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FixNCut v2.0 Enhanced Cell and Tissue Fixation for Single Cell Assays

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We use this protocol and it's working

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Abstract

The original FixNCut 1.0 protocol faced challenges in scalability bottlenecks due to the limited solubility and stability of DSP (dithiobis-succinimidyl propionate) NHS-esters in aqueous environments. Additionally, preparing the working solution required careful handling and experience, which restricted its application in large-scale, parallel experiments. This enhancement of original FixNCut (OzSoup v1) overcomes these limitations by a dual complementary system combining DSP for crosslinking and cold methanol for fixing and permeabilizing. This combination improves cell integrity, minimizes clumping, and simplifies processing. Integrated rehydration and washing steps further prepare cells for downstream molecular assays. These refinements aim to overcome limitations of the original protocol, and enhance its reliability, scalability, and versatility, making it more accessible for diverse experimental workflows while inviting broader validation and application.

Guidelines

This protocol outlines a method for improving sample fixation and preparation through a combined use of DSP (dithiobis-succinimidyl propionate) and methanol.

DSP acts as a crosslinking agent, stabilizing proteins, while methanol complements this by providing fixation and permeabilization properties. Cold methanol is introduced to stabilize DSP during the fixation process, enhancing the overall effectiveness and reliability of the fixative.

This combination aims to address the instability of NHS-esters in aqueous environments, improving cell structural integrity and reducing clumping during downstream applications.

Following fixation, rehydration and washing steps are incorporated to prepare samples for further processing and to reduce clump formation. These steps are particularly relevant for maintaining sample quality in single cell assays.

Additional steps, such as the inclusion of DNAse I during tissue digestion, can be employed to mitigate DNA contamination and enhance sample preparation.

Periodic review and optimization of the protocol are recommended to ensure adaptability to specific experimental conditions and to maintain consistency across different applications.

Materials

Biological Materials

Tissue samples and cell suspensions can both be used.

For cells, the protocol is set for up to 4 × 10^6 cells. For more cells, scale up the fixation accordingly.

Tissues larger than 3 mm in diameter or edge length need to be partitioned into smaller pieces to facilitate fixative penetration.

Reagents

Fixation and Neutralization

1. DSP (dithiobis(succinimidyl propionate)), Lomant's Reagent **Thermo Fisher Catalog #**22586 also known as Lomant's Reagent.
2. DMSO, Anhydrous **Thermo Fisher Catalog #**D12345 DMSO, Anhydrous (Thermo Fisher, Molecular Probes, catalog number: D12345)
3. Methanol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**34860 Methanol, ≥99.9% (Sigma Aldrich, catalog number: 34860)
4. 1M Tris-HCl pH 7.5 **Thermo Fisher Scientific Catalog #**15567027 UltraPure 1 M Tris-HCl Buffer, pH 7.5 (Thermo Fisher, Invitrogen, catalog number: 15567027)

Rehydration, wash and tissue digestion

1. SSC Buffer (20X) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**S6639
2. Protector RNase Inhibitor **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**3335402001 or RiboLock RNase Inhibitor **Thermo Fisher Scientific Catalog ##**EO0381
3. Bovine Serum Albumin solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**A1595-50ML Bovine Serum Albumin 10% that is sterile filtered and cell-culture tested [Sigma Aldrich A1595 or 126615]
4. Liberase TM **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**000000005401119001 Liberase TM Research Grade, 10 mg (Roche, catalog number: 5401119001)
5. Nuclease-Free Water **Thermo Fisher Scientific Catalog #** AM9939
6. PBS - Phosphate-Buffered Saline (10X) pH 7.4 RNase-free **Thermo Fisher Scientific Catalog #**AM9624
7. Nuclei EZ lysis buffer **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**EZ PREP NUC-101

Miscellaneous:

- Sterile and RNAse-free Tissue Culture Dish, 6cm or 10cm from Corning, Falcon or similar.
- Forceps
- Razor Blades
- Wide-Bore Pipette Tips
- pluriStrainer Mini 40 and 70 µm (Cell Strainer)

- DNA LoBind Tubes 1.5 mL [022431021]
- DNA LoBind Tubes 2.0 mL [022431048]
- Ice bucket/blocks

Equipment:
- Automated Cell counter, for example Luna-FX7
- Tabletop centrifuge with swing rotor adapter, for example Eppendorf 5810R.

