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

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Abstract

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, 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 (**F**ormaldehyde-fixed **I**ntracellular **T**arget-**S**orted **A**ntigen **R**etrieval) method in which protein can be isolated and characterized from fixed, enriched cell subpopulations. The current study improves 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 using an enzyme-linked immunosorbent assay (ELISA) panel with human, adipose-derived stem cells stored for up to one month.

Keywords

FITSAR; protein characterization; paraformaldehyde; multiplex ELISA

Cell type-specific protein characterization is essential for accurate understanding of normal and pathological biology. Ability to extract protein from fixed cells increases flexibility in sample preparation and potential for intracellular target-based enrichment. We previously established the FITSAR (**F**ormaldehyde-fixed **I**ntracellular **T**arget-**S**orted **A**ntigen **R**etrieval) method, in which protein was isolated and characterized from fixed, enriched cell populations (1). Using adipose-derived stem cells (ASCs) as our cell type of interest, this study expands and improves upon FITSAR via two objectives: 1) validate protein isolation

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from fixed and stored single cell suspensions and 2) demonstrate detection of isolated proteins on enzyme-linked immunosorbent assay (ELISA) arrays.

A multi-donor, human ASC superlot (Zen-Bio Inc., Research Triangle Park, NC; #36, passage 5) was used for all experiments. ASCs were cultured in medium containing DMEM/F12, 1% antibiotic/antimycotic (HyClone, Logan, UT), 10% fetal bovine serum (Zen-Bio Inc.), 1 ng/mL fibroblast growth factor, 5 ng/mL epidermal growth factor, and 0.25 ng/mL transforming growth factor- β 1 (R&D Systems, Minneapolis, MN) (2) and were maintained at 37°C, 5% CO₂.

To ensure reproducible protein isolations from cells chemically fixed in 4% paraformaldehyde (PFA) as compared to fresh samples, protein extraction protocols were assessed by total protein amount (BCA Assay, Pierce, ThermoFisher Scientific, Waltham, MA) and quality (Coomassie Blue-stained polyacrylamide gel electrophoresis (PAGE) gel, MP Biomedicals LLC, Santa Ana, CA) (N=3). Fresh and PFA-fixed ASCs were either lysed on ice or by boiling (N=3). Lysis buffer was composed of Tris-HCl, sodium dodecyl sulfate (SDS; Fisher Scientific, Hampton, NH), and protease/phosphatase inhibitors (Pierce) (details in Table 1, Supplementary Protocol) (3). No significant difference in protein yield or banding was observed between the gold standard approach (fresh ASCs lysed on ice, “Fr-i”) (4) and the fixed/boiled method (“PFA-b”) ($P > 0.05$) (Figure 1A, Supplementary Figure 1S). Additionally, findings were similar when using formalin (4% PFA + 1.5% methanol) or pure 4% PFA fixatives (Supplementary Figure 2S). As corroborated in formalin-fixed paraffin-embedded (FFPE) literature, 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 user flexibility with the FITSAR process. PFA-fixed ASCs were suspended in phosphate buffered saline 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 collected protein is generally representative of the sample’s proteome, and any loss is homogeneously distributed across protein types.

