Processing fixed and stored adipose-derived stem cells for quantitative protein array assays

Jessica S. Sadick¹ and Eric M. Darling^{1,2,3,4}

¹Department of Molecular Pharmacology, Physiology, and Biotechnology,

²Center for Biomedical Engineering, ³School of Engineering, and

⁴Department of Orthopaedics, Brown University, Providence, RI

BioTechniques 63:275-280 (December 2017) doi 10.2144/000114620

Keywords: FITSAR; protein characterization; paraformaldehyde; multiplex ELISA

Supplementary material for this article is available at www.BioTechniques.com/article/114620.

Accurately characterizing cellular subpopulations is essential for elucidating the mechanisms underlying normal and pathological biology. Isolation of specific cell types can be accomplished by labeling unique cell-associated proteins with fluorescent antibodies. Cell fixation is commonly used to prepare these samples and allow for long-term storage, but this poses challenges for subsequent protein analysis. We previously established the FITSAR (formaldehyde-fixed intracellular target-sorted antigen retrieval) method, in which protein can be isolated and characterized from fixed, enriched cell subpopulations. Here, we improve on this method by allowing compatibility with highly sensitive multiplex protein arrays and demonstrating applicability to long-term stored samples. Feasibility experiments demonstrated parallel detection of cell adhesion molecules (CAMs) using an enzyme-linked immunosorbent assay (ELISA) panel with human adipose-derived stem cells (ASCs) stored for up to 1 month.

Cell type-specific protein characterization is essential for accurate understanding of normal and pathological biology. The ability to extract protein from fixed cells increases flexibility in sample preparation and the potential for intracellular target-based enrichment. We previously established the FITSAR (formaldehyde-fixed intracellular target-sorted antigen retrieval) method, in which protein is isolated and characterized from fixed enriched cell populations (1). Using adipose-derived stem

cells (ASCs) as our cell type of interest, we expand and improve upon FITSAR with two objectives: (i) to validate protein isolation from fixed and stored single-cell suspensions and (ii) to demonstrate detection of isolated proteins by enzyme-linked immunosorbent assay (ELISA) arrays.

A multi-donor human ASC superlot (passage 5) (#36; Zen-Bio Inc., Research Triangle Park, NC) was used for all experiments. ASCs were cultured in medium containing DMEM/F12 (Cat.

#SH30023.01; HyClone, Logan, UT), 1% antibiotic/antimycotic (Cat. #SV30079.01; HyClone), 10% fetal bovine serum (FBS) (Cat. #SER-500; Zen-Bio Inc.), 1 ng/mL fibroblast growth factor (FGF) (Cat. #233-FB), 5 ng/mL epidermal growth factor (EGF) (Cat. #236-EG), and 0.25 ng/mL transforming growth factor- β 1 (TGF- β 1) (Cat. #240-B) (all growth factors from R&D Systems, Minneapolis, MN) (2) and were maintained at 37°C, 5% CO₂.

To ensure reproducible protein isolation from cells chemically fixed in 4% paraformaldehyde (PFA) (Cat. #28906; ThermoFisher Scientific, Waltham, MA) as compared with fresh samples, protein extraction protocols were assessed by total protein amount (Pierce BCA Assay Kit, Cat. #23225; ThermoFisher Scientific) and quality on a Coomassie Blue (MP Biomedicals LLC, Santa Ana, CA)-stained PAGE gel (precast gel, Cat. #456-9033; Bio-Rad, Hercules, CA) ($n = 3$). Fresh and PFA-fixed ASCs were lysed either on ice or by boiling ($n = 3$). Lysis buffer was composed of Tris-HCl and SDS, as well as protease/phosphatase inhibitors (Halt Protease and Phosphatase Single-Use Inhibitor Cocktail, 100 $\times$; Cat. #78442, ThermoFisher Scientific) (details in Table 1 and the Supplementary Protocol) (3). No significant difference in protein yield or banding was observed between the gold standard approach using fresh ASCs lysed on ice (Fr-i) (4) and the fixed/boiled method (PFA-b) ($P > 0.05$) (Figure 1A, Supplementary Figure S1). Additionally, findings were similar when using formalin (4% PFA + 1.5% methanol) or pure 4% PFA fixatives (Supplementary Figure S2). As corroborated by the literature regarding formalin-fixed paraffin-embedded (FFPE) samples, successful protein extraction from fixed samples requires exposure to formaldehyde scavenging and denaturing reagents (high concentrations of both Tris-HCl and SDS) in addition to high heat to promote protein untangling and methylene bridge thermal hydrolysis (3,5,6).

