ProFACT PROTEOMICS
MAKING A FUNCTIONAL DIFFERENCE

Global Protein Analysis Using SeraFILE™, a Novel Proteomic Profiling Tool

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ABSTRACT

SeraFILE™ is a novel proteomic profiling tool designed for rapid quantification and functional analysis of protein samples. SeraFILE™, a proprietary surface library and associated automated protocols, was developed to characterize changes in the global proteomic composition of prokaryotic and eukaryotic samples. SeraFILE™ enables researchers to 'shuffle the deck' and visualize all of the proteins in a given sample based on their interaction with the surface library. Each of the eleven SeraFILE™ matrices have different functionalities, facilitating protein separation based on several protein characteristics concurrently. Protein samples are applied and weakly bound to the 11 SeraFILE™ surfaces and subjected to a mild, consecutive, multiple elution scheme. When all matrices are used in combination, the resultant 77 biologically active sub-proteomes offer a comprehensive signature of the starting sample. Serum studies confirm the value of SeraFILE™ in various types of profiling applications. SeraFILE™ does not require any depletion step to resolve low abundance proteins and nothing is removed from the primary sample. Some surfaces bind high abundance proteins while others leave them intact. For instance, albumin is removed in certain sub-proteomes, revealing low abundance serum proteins otherwise masked by albumin. In addition, the distribution of protein in sub-proteomes recovered from each matrix is reproducible independent of starting concentration. Functional profiles of the serum enzyme, alkaline phosphatase, verify that reproducible patterns of native activity are also generated.

SeraFILE™ offers a unique platform for Rational Proteome Prospecting™ and biomarker discovery. SeraFILE™ experiments were conducted with yeast lysates and patient matched tumor samples to study the Ubiquitin Proteasome Pathway. Preliminary data suggests that SeraFILE™ has the potential to shed light on regulatory factors and alternate conformers specific to certain cancers. Our multidimensional approach allows researchers to correlate changes in native enzyme function to corresponding simplified protein pools. These functional profiles may facilitate detection and identification of small molecule inhibitors and/or co-factors associated with the protein target of interest, thus offering a link to pre-clinical drug development.

Introduction

•SeraFILE™ is a high-throughput method that reduces protein pool complexity while maintaining native proteins that are still functionally active. These protein pools can then be profiled by traditional electrophoretic or bioassay methods.

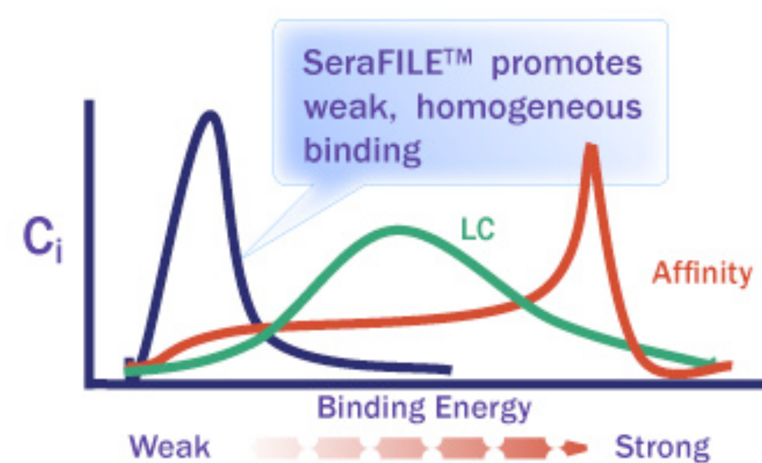
•Serum and specific tissues are a source of overexpressed and/or modified proteins unique to the disease state.

•Current serum proteomics applications have a suite of limitations, including the following 1) many protocols used in serum biomarker research purposely discard abundant serum proteins 2) discarded proteins may bind diagnostically relevant proteins in a given sample and 3) finding a single protein biomarker representative of the diseased state has proven difficult.

•SeraFILE™ may overcome these challenges in numerous ways. Certain surfaces remove albumin while others leave it intact, so all proteins in a serum sample can be resolved in the presence/absence of abundant serum proteins. Since proteins profiled by SeraFILE™ are still functionally active, they can be analyzed by a variety of downstream methods. The utility of SeraFILE™ allows for a more comprehensive understanding of the serum proteome in both the normal and diseased state.

•Sample heterogeneity is a recognized problem in clinical proteomics. SeraFILE™ is well suited to address the complex normalization issues involved in tissue/tumor proteomics applications.