Protocol materials

CryoStor CS10 100ML **Fisher Scientific** **Catalog #**NC9930384

Mr. Frosty Freezing Container, 2mL tubes, Nalgene Mr. Frosty Freezing Container for 1-2mL cryogenic tubes, PC, clear w/ blue lid, 1/Cs. **Thermo Fisher** **Catalog #**5100-0001

DSP (dithiobis(succinimidyl propionate)), Lomant's Reagent **Thermo Fisher** **Catalog #**22585

SSC Buffer (20X) **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**S6639

RiboLock RNase Inhibitor **Thermo Fisher Scientific** **Catalog ##**EO0381

Protector RNase Inhibitor **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**3335402001

DNAse I **Roche** **Catalog #**04716728001

Liberase TM **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**000000005401119001

DSP (dithiobis(succinimidyl propionate)), Lomant's Reagent **Thermo Fisher** **Catalog #**22586

Nuclei EZ lysis buffer **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**EZ PREP NUC-101

1M Tris-HCl pH 7.5 **Thermo Fisher Scientific** **Catalog #**15567027

Bovine Serum Albumin solution **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**A1595-50ML

Nuclease-Free Water **Thermo Fisher Scientific** **Catalog #** AM9939

DMSO, Anhydrous **Thermo Fisher** **Catalog #**D12345

PBS - Phosphate-Buffered Saline (10X) pH 7.4 RNase-free **Thermo Fisher Scientific** **Catalog #**AM9624

Methanol **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**34860

Safety warnings

DSP is a reactive crosslinker that can cause eye and skin irritation and may be harmful if ingested or inhaled. Proper handling requires the use of personal protective equipment (PPE), such as gloves, goggles, and lab coats, and working in a well-ventilated area or fume hood.

Methanol, frequently used alongside DSP, is a highly flammable and toxic substance. Its misuse can lead to irreversible and permanent blindness, organ damage, or other severe health effects if ingested, inhaled, or absorbed through the skin. Extreme caution is required, and strict adherence to safety protocols is essential when handling methanol.

Both DSP and methanol waste should be treated as hazardous materials. Methanol waste, in particular, must be collected in clearly labeled, compatible containers and disposed of according to local regulations for hazardous waste to prevent environmental contamination and health risks.

Before start

All washes and centrifugations need to be done at 4 °C unless otherwise specified.

Therefore, precool the centrifuge according and prepare buffers and place it On ice.

50x Fixation Concentrate Stock Preparation (50 mg/mL)

30m

1 Thoroughly equilibrate the DSP mylar package to room temperature for 00:30:00 prior to first opening. 30m

DSP (dithiobis(succinimidyl propionate)), Lomant's Reagent **Thermo Fisher Catalog #**22585

> Note
>
> **Critical:** Only open the package after equilibration. The NHS-ester of DSP is susceptible to hydrolysis upon contact with humid atmosphere and therefore moisture-sensitive. Thorough equilibration is crucial in preventing condensation and premature inactivation of DSP.

2 Dissolve 50 mg DSP in 1 mL high quality DMSO, Anhydrous **Thermo Fisher Catalog #**D12345 for best performance. Final concentration is [M] 50 mg/mL .

3 Each 10 µL aliquots can be stored in cryogenic vials with compression O-rings to prevent moisture absorption. Avoid freeze-thaw cycles by limiting the size of aliquots (up to 100 µL).

> Note
>
> Discard any opened but unused stock.

DSP-Methanol Fixative Preparation (1 mg/mL)

4 Mix 12.5 µL of 50x DSP ([M] 50 mg/mL) with 587.5 µL of pre-cooled cold methanol at 4 °C in a 1.5 or 2mL tube to achieve a total volume of 600 µL and final DSP concentration of [M] 1 mg/mL . Each tube can be used for one samples with up to 4×10^6 cells.

Note

Compared to aqueous condition, methanol offers a controlled medium for DSP dissolution and reactions, reducing NHS ester hydrolysis, especially in its anhydrous form. However, DSP solutions in methanol should still be freshly prepared for best results. Avoid making large master mix for multiple samples, instead, prepare multiple aliquots in parallel.