Being able to evaluate stored, fixed samples would increase the user flexibility of the FITSAR process. PFA-fixed

METHOD SUMMARY

Here, we present a method in which protein from stored formaldehyde-fixed samples may be assessed on high-throughput, multiplex, enzyme-linked immunosorbent assay (ELISA) arrays.

Figure 1. Protein extraction amount and quality are similar between fresh and formaldehyde-fixed/stored samples when using appropriate cell lysis buffer and exposure to high heat. (A) Total protein amount isolated from fresh (Fr) and PFA-fixed (PFA) adipose-derived stem cells (ASCs) was compared under two lysis conditions (i: on ice; b: by boiling). Data are shown as mean $\pm$ SD from three independent experiments. A Kruskal-Wallis test with post-hoc Tukey test was performed to determine significance (* denotes significantly different from unmarked groups, $P < 0.05$). The Coomassie Blue-stained PAGE gel (shown below) indicated protein quality through a comparison of banding profiles. Low protein yields and increased aggregation of proteins (>260 KDa) in the PFA-i condition underscore the importance of the lysis process used to isolate proteins from fixed samples. (B) Total isolated protein was compared across PFA-fixed ASCs stored for 0–28 days. Data are shown as mean $\pm$ SD from one independent experiment run in biological triplicate. A one-way ANOVA with Dunnett's test with Day 0 (D0) as the point of comparison was performed to determine significance (* denotes a statistically significant difference from D0, $P < 0.02$). Protein quality was assessed by Coomassie Blue-stained PAGE gel as shown in the lower panel.

ASCs were suspended in PBS and stored at 4°C for 0–28 days. After storage, samples were lysed by boiling and assessed for total extracted protein yield and quality ($n = 3$). Fixed cells could be stored for up to 7 days before undergoing a minor but statistically significant decrease in total protein yield ($P < 0.02$) (Figure 1B). The ~15%–20% decrease between days 0–14 and 0–28 did not produce visible changes in protein banding profiles among samples isolated across all storage conditions (Figure 1B). This suggested that the collected protein is generally representative of the sample's proteome and that any loss is homogeneously distributed across protein types.

ELISA arrays, a platform in which multiple proteins of interest (POIs) may be detected simultaneously at picomolar concentrations, were chosen to demonstrate the expanded compatibility of FITSAR with other protein analysis techniques. ELISA arrays are incompatible with the SDS in the original FITSAR sample processing steps, since detergents interfere with antigen–antibody interactions by forming adducts with protein residues (7). Therefore, the FITSAR sample processing method was adjusted to include an SDS-exclusion step using commercially available, detergent-removing resin spin columns (Pierce Detergent Removal Spin Columns, Cat. #87776/#87777; ThermoFisher Scientific). Successful SDS removal from fixed cell lysates was verified using the Stains-all Assay (Cat. #213512500; Acros Organics, Geel, Belgium) (8,9), and protein yield was quantified. More than 99.99% of

the SDS was removed, as evidenced by the lack of a characteristic SDS absorbance peak at 440–450 nm wavelength for all processed samples (Figure 2A). Protein loss in the columns varied depending on column size. For small columns (10–25 μ L sample volume), the yield of eluted protein was not significantly different from the amount of loaded protein ($P = 0.29$) ($n = 3$) (Supplementary Figure S3A). For large columns (25–100 μ L sample volume), the yield of eluted protein was significantly less than the amount of loaded protein ($P < 0.001$) ($n = 3$) (Supplementary Figure S3B). Regardless of size, these spin columns function by providing a hydrophobic microenvironment in the resin cavity into which small nonpolar compounds, such as SDS, infiltrate and subsequently form an inclusion complex (7). Non-specific adsorption or entrapment of protein is expected to be distributed evenly across protein types, resulting in a detergent-free protein lysate that is representative of the original sample proteome.

As a proof-of-concept, SDS-purified ASC storage protein lysates ($n = 3$) were assessed using a multiplex human cell adhesion molecule (CAM) ELISA array (QAH-CAM-1; RayBiotech, Norcross, GA). Isolation of cell-surface/membrane-bound proteins from fixed samples is notoriously variable compared with proteins isolated from the cytosol or nucleus (10,11), thus providing a challenging test of the reliability and rigorousness of the processing involved in the updated FITSAR method. Each CAM ELISA array consisted of 17 POIs (Table 2) printed in

Figure 2. Detergent-free protein lysates were successfully detected by a cell adhesion molecule (CAM) ELISA array. (A) Representative absorbance spectra of the Stains-all Assay comparing SDS-positive (blue) and -negative (purple) controls to column-processed adipose-derived stem cell (ASC) storage protein lysates (shades of red). The lack of an absorbance peak at 440–450 nm indicates successful removal of SDS detergent. (B) Quantified protein expression of CAMs expressed in ASC lysates, comparing fresh and fixed/stored samples. Data are shown as mean $\pm$ SD from one independent experiment run in biological triplicate (technical quadruplicate). Within each protein of interest (POI), one-way ANOVA was performed to determine statistically significant differences.