SeraFILE™ Technology



ProFACT Proteomics' multi-disciplinary team of scientists have invented and characterized a novel proteomic profiling tool. SeraFILE™ (patent pending) is a surface library for sorting proteins for differential analysis. The SeraFILE™ surface library is not based on conventional liquid chromatography stationary phases. Whereas conventional LC (green) suffers from a heterogeneous mix of binding energies, and Affinity (red) has exceedingly high binding energy, SeraFILE™ promotes weak, homogeneous binding - ideal for proteomic investigation. Selectivity modulation comes from the structural morphology and spatial distribution of immobilized electrolytes, thus generating protein pools with both differential constituents and activity states.

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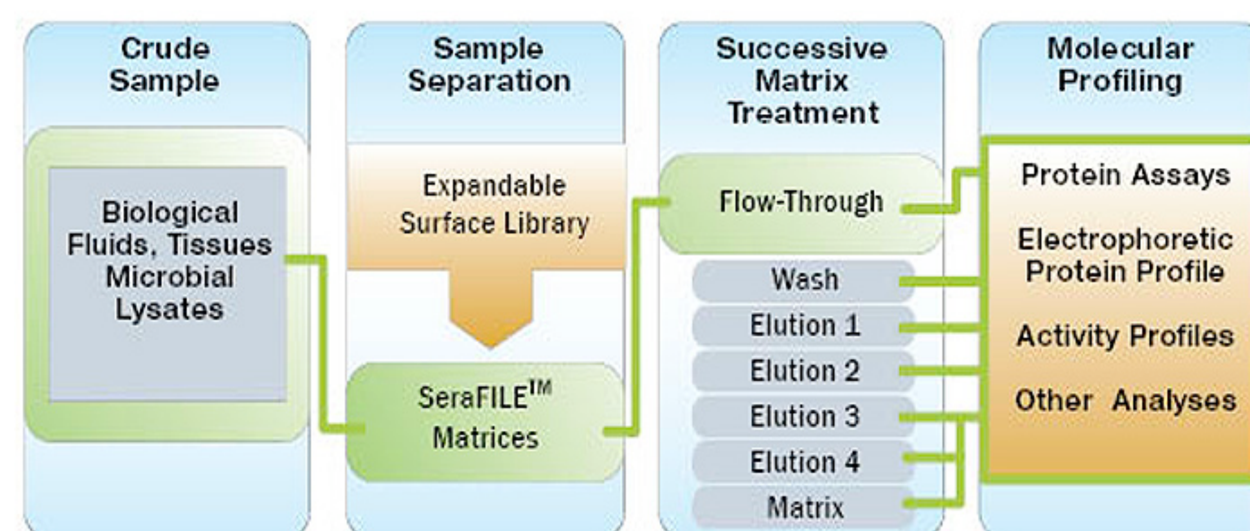
Protein Profiling with SeraFILE™



Methods

Protein Sample Preparation
•The protein content of all experimental samples was quantified using either BCA or Bradford chemistry
•Serum proteins were diluted to the appropriate starting concentration in SeraFILE™ buffer prior to surface application
•Tissue samples were homogenized, proteins were harvested, and lysates were diluted in SeraFILE™ buffer prior to application

SeraFILE™ Application
See schematic (right)

Protein Recovery
•All samples were read directly in triplicate at A 280nm on a NanoDrop ND-1000 spectrophotometer
•Protein concentration was assumed to be 1 mg/ml=1 AU

SDS-PAGE
Samples were analyzed on 10% Bio-Rad Criterion Tris-Glycine pre-cast gels (Hercules, CA)