ELISA arrays, a platform in which multiple proteins of interest (POI) may be detected simultaneously at picomolar concentrations, were chosen to demonstrate the expanded compatibility of FITSAR with other protein analysis techniques. However, this technology is incompatible with SDS since detergents interfere with antigen-antibody interactions by forming adducts with protein residues (7). Therefore, sample processing methods were adjusted to include an SDS exclusion step using commercially available, detergent-removing resin spin columns (Pierce). Successful SDS removal from fixed cell lysates was verified using the Stains-All Assay (Acros Organics, Geel, BE) (8,9), and protein yield was quantified. Greater than 99.99% of 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 to the columns varied depending on column size. For small columns (10–25 μ L sample volume), eluted protein was not significantly different from loaded protein ($P = 0.29$) ($N = 3$) (Supplementary Figure S3A). For large columns (25–100 μ L sample volume), eluted protein was significantly less than 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, like 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 multiplexed, 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 to proteins isolated from the cytosol or nucleus (10,11), thus providing a challenging test of the reliability/rigorousness of the processing involved in the updated FITSAR method. Each CAM ELISA array consisted of 17 POI (Table 2) printed in technical quadruplet. A 6-point standard curve provided means for quantification. Of the 17 CAM POI available, 8 were potentially expressed in ASCs, given species, origin, and passage number (12–14). Of these 8 POI, 6 were detected, with 5/6 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 -28 fixed samples compared to 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 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 assessment of fixed, stored samples and use of low protein, high throughput ELISA arrays. The improvements to sample processing, specifically detergent removal, also provides potential compatibility with techniques like mass spectrometry and proteomics. 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 use of sensitive, quantitative assays.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Sadick JS, Boutin ME, Hoffman-Kim D, Darling EM. Protein characterization of intracellular target-sorted, formalin-fixed cell subpopulations. *Sci Rep*. 2016; 6:33999. [PubMed: 27666089]
2. Estes BT, Diekman BO, Guilak F. Monolayer cell expansion conditions affect the chondrogenic potential of adipose-derived stem cells. *Biotechnol Bioeng*. 2008; 99:986–995. [PubMed: 17929321]
3. Kawashima Y, Kodera Y, Singh A, Matsumoto M, Matsumoto H. Efficient extraction of proteins from formalin-fixed paraffin-embedded tissues requires higher concentration of tris(hydroxymethyl)aminomethane. *Clinical Proteomics*. 2014; 11:4. [PubMed: 24484752]
4. Mahmood T, Yang PC. Western blot: technique, theory, and trouble shooting. *N Am J Med Sci*. 2012; 4:429–434. [PubMed: 23050259]
5. Magdeldin S, Yamamoto T. Toward deciphering proteomes of formalin-fixed paraffin-embedded (FFPE) tissues. *PROTEOMICS*. 2012; 12:1045–1058. [PubMed: 22318899]
6. Addis M, Tanca A, Pagnozzi D, Crobu S, Fanciulli G, Cossu-Rocca P, Uzzau S. Generation of high-quality protein extracts from formalin-fixed, paraffin-embedded tissues. *PROTEOMICS*. 2009; 9:3815–3823. [PubMed: 19637237]
7. Antharavally BS, Mallia KA, Rosenblatt MM, Salunkhe AM, Rogers JC, Haney P, Haghdoost N. Efficient removal of detergents from proteins and peptides in a spin column format. *Anal Biochem*. 2011; 416:39–44. [PubMed: 21640699]
8. Rupprecht KR, Lang EZ, Gregory SD, Bergsma JM, Rae TD, Fishpaugh JR. A precise spectrophotometric method for measuring sodium dodecyl sulfate concentration. *Anal Biochem*. 2015; 486:78–80. [PubMed: 26150094]
9. Rusconi F, Valton E, Nguyen R, Dufourc E. Quantification of sodium dodecyl sulfate in microliter-volume biochemical samples by visible light spectroscopy. *Anal Biochem*. 2001; 295:31–37. [PubMed: 11476542]
10. Nirmalan NJ, Harnden P, Selby PJ, Banks RE. 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*. 2009; 217:497–506. [PubMed: 19156775]
11. Crockett DK, Lin Z, Vaughn CP, Lim MS, Elenitoba-Johnson KJS. Identification of proteins from formalin-fixed paraffin-embedded cells by LC-MS/MS. *Laboratory Investigation*. 2005; 85:1405–1415. [PubMed: 16155593]
12. Zuk P, Zhu M, Ashjian P, De Ugarte D, Huang J, Mizuno H, Alfonso Z, Fraser J, et al. Human Adipose Tissue Is a Source of Multipotent Stem Cells. *Mol Biol Cell*. 2002; 13:4279–4295. [PubMed: 12475952]
13. Katz AJ, Tholpady A, Tholpady SS, Shang H, Ogle RC. Cell Surface and Transcriptional Characterization of Human Adipose-Derived Adherent Stromal (hADAS) Cells. *Stem Cells*. 2005; 23:412–423. [PubMed: 15749936]
14. Mitchell JB, McIntosh K, Zvonic S, Garrett S, Floyd ZE, Kloster A, Halvorsen YD, Storms RW, et al. Immunophenotype of Human Adipose-Derived Cells: Temporal Changes in Stromal-Associated and Stem Cell-Associated Markers. *Stem Cells*. 2006; 24:376–385. [PubMed: 16322640]
15. Berg D, Hipp S, Malinowsky K, Böllner C, Becker KF. Molecular profiling of signalling pathways in formalin-fixed and paraffin-embedded cancer tissues. *Eur J Cancer*. 2010; 46:47–55. [PubMed: 19914823]