Table 1. Processing parameters for cell lysis.

Processing parameters	Fr-i	PFA-i	Fr-b	PFA-b
While in suspension, ASCs were left in PBS.	✓		✓	
While in suspension, ASCs were fixed in 4% PFA for 10 min at room temperature.		✓		✓
ASCs were lysed in a 4°C ice bath for 30 min.	✓	✓		
ASCs were lysed in a boiling water bath at 100°C for 30 min, followed by 60°C for 2 h.			✓	✓
Lysis buffer was composed of 300 mM Tris-HCl, pH 8 + 2% SDS + 2× protease/phosphatase inhibitor.	✓	✓	✓	✓
Check marks indicate process used in preparing samples. ASCs: adipose-derived stem cells; PFA: paraformaldehyde; Fr-i: fresh/ice; PFA-i: PFA-fixed/ice; Fr-b: fresh/boiled; PFA-b: PFA-fixed/boiled.				

technical quadruplets. A six-point standard curve was used for quantification. Of the 17 CAM POI available, 8 were potentially expressed in the ASCs, given their species, origin, and passage number (12–14). Of these 8 POIs, 6 were detected, with 5 of the 6 proteins having no significant difference in expression level among fresh, fixed, and fixed/stored samples ($P > 0.05$) (Figure 2B). Only ICAM1 expression changed significantly, being higher in Day 7 and Day 28 fixed samples compared with Day 0 fresh and fixed samples ($P < 0.02$). This discrepancy is most likely due to the variability associated with these types of microarrays (15). However, another possibility is unforeseen bias favoring ICAM1 isolation during processing (i.e., ICAM1 extraction was more efficient than extraction of other proteins, resulting in an artificially inflated value after normalization to total protein levels). For all other proteins assessed, no differences from the gold standard approach was observed.

Our enhanced technique allows the assessment of stored fixed samples and the use of low-protein, high-throughput ELISA

Table 2. Proteins of interest (POIs) available on cell adhesion molecule (CAM) ELISA arrays.

POI	Proteins expressed in passaged ASCs (P3–6)	
	Expected	Observed
ALCAM	✓	✓
BCAM		
CEACAM1		
E-Cadherin		
EpCAM		
E-Selectin	?	✓
ICAM1	✓	✓
ICAM2		
ICAM3		
L-Selectin	✓	✓
NCAM1		
NrCAM		
P-Cadherin		
P-Selectin	?	X
PECAM1	?	X
VCAM1	?	✓
VE-Cadherin	✓	✓

Check marks indicate anticipated or observed expression in passaged adipose-derived stem cells (ASCs). Question marks indicate conflicting reports for expression in passaged ASCs. X marks indicate lack of observed expression (12–14).

arrays. The improvements to sample processing, specifically detergent removal, also provide potential compatibility with techniques such as mass spectrometry. By pairing these improvements with our FITSAR method, cell subpopulations of interest can be isolated and their protein expression profiles analyzed with flexibility in timing and the use of sensitive, quantitative assays.

A NEW Era in Pipetting

BRAND® Liquid Handling Station

✓COMPACT ✓EFFICIENT ✓AFFORDABLE

**Bring Automation to
Your Benchtop**

Request a demo today!

The new BRAND® Liquid Handling Station (LHS) is an extremely compact, versatile and easy to program pipetting robot. Free yourself from the monotonous and error prone tasks of manual liquid transfers.

BRANDTECH®
SCIENTIFIC, INC.

888-522-2726 | www.brandtech.com/LHS

Author contributions

J.S.S. and E.M.D. designed the study and wrote the manuscript. J.S.S. conducted all of the experimental work.

Acknowledgments

This work was supported by the National Institutes of Health (R01AR06304 to E.M.D.) and National Science Foundation (CAREER CBET 1253189 and EAGER CBET 1547819 to E.M.D., GRFP 2014183678 to J.S.S.). This paper is subject to the NIH Public Access Policy.

Competing interests

The authors declare no competing interests.

