to the new beaker once all liquids are transferred.

8 Weigh out 200 g of Nycodenz AG® **Proteogenix Catalog #**1002424 powder on a weighing boat.

9 Gently transfer the Nycodenz powder to the glass beaker. The weight ratio of iohexol to urea solution is now 2:3

10 Wrap the top of the beaker with parafilm to reduce water evaporation.

11 Heat up the beaker to 60 °C and gently mix the solution with the stirring bar until all powder is dissolved.

11.1 Nicodenz can take a long time (3-6 hours) to dissolve.

11.2 The solution can be left overnight if it is not heated (room temperature) and the beaker is well covered with parafilm.

12 Take a small sample (~ 50 µL) from the beaker and measure the refractive index with an abbe refractometer.

Equipment	
Abbe refractometer	NAME
Abbe refractometer	TYPE
Azzota	BRAND
AR-1	SKU

13 In order to adjust the refractive index of the LIMPID solution, add Nycodenz powder or 50% (w/w) urea solution. Use the calibration curve (supplementary figure 1) for rough estimates.

13.1 Nycodenz will increase the refractive index (up to ~1.57).

13.2 50% urea solution will decrease the refractive index (down to ~1.41).

14 If you see crystalized urea in any steps of this section, it is a sign that too much evaporation has occurred and the urea concentration is higher than [M] 50 Mass Percent . In such a case, use a 500 mL glass container with a cap instead of a beaker. Use a powder funnel to minimize the loss and heat up the container no higher than 40 °C .

Synthesis of SSC-LIMPID

15 Prepare 200 ml of SSC buffer by diluting x ml of 20X SSC **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**S6639-1L buffer with y ml of deionized MilliQ water **Contributed by users** .

15.1 For 5xSSC-LIMPID: x = 50 ml, y = 150 ml

15.2 For 2xSSC-LIMPID: x = 20 ml, y = 180 ml

16 Place a 500 ml glass beaker on a large weight balance and tare.

17 Transfer 200 g of SSC buffer to the glass beaker. Remove the beaker from the balance.

18 Weigh out the same weight (200 g) of Urea **P212121** powder and gently transfer the urea powder to the glass beaker. This will make a roughly [M] 50 Mass Percent urea solution, which will clear the sample (Supplementary Fig. 5 a-b).

19 Add a magnetic stirring bar in the beaker.

20 Wrap the top of the beaker to reduce water evaporation.

21 Heat up the beaker to 60 °C and gently mix the solution with a stirring bar until all powder is dissolved.

22 Once all urea powder is dissolved, cool down the solution and transfer 300 g of 50% (w/w) urea solution to an empty 500 ml glass beaker.

22.1 Place the empty glass beaker on the large weight balance and slowly pour 50% (w/w) urea + SSC solution until 300 g is transferred.

22.2 Transfer remaining 50% (w/w) urea + SSC solution to a glass container with a cap. Close the cap and reduce moisture loss by wrapping the cap with parafilm.

22.3 Transfer the magnetic stirring bar to the new beaker once all liquids are transferred.

23 Weigh out 200 g of Nycodenz AG® **Proteogenix Catalog #**1002424 powder on a weighing boat.

24 Gently transfer the Nycodenz powder to the glass beaker. The weight ratio of iohexol to urea solution is now 2:3.

25 Wrap the top of the beaker with parafilm to reduce water evaporation.

26 Heat up the beaker to 60 °C and gently mix the solution with the stirring bar until all powder is dissolved.

26.1 Nicodenz can take a long time (3 - 6 hours) to dissolve.

26.2 The solution can be left overnight if it is not heated (room temperature) and the beaker is well covered with parafilm.

27 Take a small sample (~50 µl) from the beaker and measure the refractive index with an Abbe refractometer.

Equipment

Abbe refractometer

Abbe refractometer	TYPE
Azzota	BRAND
AR-1	SKU

28 In order to adjust the refractive index of the SSC-LIMPID, add Nycodenz powder or 50% (w/w) urea solution. Use the calibration curve (supplementary figure 1) for rough estimates.

28.1 Nycodenz will increase the refractive index (up to ~1.57).

28.2 50% (w/w) urea + SSC solution will decrease the refractive index (down to ~1.41).

29 If you see crystalized urea in any steps of this section, it is a sign that too much evaporation has occurred and the urea concentration is higher than [M] 50 Mass Percent. In such a case, use a 500 mL glass container with a cap instead of a beaker. Use a powder funnel to minimize the loss and heat up the container no higher than 40 °C.

Methanol dehydration/delipidation

30 Prepare [M] 4 % (v/v) paraformaldehyde (PFA) solution with 10 mL of [M] 16 % (v/v) Paraformaldehyde, 16% (wt/vol) **Electron Microscopy Sciences Catalog #**15710, 4 mL of 10x PBS **Thermo Fisher Scientific Catalog #**AM9624 and 26 mL of deionized water.