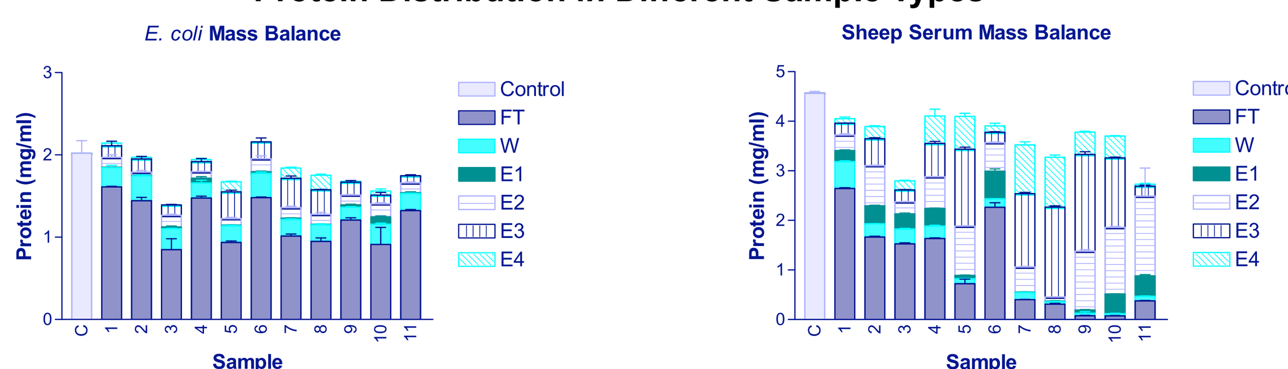
Alkaline Phosphatase Assay[1]
•Samples were adjusted to pH 9.5 prior to analysis
•60 µl samples were aliquoted in duplicate in 1.5 ml microcentrifuge tubes and incubated at room temperature for 20 minutes with 50 µl of PNPP substrate (Sigma Aldrich, Bellefonte, PA) and the reaction was stopped with 7.5 µl of 3M NaOH
•Samples were read at A 400 nm on a NanoDrop ND-1000 spectrophotometer (Wilmington, DE)
•Alkaline phosphatase activity was calculated based on a standard curve generated with commercial grade pure enzyme

Proteasome Activity Assay
•SeraFILE™-derived protein sub-fractions were mixed with 25mM HEPES ,0.5mM EDTA assay buffer, pH 7.5 containing 40µM SUC-Leu-Leu-Val-Tyr-AMC (Boston Biochem, Cambridge, MA)
•Chymotrypsin-like activity was determined using a Turner 7000 fluorometer (Sunnyvale, CA)

Data Analyses
•Gel images were analyzed using TotalLab software (Nonlinear Dynamics, Durham, NC)
•Statistical analyses were performed using GraphPad Prism software (San Diego, CA)

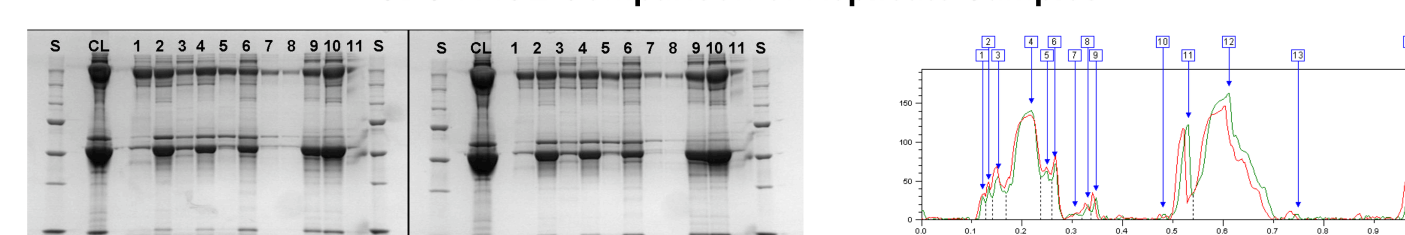
Reproducible Fractionation

Protein Distribution in Different Sample Types



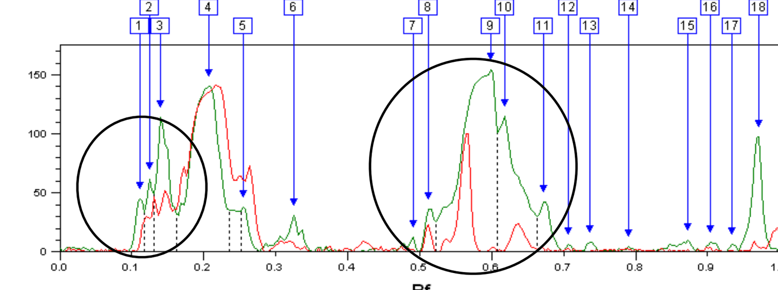
SeraFILE™ can be used to profile a variety of sample types. Distinct protein fractionation profiles are generated after treatment with each matrix. RIGHT: *E.coli* protein recovery after SeraFILE™ profiling. LEFT: Sheep serum protein distribution.

