INTACT Proteomics in *Xenopus*

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Analysis of the molecular mechanisms driving cell specification, differentiation, and other cellular processes can be difficult due to the heterogeneity of tissues and organs. Therefore, it is critical to isolate pure cell populations in order to properly assess the function of certain cell types in the context of a tissue. This protocol describes use of the INTACT (isolation of nuclei tagged in specific cell types) method in *Xenopus*, followed by proteomics analysis of nuclear protein complexes. The INTACT protocol utilizes two transgenes: (1) a three-part nuclear targeting fusion (NTF) consisting of a nuclear envelope protein (Nup35) that targets the NTF to the nuclear membrane, an enhanced green fluorescent protein (EGFP) cassette for NTF visualization in live animals, and a biotin ligase receptor protein (BLRP) that provides a substrate for the biotinylation of the NTF, and (2) the *E. coli* ligase BirA (which biotinylates the NTF) tagged to mCherry (for visualization). Either or both transgenes are driven by a tissue-specific promoter, making this protocol easily adaptable to proteomics analyses of immunoprecipitated complexes from INTACT-isolated nuclei of multiple tissue types to determine the composition of protein complexes in pure cell populations.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

Acetonitrile, HPLC grade (Fisher A998)

BCA Protein Assay Kit (Pierce) (Thermo Fisher Scientific 23225)

BirA-mCherry transgene (Schaffer et al. 2010)

Synthesize capped BirA-mCherry mRNA constructs using the mMessage mMachine Transcription Kit (Thermo-Fisher Scientific AM1344 for T7, AM1348 for T3, or AM1340 for SP6) according to the manufacturer's protocol. Note that the enzyme used for transcription will depend on the RNA transcription promoter present in the mRNA construct used (i.e., T7, T3, or SP6).

Dulbecco's phosphate-buffered saline (DPBS) (1×, pH 7.4) (Thermo Fisher Scientific 14040117)

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From the *Xenopus* collection, edited by Hazel L. Sive.

Cite this protocol as *Cold Spring Harb Protoc*; doi:10.1101/pdb.prot098384

Ethanol (100%)
Formic acid, LC-MS/MS grade (99%+) (Pierce 28905)
Methanol
Nuclear purification buffer (NPB) <R> (at 4°C)

In addition, prepare NPBb (NPB + 0.5% BSA) and NPBt (NPB + 0.1% Triton X-100).

Nuclear targeting fusion (NTF) transgene (Amin et al. 2014)

The NTF consists of three parts: a nuclear envelope protein (Nup35) that targets the NTF to the nuclear membrane, an EGFP cassette for NTF visualization in live animals, and a biotin ligase receptor protein (BLRP) that provides a substrate for the biotinylation of the NTF.

Clone the NTF transgene downstream of a tissue-specific promoter of choice (e.g., mlc2p or cardiac actin). For example, fusion downstream of the mlc2p regulatory element would drive NTF expression specifically in cardiomyocytes after stage 26 (Latinkic et al. 2004).

OptiPrep density gradient medium (Sigma-Aldrich D1556)

Prepare a solution of 30% OptiPrep in NPB.

Polyacrylamide gels and reagents for denaturing gel electrophoresis (Thermo Fisher Scientific)

- NuPAGE 4%–12% Bis-Tris protein gels (NP0321)
- NuPAGE antioxidant (NP0005)
- NuPAGE LDS sample buffer (4×) (NP0008)
- NuPAGE MOPS running buffer (20×) (NP0001)
- NuPAGE sample reducing agent (10×) (NP0009)

RIPA buffer for *Xenopus* <R>
Streptavidin-conjugated magnetic Dynabeads (Invitrogen M-270)
Trypsin stock (0.5 µg/µL), sequencing grade (Promega V5111)

Store stock solution at −80°C and limit to <5 freeze/thaw cycles.

Immediately before use, prepare a working solution of 12.5 ng/µL trypsin in 20 µL of 50 mM ammonium bicarbonate.

Xenopus and reagents for transgenesis using REMI or method of choice (Amaya and Kroll 1999; Allen and Weeks 2005; Yergeau and Mead 2007)

Equipment

Cell strainer (100 µM) (Sigma-Aldrich CLS431752)
Centrifuge, refrigerated
Denaturing gel electrophoresis equipment
Embryo injector
Heat blocks at 37°C and 95°C
Liquid chromatography system and mass spectrometer

We recommend using a Dionex Ultimate 3000 nanoRSLC system coupled to an LTQ-Orbitrap Velos mass spectrometer.

Liquid nitrogen
Magnet (Thermo Fisher Scientific 12321D)
Mortar and pestle, ceramic (Thermo Fisher Scientific FB961A-3 and FB961K-M)
Polytetrafluoroethylene tissue grinder
Razor blades
Rotator
Sonicator (Bioruptor [Diagenode])
Stopcock, two way (Thermo Fisher Scientific 6460)
Styrofoam cooler
Syringe needle
Transfer pipettes, plastic (Thermo Fisher Scientific 242)

Tubes, polypropylene (conical, 15- and 50-mL)
Tubes, polypropylene (rounded bottom, 2-mL)
Windex

METHOD

Generating INTACT Nuclei in *Xenopus*

This section describes generation of transgenic Xenopus *embryos harboring the NTF transgene under control of a tissue-specific promoter and injected with or without BirA-mCherry mRNA.*

1. Generate transgenic *Xenopus* containing the NTF transgene using REMI or the transgenesis method of choice (Amaya and Kroll 1999; Allen and Weeks 2005; Yergeau and Mead 2007).