31 Fix fresh samples in PFA solution at 4 °C overnight.

31.1 Fixation condition and time will differ by sample size and properties. This protocol is optimized for pre-processing day 5 quail. Mouse brain was fixed with a perfusion fixation protocol, and trigeminal ganglia was fixed for 3 hours at 4 °C.

32 Wash sample 3 x 5 minutes with 1xPBST.

33 Remove all fluids and immerse the sample in increasingly concentrated Methanol **P212121 Catalog #**PA-33900HPLCCS4L solution (2 mL).

33.1 10 minutes in 25% methanol / 75% 1xPBST solution

33.2 10 minutes in 50% methanol / 50% 1xPBST solution

33.3 10 minutes in 75% methanol / 25% 1xPBST solution

33.4 10 minutes in 100% methanol

34 Wash the sample with methanol for 10 minutes.

35 Incubate at Room temperature overnight in methanol and the sample will become delipidated.

36 Wash the sample with methanol for 10 minutes. (Sample can be stored at -20 °C for long term storage)

37 Rehydrate sample by washing it with increasingly concentrated 1xPBST solutions

37.1 10 minutes in 75% methanol / 25% 1xPBST solution On ice

37.2 10 minutes in 50% methanol / 50% 1xPBST solution On ice

37.3 10 minutes in 25% methanol / 75% 1xPBST solution On ice

37.4 10 minutes in 100% 1xPBST On ice

38 Wash the sample with 1xPBST for 10 minutes On ice

Sample Bleaching

39 Prepare bleaching solution. For 10ml of bleaching solution: 1 mL of 5x SSC, 50 µL of Hydrogen Peroxide, 30% **Fisher Scientific Catalog #**H325-500 , 40 µL of

Formamide (deionized) **Fisher Scientific Catalog #**BP228100 , 8.91 mL of deionized H2O.

40 Incubate sample in the bleaching solution at Room temperature for 2 hours

40.1 A bright white light source can be added to increase bleaching.

41 Wash the sample twice with 1xPBST for 10 minutes

Air bubble removal

42 Boil 75 mL of 1xPBS.

43 Once the solution is boiling, stop the heating and cool it down with ice. It is important to not disturb the solution. Move it carefully to the ice bucket.

44 Use an infrared thermometer gun to indirectly measure the temperature.

45 Once the solution has cooled down to 36-40 °C, carefully transfer it to a 50 mL plastic tube. Fill the tube up to the top. It is important to completely fill the tube without any air pockets.

46 Transfer the sample to the tube.

47 Close the cap and wrap it with parafilm

48 Cool down the sample further to 4 °C

49 Visually check the bubbles inside the sample. If the bubbles are not completely removed, repeat step 1-7 until all bubbles are removed (Supplementary Fig. 7 b).

HCR hybridization and amplification for large samples with cavities

50 Prepare formamide solution

50.1 For 50%: 5 mL of formamide, 4 mL of deionized water and 1 mL of 20xSSC

50.2 For 30%: 3 mL of formamide, 6 mL of deionized water and 1 mL of 20xSSC.

50.3 For 15%: 1.5 mL of formamide, 7.5 mL of deionized water and 1 mL of 20xSSC.

51 Incubate sample in 2 mL of 15% formamide solution at 37 °C for 30 minutes

51.1 Take extra precaution on the cavity. Revert back to a lower concentration solution if the cavity is or about to collapse. Smaller increments of concentrations are recommended to alleviate osmotic pressure.

52 Replace the 15% formamide solution with 2 mL of 30% formamide solution and incubate at 37 °C for 30 minutes

53 Replace the 30% formamide solution with 2 mL of 50% formamide solution and incubate at 37 °C for 3 hours

54 Thaw 100% HCR Probe Hybridization Buffer **Molecular Instruments** from the -20 °C storage and prepare 33% and 66% hybridization buffers

54.1 For 33%: 1 mL of hybridization solution, 2 mL of 5xSSC buffer.

54.2 For 66%: 2 mL of hybridization solution, 1 mL of 5xSSC buffer.

55 Incubate sample in 2 mL of 33% hybridization buffer at 37 °C for 30 minutes

55.1 Take extra precaution on the cavity. Revert back to a lower concentration solution if the cavity is or about to collapse. Smaller increments of concentrations are recommended to alleviate osmotic pressure.

56 Replace 33% hybridization buffer with 2 mL of 66% hybridization buffer and incubate at 37 °C for 30 minute

57 Replace 66% hybridization buffer with 2 mL of 100% hybridization buffer and incubate at 37 °C overnight

58 Prepare a hybridization solution by mixing x pmol of hybridization probes and y ml of hybridization buffer.

58.1 For a small sample, use the recommended recipe from the paper:
x = 2, y = 0.5.

58.2 For larger samples, increase volume of hybridization buffer and increase the moles of probes proportionally. (e.g., x = 4, y = 1 or x = 6, y = 1.5)

58.3 For a reference, we used 1 mL of hybridization buffer (4 pmols of probes) for the day 5 quail (~ 1 cm and larger) sample.