METHOD SUMMARY

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

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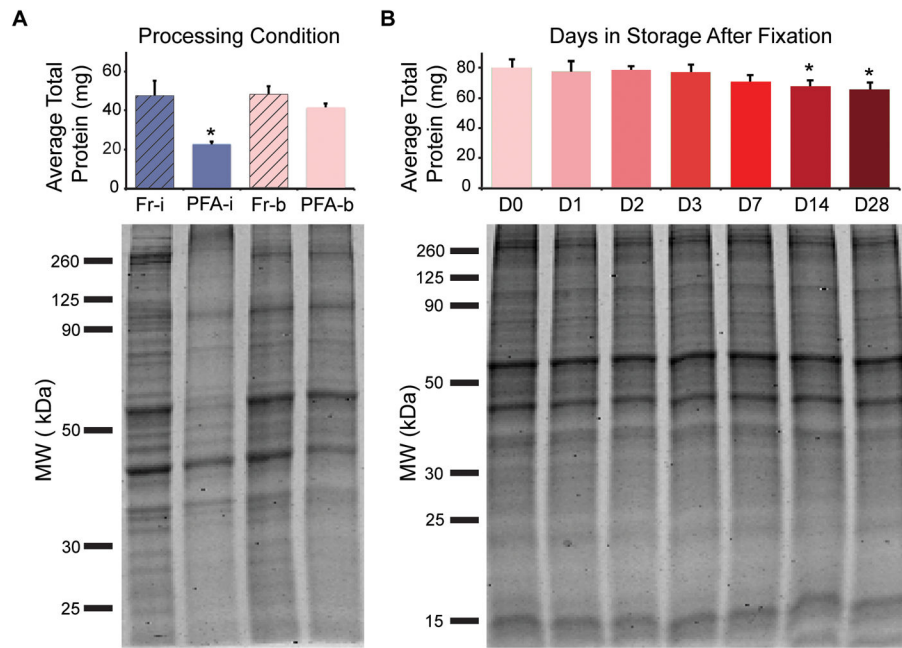


Figure 1. Protein extraction amount and quality are similar between fresh and formaldehyde-fixed/stored samples when using appropriate cell lysis buffer and exposure to high heat (A) Total protein amount isolated from fresh (Fr) and PFA-fixed (PFA) ASCs was compared under two lysis conditions (i, on ice; b, by boiling). Data represented as mean \pm standard deviation (s.d.) from three, independent experiments. A Kruskal-Wallis test with post-hoc Tukey test was performed to determine significance (* significantly different from unmarked groups, $P < 0.05$). Coomassie Blue-stained PAGE gels indicated protein quality through comparison of banding profiles. Low protein yields and increased aggregation of proteins (>260 KDa) in the “PFA-i” condition underscore importance of 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 represented as mean \pm s.d. from one independent experiment run in biological triplicate. A one-way ANOVA with Dunnett’s test (D0 as the point of comparison) was performed to determine significance (* significantly different from D0, $P < 0.02$). Protein quality assessed by Coomassie Blue-stained PAGE gels.