   Stable transgenic lines assure uniform expression; however, this method has been used successfully with transient (mosaic) transgenic animals (Amin et al. 2014).

2. Inject 1 ng of *BirA-mCherry* mRNA into the transgenic *Xenopus* embryos at the 1-cell stage. As a negative control, generate NTF-transgenic embryos that do not receive *BirA-mCherry* injection.

   Injection at the 1-cell stage ensures distribution of BirA-mCherry throughout the entire developing embryo.

3. Culture the embryos to the desired stage of development.

Isolating INTACT Nuclei

This section describes the separation and isolation of nuclei from the NTF/BirA-mCherry transgenic embryos.

4. Harvest 100 embryos in 1× DPBS in a 2-mL round-bottom tube. Wash the embryos three times with 1× DPBS by gently resuspending them using a plastic transfer pipette and then letting them settle to the bottom of the tube.

   The required number of embryos should be determined empirically based on the percentage of cells in the embryos expressing the NTF. For most applications, 100 embryos should be sufficient; however, in instances where the NTF is only driven in a few cells (e.g., cardiac valve cells), more embryos will be required. Enough embryos should be harvested to recover 30 µg of nuclear protein as measured by the BCA assay (Step 30).

5. Using a syringe needle, poke four holes in the cap of a 50-mL conical tube. Remove the cap and secure the tube into a rack in a Styrofoam cooler. Fill the cooler and tube with liquid nitrogen.

6. Using a plastic transfer pipette, drop the embryos into the conical tube containing liquid nitrogen, minimizing the volume of transferred DPBS. Once the embryos have been transferred, screw the cap onto the tube tightly. Remove the tube from the cooler (using a paper towel or cryo-glove for protection) and invert to drain the liquid nitrogen. Store the frozen tissue at −80°C.

7. Wash a ceramic mortar and pestle once each with the following: Windex, ethanol, methanol, and ddH_2O. Wipe dry with a paper towel. Carefully pour liquid nitrogen into the mortar and let it evaporate to cool the ceramic.

8. Carefully pour liquid nitrogen into the ceramic mortar, add the embryos, and grind the frozen embryos to a powder using a cold pestle. Collect the frozen powder in a 15-mL conical tube.

9. Resuspend the frozen powder in 6 mL of NPB. Thaw the powder in NPB for 10 min on ice.

10. Transfer the lysate to a polytetrafluoroethylene tissue grinder and homogenize with 40 strokes.

11. Pass the lysate through a 100-µm cell strainer into a 50-mL conical tube.

12. Centrifuge the lysate at 1000*g* for 10 min at 4°C to collect crude nuclei.

13. Resuspend the nuclei in 6 mL of 30% Optiprep in NPB. Centrifuge at 1000*g* for 10 min at 4°C to harvest the enriched nuclei.

    A small portion of this suspension can be analyzed by phase-contrast or stained with DAPI to determine nuclear purity.

14. Wash the nuclei three times by gentle resuspension in 6 mL of NPB per wash.

    Be sure to remove all traces of Optiprep before proceeding with subsequent steps.

Affinity-Isolating INTACT Nuclei

This section describes streptavidin-based isolation of nuclei. The addition of a large volume (9 mL) of NBPt ensures that contaminating cell types are washed away while the nuclei are preserved.

Perform Steps 15–26 at 4°C.

15. Resuspend the nuclei in 1 mL of NPB.
16. Incubate the nuclei with 50 µL of streptavidin-conjugated magnetic beads. Rotate for 30 min.

    Proceed to Steps 17–19 during this incubation.

17. At the start of the incubation in Step 16, preload P1000 tips with 1 mL of NPBb per tip. Lay the preloaded tips on their sides.
18. After 20 min of incubation, insert a pre-loaded tip vertically into a two-way stopcock and attach it to a magnet. Open the stopcock and drain the NPBb from the P1000 tip.
19. Add 1.2 mL of NPBt to the broad opening of the stopcock assembly.
20. After the 30-min incubation (Step 16), add 9 mL of NPBt to the nuclei/bead mixture.
21. Resuspend the mixture in a 10-mL pipette and insert it vertically into the broad opening of the stopcock assembly.
22. Slowly release the mixture through the stopcock assembly (~1 mL/30 sec).

    The bead-bound nuclei should be collected to the side of the P1000 tip by the magnet. The flow-through can be retained and processed through the remainder of the protocol (Steps 27–36) as a non-bead-bound control.

23. Remove the magnet and collect the bead-bound nuclei in a fresh tube in 1 mL of NPB.
24. Dilute the NPB/nuclei mixture (1 mL) to 10 mL with NPBt.
25. Add 1.2 mL of NPBt to the stopcock assembly.
26. Repeat Steps 21–23 with a new tip-magnet assembly.