59 Transfer the sample to the prepared hybridization solution.

60 Incubate the sample in the hybridization solution at 37 °C for 2 days. Rotate or shake samples for better staining.

61 Thaw HCR Probe Wash Buffer **Molecular Instruments** from -20 °C storage

62 Wash sample with 2 mL of wash buffer at 37 °C for 4x15 minutes. Rotate or shake samples.

63 Wash the sample twice with 2 mL of 5xSSCT for 10 minutes.

64 Incubate sample in 2 mL of HCR Amplification Buffer **Molecular Instruments** for 30 minutes.

65 Prepare amplification probes in 600 µL tubes

65.1 For small samples, use the recommended recipe from the paper:
30 pmols of probes for 500 µL of amplification buffer.

65.2 For larger samples, increase volume of hybridization buffer and increase the moles of probes proportionally.

65.3 For a reference, we used 1 mL of amplification buffer. 48 pmols of probes were sufficient to give a strong signal.

66 Make snap-cool hairpins by running a heat-block program (Techne TC-3000 PCR Thermal Cycler) on the probes

66.1 90 seconds at 95 °C

66.2 30 minutes at 25 °C

67 Spin down the 600 µL tubes with a centrifuge.

68 Prepare an amplification solution by transferring all probes to the fresh 1 mL amplification buffer.

69 Transfer the sample to the amplification solution.

70 Wrap the sample container with aluminum foil and incubate it at 4 °C overnight. Rotate or shake the sample.

71 After the overnight incubation, warm up the sample to Room temperature and incubate for 2 hours. Rotate or shake the sample.

72 Wash the sample twice with 2 mL of 5xSSCT for 10 minutes.

73 If desired, stain the sample with a
4,6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) **Thermo Fisher Scientific Catalog #**D1306
stain

73.1 Prepare Dapi staining solution: 1:1000 dilution of [M] 300 micromolar (µM) Dapi in 2 mL of 5xSSCT.

73.2 Incubate the sample in Dapi at Room temperature overnight.

73.3 Wash the sample twice with 2 mL of 5xSSCT for 10 minutes.

74 If desired, the sample can be stained with antibodies

74.1 Prepare antibody staining solution by following the manufacturer recommended dilution. E.g., 1:20 dilution of antibody in a blocking buffer.

74.2 Block sample in 1xPBS buffer with 1.5% bovine serum albumin (BSA), 0.1% tween at 4 °C for overnight.

74.3 Stain in antibody staining solution following manufacturer's suggested incubation temperature.

Tissue clearing

75 Remove as much fluids from the sample as possible.

75.1 It is recommended that no strong force is exerted on the sample during fluid aspiration.

75.2 It is acceptable to leave some fluids and preserve the sample integrity.

76 Transfer LIMPID (H2O or SSC variant) to the sample-holding container.

76.1 The volume of added LIMPID is recommended to be at least 3 times the volume of the sample.

77 Mix the solution by placing the sample container on a rotator or shaker for 5 minutes.

78 Remove LIMPID and transfer fresh LIMPID to the sample container.

79 Mix the solution by placing the sample container on a rotator or shaker for 5 minutes.

80 Examine the sample and its transparency

80.1 It is expected that a thick sample is not completely cleared.

80.2 Samples with chromophores (e.g., blood) will be tinted with the color. If needed, please refer to sample bleaching section above to bleach the sample.

80.3 Samples with some visible opaqueness can still generate good optically sectioned 3D volumes.

81 If more clearing is desired, remove the LIMPID and transfer fresh LIMPID to the sample container. Incubate for 15 minutes on a rotator or shaker.

82 Examine the sample and its transparency.

83 If more clearing is desired, remove the LIMPID and transfer fresh LIMPID to the sample container. Incubate for 30 minutes on a rotator or shaker at 36 °C.

83.1 Further incubation can increase transparency of the tissue but with diminishing returns.

83.2 The transparency is expected to max out at 1 hour incubation at 36 °C. This can vary greatly depending on the sample.

83.3 Note that long incubation may lead to probe leakage especially when not using SSC-LIMPID.

84 Transfer the sample to a chambered tissue slide. A metal slide (Supplementary Fig. 6 a, method) was used for our samples.

85 Add LIMPID to slightly overfill the chamber (Supplementary Fig. 6 a).

86 Place a coverslip on top of the chamber.

87 Gently push the coverslip with a round tip tweezer or a tweezer with rubber tip to squeeze out excess LIMPID from the chamber.

88 Gently remove excess LIMPID from the slide with a Kimwipe.

88.1 Localized evaporation of LIMPID on the edge of the coverslip will create sealing of the chamber (Supplementary Fig. 6 b) that can easily be broken later.

88.2 Small magnets can be used to secure the coverslip (Supplementary Fig. 6 b).

89 The sample is ready for imaging.

Protocol references

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