SDS-PAGE Comparison of Replicate Samples



LEFT: SDS-PAGE gels of replicate E2 samples for all eleven surfaces show the same protein profile when an automated 96 well plate method is used. SeraFILE™ proteomic pools are shown in duplicate for each surface (Sample A and Sample B). Lanes marked S represent Bio-Rad Precision Plus Protein Standards (Hercules, CA). Lanes marked CL represent the serum control. **RIGHT: TotalLab comparison of E2 pools between sample A (red) and sample B (green).**

Resolution of Low Abundance Proteins



LEFT: SeraFILE™ treatment resolves serum proteins masked by high abundance proteins like albumin. The figure shows a comparison of E2 SeraFILE™ proteomic profiles generated from surface 1 (red) and 9 (green). The serum albumin region, represented as a large green peak with an Rf value of 0.5-0.65, inhibits resolution of less abundant peaks (red) in the same region.

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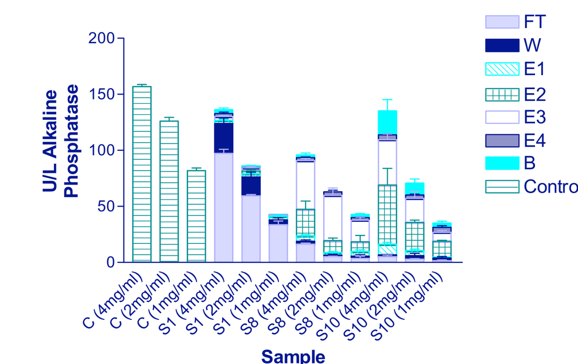
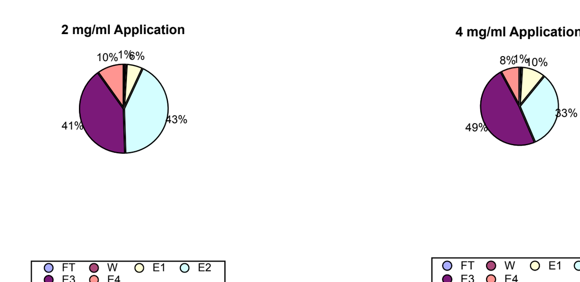
Native Activity Profiling

Alkaline Phosphatase Activity



LEFT: Enzyme activity distribution in sub-proteomes generated with SeraFILE™ Surfaces 1, 8, and 10 in three different protein application concentrations (4, 2, and 1 mg/ml respectively). Despite initial load concentration, the distribution of activity recovered from the matrices in each fraction is the same for each surface tested. Over an application concentration range of 1-4 mg/ml protein, a reproducible pattern of total enzyme activity recovery is observed in each matrix. Each application concentration was assayed in quadruplicate for each surface.