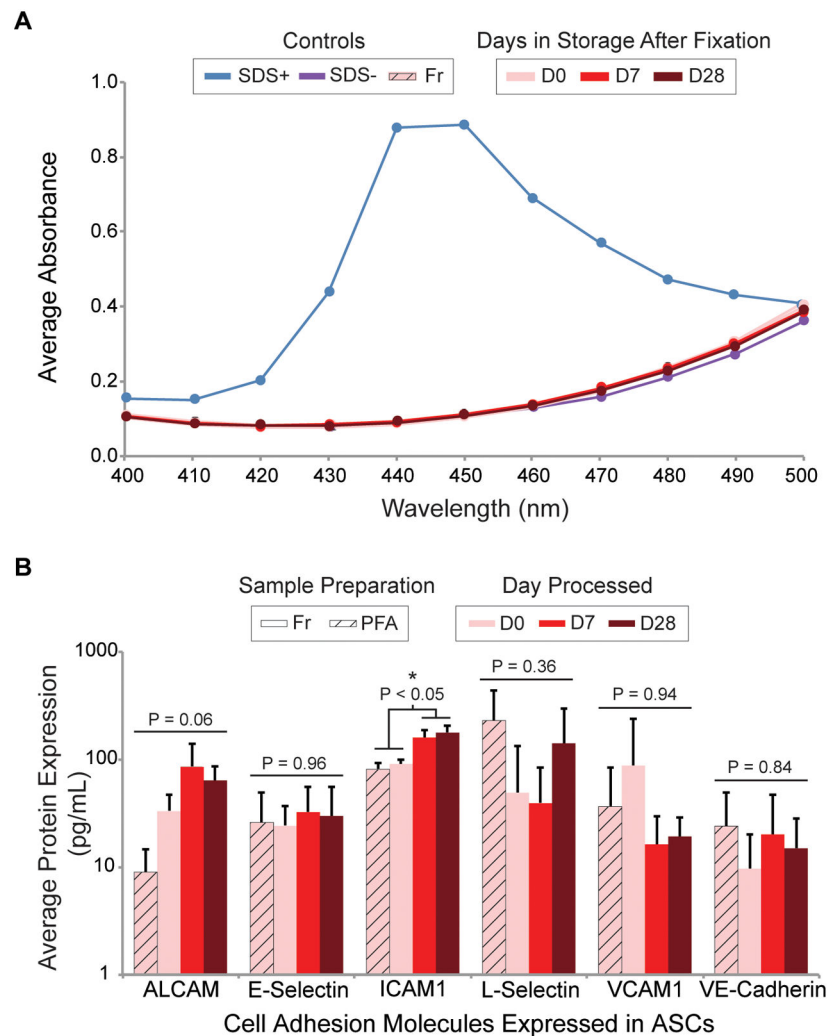


Figure 2. Detergent-free protein lysates were successfully detected on CAM ELISA array (A) Representative Stains-All Assay absorbance spectra comparing SDS positive (blue) and negative (purple) controls to column-processed ASC storage protein lysates (shades of red). Lack of 440–450 nm peak indicates successful removal of SDS detergent. (B) Quantified protein expression of CAMs expressed in ASC lysates comparing fresh and fixed/stored samples. Data represented as arithmetic mean \pm s.d. from one independent experiment run in biological triplicate (technical quadruplicate). Within each POI, one-way ANOVAs were performed to determine significance.

Table 1
Processing parameters for cell lysis

Check marks indicate process used in preparing samples. Fr-i, fresh/ice; PFA-i, PFA-fixed/ice; Fr-b, fresh/boiled; PFA-b, PFA-fixed/boiled.

Processing Parameters	Fr-i	PFA-i	Fr-b	PFA-b
While in suspension, ASCs were left in phosphate buffered saline (PBS).	✓		✓	
While in suspension, ASCs were fixed in 4% PFA for 10 minutes at room temperature.		✓		✓
ASCs were lysed in a 4°C ice bath for 30 minutes.	✓	✓		
ASCs were lysed in a boiling water bath at 100°C for 30 minutes followed by 60°C for 2 hours.			✓	✓
Lysis buffer was composed of 300 mM Tris-HCl (pH 8) + 2% SDS + 2X protease/phosphatase inhibitor.	✓	✓	✓	✓

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Table 2
POI available on CAM ELISA arrays

Check marks indicate anticipated or observed expression in passaged ASCs, question marks indicate conflicting reports for expression in passaged ASCs, and X marks indicate lack of observed expression (12–14).

POI	Proteins expressed in passaged ASCs (P3–6)	
	<i>Expected</i>	<i>Observed</i>
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	✓	✓