Fixation

30m

5 Gradually add the DSP-methanol fixative to cells and gently resuspend multiple times with a wide-bore pipette, or to tissue samples (cut into 3×3×3 mm pieces or smaller). Swirl gently to ensure mixing.

6 Fix On ice for 00:30:00, swirling occasionally. 30m

Note

If necessary, increase the fixation time incrementally, starting with 30-minute intervals to determine the optimal duration for your specific sample type. Similarly, in necessity, increase DSP concentration gradually, such as from 1 mM to 5 mM, while monitoring the solubility and sample quality. Document the outcomes of these adjustments carefully to optimize fixation conditions for your experimental needs.

Fixative Neutralization

30m

7 Centrifuge the tube and remove DSP/Methanol from cell pellet and perform 1x wash with 1 mL methanol. Ensure thorough washing after fixation to remove excess DSP. Carefully remove and discard methanol.

8 Add 1 mL Methanol supplemented with 20 µL of [M] 1 Molarity (M) Tris-HCl pH 7.5 ([M] 20 millimolar (mM) final) and let sit for at least 00:30:00 (larger chunks required longer times). 30m

Note

Excess reactive DSP, being an amine-reactive crosslinker, must be quenched with Tris-HCl buffer before proceeding. The quantity must be adjusted if more fixative was used for larger tissue or higher number of cells.

9 Store fixed samples at -20 °C or better -80 °C for up to 4 weeks (longer is possible) or proceed immediately to rehydration.

Rehydration

15m

10 Prior to rehydration, prepare 3X SSC by diluting 3 mL of SSC Buffer (20X) **Merck MilliporeSigma (Sigma-Aldrich) Catalog** #S6639 with 17 mL of Nuclease free water.

11 Thaw stored samples and equilibrated to 4 °C.

12 Centrifuge at 850 rcf for 00:05:00, discard supernatant. 5m

13 Add 1 mL of Wash-Resuspension Buffer (0.5% BSA in 3X SSC) with (0.2-0.5 U/µL) Protector RNase Inhibitor **Merck MilliporeSigma (Sigma-Aldrich) Catalog** #3335402001 or RiboLock RNase Inhibitor **Thermo Fisher Scientific Catalog** ##EO0381

14 Rehydrate On ice for 00:05:00 and centrifuge at 500 rcf for , 00:05:00 at 4 °C. 10m

For Tissue: Digestion and Washing

1h 30m

15 Digest using 1 mL of [M] 200 µg/µL Liberase in PBS for 00:30:00 - 00:45:00 at 37 °C with agitation. Pipette mix every 00:15:00. Optional: DNase I can be prepared from DNAse I **Roche Catalog** #04716728001 use at 1:1000 dilution. 1h 30m

Note

The selection of Liberase type, concentration, and digestion conditions (e.g., incubation time) must be optimized for the specific sample type being processed. This ensures effective tissue dissociation while minimizing damage to cellular or molecular targets. Always validate these parameters with pilot experiments using identical sample characteristics to refine reproducibility and performance. For more details: **Liberase Research Grade Purified Enzyme Blends**

16 Filter the cell suspension through a 70-µm cell strainer, e.g. pluriStrainer mini 70 µm (cell strainer), to remove aggregates and debris. Wash the filtered cells three times with PBS containing 1% BSA, then resuspend the washed cells in PBS with 0.04% BSA.

For Cells: Preparation and Analysis

17 Filter the cell suspension through a 40-µm mesh, e.g. pluristrainer mini 40 µm (cell strainer) to remove aggregates and debris, then count the cells and adjust their concentration to 1000–1500 cells/µL for subsequent analysis.

18 Proceed with the chosen analysis method, ensuring sample preparation aligns with platform requirements.

Storage and Preservation

19 Optionally, for later use or shipment, freeze cells in CryoStor CS10 100ML **Fisher Scientific Catalog #**NC9930384 using a controlled rate freezing container, for example

Mr. Frosty Freezing Container, 2mL tubes, Nalgene Mr. Frosty Freezing Container for 1-2mL cryogenic tubes, PC, clear w/ blue lid, 1/Cs. **Thermo Fisher Catalog #**5100-0001

20 Store at -80 °C until use. Thaw samples in a 37 °C water bath and wash twice with PBS + 0.5-1% BSA.

Protocol references

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