References

1. **Sadick, J.S., M.E. Boutin, D. Hoffman-Kim, and E.M. Darling.** 2016. Protein characterization of intracellular target-sorted, formalin-fixed cell subpopulations. *Sci. Rep.* 6:33999.
2. **Estes, B.T., B.O. Diekmann, and F. Guilak.** 2008. Monolayer cell expansion conditions affect the chondrogenic potential of adipose-derived stem cells. *Biotechnol. Bioeng.* 99:986-995.
3. **Kawashima, Y., Y. Kodera, A. Singh, M. Matsumoto, and H. Matsumoto.** 2014. Efficient extraction of proteins from formalin-fixed paraffin-embedded tissues requires higher concentration of tris(hydroxymethyl)aminomethane. *Clin. Proteomics* 11:4.
4. **Mahmood, T. and P.C. Yang.** 2012. Western blot: technique, theory, and trouble shooting. *N. Am. J. Med. Sci.* 4:429-434.
5. **Magdeldin, S. and T. Yamamoto.** 2012. Toward deciphering proteomes of formalin-fixed paraffin-embedded (FFPE) tissues. *Proteomics* 12:1045-1058.
6. **Addis, M.F., A. Tanca, D. Pagnozzi, S. Crobu, G. Fanciulli, P. Cossu-Rocca, and S. Uzzau.** 2009. Generation of high-quality protein extracts from formalin-fixed, paraffin-embedded tissues. *Proteomics* 9:3815-3823.
7. **Antharavally, B.S., K.A. Mallia, M.M. Rosenblatt, A.M. Salunkhe, J.C. Rogers, P. Haney, and N. Haghdoust.** 2011. Efficient removal of detergents from proteins and peptides in a spin column format. *Anal. Biochem.* 416:39-44.
8. **Rupprecht, K.R., E.Z. Lang, S.D. Gregory, J.M. Bergsma, T.D. Rae, and J.R. Fishpaugh.** 2015. A precise spectrophotometric method for measuring sodium dodecyl sulfate concentration. *Anal. Biochem.* 486:78-80.
9. **Rusconi, F., E. Valton, R. Nguyen, and E. Dufourc.** 2001. Quantification of sodium dodecyl sulfate in microliter-volume biochemical samples by visible light spectroscopy. *Anal. Biochem.* 295:31-37.
10. **Nirmalan, N.J., P. Hamden, P.J. Selby, and R.E. Banks.** 2009. Development and validation of a novel protein extraction methodology for quantitation of protein expression in formalin-fixed paraffin-embedded tissues using western blotting. *J. Pathol.* 217:497-506.
11. **Crockett, D.K., Z. Lin, C.P. Vaughn, M.S. Lim, and K.J.S. Elenitoba-Johnson.** 2005. Identification of proteins from formalin-fixed paraffin-embedded cells by LC-MS/MS. *Lab. Invest.* 85:1405-1415.
12. **Zuk, P.A., M. Zhu, P. Ashjian, D. De Ugarte, J. Huang, H. Mizuno, Z. Alfonso, J. Fraser, et al.** 2002. Human adipose tissue is a source of multipotent stem cells. *Mol. Biol. Cell* 13:4279-4295.
13. **Katz, A.J., A. Tholpady, S.S. Tholpady, H. Shang, and R.C. Ogle.** 2005. Cell surface and transcriptional characterization of human adipose-derived adherent stromal (hADAS). *Stem Cells* 23:412-423.
14. **Mitchell, J.B., K. McIntosh, S. Zvonic, S. Garrett, Z.E. Floyd, A. Kloster, Y.D. Halvorsen, R.W. Storms, et al.** 2006. Immunophenotype of human adipose-derived cells: temporal changes in stromal-associated and stem cell-associated markers. *Stem Cells* 24:376-385.
15. **Berg, D., S. Hipp, K. Malinowsky, C. Böllner, and K.F. Becker.** 2010. Molecular profiling of signalling pathways in formalin-fixed and paraffin-embedded cancer tissues. *Eur. J. Cancer* 46:47-55.

Received 21 September 2017; accepted 06 November 2017.

Address correspondence to Eric M. Darling, 175 Meeting Street, Brown University, Providence, RI 02912. E-mail: eric_darling@brown.edu

To purchase reprints of this article, contact: biotechniques@fosterprinting.com

ISOTYPE SWITCHING

Customize your antibodies

pFUSE-CLlg and pFUSE-CHlg plasmids are designed to change a monoclonal antibody (mAb) from one isotype to another therefore enabling the generation of antibodies with the same antigen affinity but with different effector functions (increased or reduced ADCC and CDC). Furthermore, they can be used to produce entire antibodies from fragment antigen-binding (Fab) or single-chain variable fragment (scFv) fragments that are either chimeric, humanized or fully human.

Mix & match to make the perfect antibody

- IgG, IgA, IgM, IgD, IgE and engineered IgG CH domains
- Lucia-labeled CH domains to make luminescent mAbs
- Ig-kappa and Ig-lambda CL domains