Proteomic Profiling INTACT Nuclei

This section describes the lysis of nuclei, isolation and separation of protein complexes, digestion of proteins into peptides for mass spectrometry, and preliminary data analysis.

27. Resuspend the bead-bound nuclei in 100 µL of RIPA buffer. Incubate for 10 min on ice to lyse the nuclei.
28. Sonicate the lysate using a Bioruptor on high for 15 min (30 sec on/30 sec off) at 4°C.
29. Place the lysate on a magnet to elute the sample from the beads.
30. Measure the protein concentration using a BCA assay.
31. Add the appropriate amount of 4× LDS buffer and reducing agent to obtain a final concentration of 0.5× buffer to 30 µg of protein sample. Incubate the sample for 10 min at 95°C.
32. Load and run each sample on a 4%–12% Bis-Tris polyacrylamide gel. Add 500 µL of antioxidant to the middle chamber before running. Load empty 1× sample buffer in all empty lanes to prevent uneven running of the gel.

    We recommend 1× MOPS running buffer, since MOPS is ideally used to separate proteins between 14 kDa and >200 kDa, ensuring a thorough separation of the majority of the proteome.

33. Using a razor blade, slice each lane into 1-mm gel slices. Group 10 slices per sample.
34. Add trypsin (12.5 ng/µL) to the gel slices. Incubate overnight at 37°C to digest the proteins into peptides.
35. Extract the peptides in 0.5% formic acid/50% acetonitrile (ACN).

36. Analyze the peptides by nanoliquid chromatography-tandom mass spectrometry.

Search the spectra using a Xenopus*-specific protein sequence database; see Greco et al. (2012) for a detailed protocol.*

DISCUSSION

Studies of cellular and molecular pathways that are required for the development and maintenance of multicellular organs and tissues are notoriously difficult due to the heterogeneity of these structures. Many strategies have been implemented to isolate pure cell populations to circumvent these issues, including physical isolation by laser microdissection (Golubeva et al. 2013) or isolation based on the expression of a tissue or cell specific marker (FACS) (Barker et al. 1975). However, these methods of isolation require that the cell type of interest expresses a unique marker or reporter construct in order to ensure isolation of a pure population of cells. In *Xenopus*, isolation of cell populations using antibody-based sorting is difficult due to the availability of antibodies against these unique markers. The INTACT (isolation of nuclei tagged in specific cell types) method has been utilized in plants, worms, and flies to isolate pure nuclear populations via in vivo biotinylation of the nuclear envelope (Deal and Henikoff 2010, 2011; Henry et al. 2012; Steiner et al. 2012). The biotinylated nuclei are then isolated using streptavidin beads, separating nuclei from the cell population of interest away from other contaminating cell types without requiring an antibody. These studies have led to analysis of gene expression and chromatin features in *Arabidopsis* root epidermis cells (Deal and Henikoff 2011), distinct cell types in the *Drosophila* brain (Henry et al. 2012), and muscle cells from *C. elegans* (Steiner et al. 2012). This protocol describes the adaptation of the INTACT method for use in *Xenopus*, and subsequent proteomics analysis of nuclear protein complexes (Amin et al. 2014). The INTACT protocol utilizes two transgenes, the NTF transgene and the *E. coli* ligase BirA tagged to mCherry. Either or both transgenes are driven under the control of a tissue-specific promoter (e.g., the *mlc2p* regulatory element fused upstream of the NTF to drive NTF expression specifically in cardiomyocytes after stage 26). The fact that the NTF can be expressed in a specific spatial and temporal manner makes this protocol easily adaptable to almost any cell or tissue type. Furthermore, proteomics analysis of immunoprecipitated complexes from INTACT-isolated nuclei of multiple tissue types can determine the composition of protein complexes in pure cell populations (Conlon et al. 2012).

A potential disadvantage of the INTACT method is that it requires the use of transgenic animals that express the NTF transgene. Ideally, stable transgenic lines would assure uniform expression of the transgene. Generation of stable transgenic lines can be technically challenging and time-consuming, however, and this method has been used successfully with transient (mosaic) transgenic animals (Amin et al. 2014).

RECIPES

Nuclear Purification Buffer (NPB)

10 mM Tris (pH 7.4)
40 mM NaCl
90 mM KCl
2 mM EDTA
0.5 mM EGTA
0.2 mM dithiothreitol (DTT)
0.5 mM phenylmethanesulfonyl fluoride (PMSF)
0.5 mM spermine (Sigma-Aldrich S3256)
0.25 mM spermidine (Sigma-Aldrich S2626)
1× Roche cOmplete Protease Inhibitor Cocktail (Sigma-Aldrich 11697498001)

Add DTT, PMSF, spermine, spermidine, and protease inhibitors to NPB immediately prior to use.
Use at 4°C.

RIPA Buffer for Xenopus

50 mM Tris (pH 8.0)
150 mM NaCl
0.5% sodium deoxycholate
1% NP-40
0.1% SDS

Store for up to 1 mo at 4°C.

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