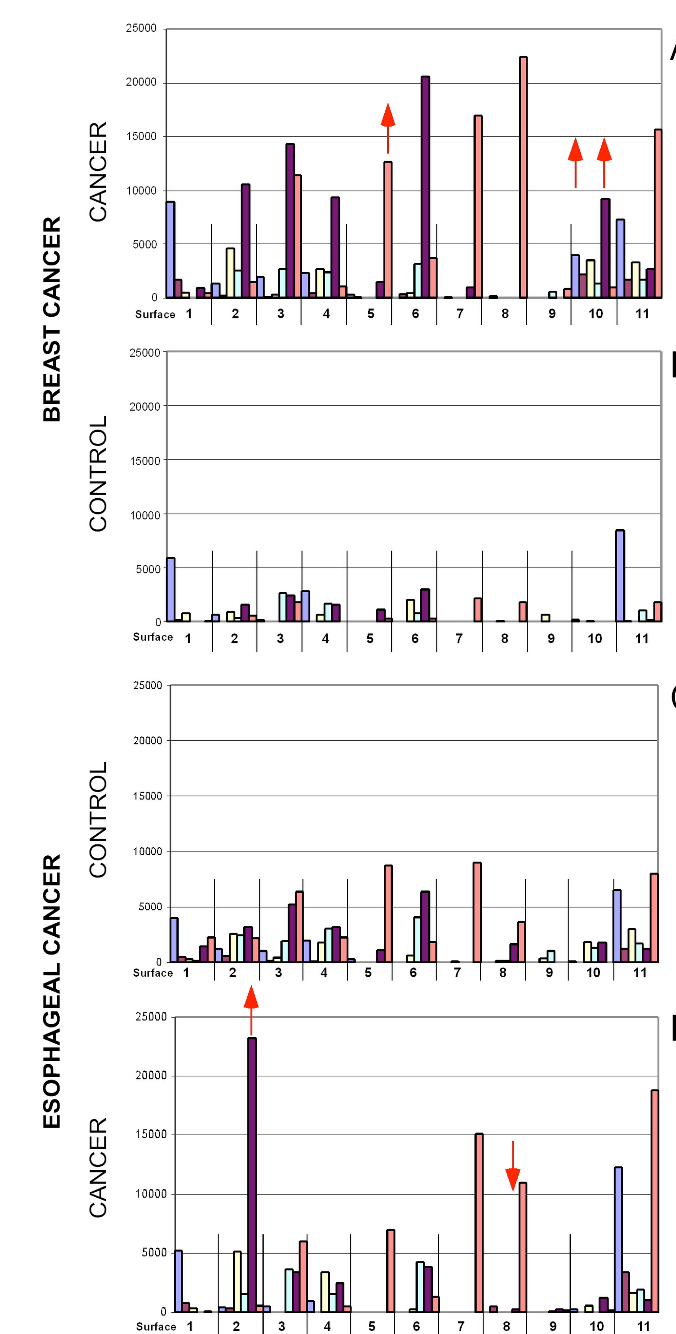
Alkaline Phosphatase Recovery



Legend 1: FT, **2:** W, **3:** E1, **4:** E2, **5:** E3, **6:** E4, **7:** B
The charts above represent the percentage of alkaline phosphatase activity recovered in each fraction for surface 10 over two application concentrations (i.e. 4 and 2 mg/ml total serum protein) in separate experiments. The activity recovery is consistent for each surface in all sub-proteomes (tested in quadruplicate) despite initial load concentrations. The data for serum samples at 1 mg/ml total protein is not shown, but is consistent with the results seen in more concentrated samples.

Clinical Applications

Proteasome Activity After SeraFILE™ Treatment



SeraFILE™-derived enzymatic activity profiles may provide a means to detect disease-specific differences. LEFT: Proteasome chymotryptic activity increased significantly in cancer samples in most sub-fractions, for both breast and esophageal cancer (A and D), consistent with previous studies. Red arrows indicate altered proteasome activity in a few notable sub-proteomes compared to control extracts (for instance flow-through fractions from matrix 1 and 11; B).

Altered proteasome activity in certain sub-fractions may have clinical relevance because 1) disease-specific inhibitors (i.e. potential drug targets) may be identified in other fractions and 2) activity profiles may function as disease-specific biomarkers.

Prostate-Specific Antigen (PSA) Serum Studies

Surface Designation	S1	S5	S6	S11	C
Number of Putative proPSA Isoforms Detected	2	4	1	2	1

Preliminary Western blot data suggests that SeraFILE™ surface architectures may be able to discriminately bind proPSA isoforms (above). Current ELISA-based diagnostics are problematic; many isoforms of PSA, including BPSA, cross-react with the immunoassay and are not specific to cancer. Serum from late stage prostate cancer patients was fractionated using SeraFILE™ matrices 1, 5, 6, and 11. One band was present in the unfractionated serum (C).

Rapid and selective enrichment of isoforms associated with cancer may provide an avenue for improved diagnostics.

Conclusions

•SeraFILE™ is an automated proteomic profiling system that generates protein pools that retain biological activity.
•Different protein profiles are observed after treatment with each individual matrix. When all matrices are used in combination, 77 sub-proteomes are generated. These protein pools are amenable to a variety of downstream analyses.
•Low abundance proteins can be unmasked using SeraFILE™ profiling technology.
•High abundance proteins, like albumin, can be removed in certain SeraFILE™ sub-proteomes. It is important to note that SeraFILE™ technology does not discard any proteins in a given sample; all protein can be accounted for in either the FT, W, E1-E4, or bound to the matrix.
•In order to assess if native enzyme activity could be retained and effectively profiled in SeraFILE™ sub-fractions, alkaline phosphatase activity was assayed in sheep serum protein pools. Despite initial protein load concentration, the distribution of protein and enzyme activity recovered from the matrices in each fraction is the same for each surface tested. Over an application concentration range of 1-4 mg/ml protein, a reproducible pattern of total protein and activity recovery is observed in each matrix.
•Clinical proteomics applications are encumbered by standardization protocol difficulties, so samples may not be adequately compared. For example, biopsies of tumor cells often include some portion of the normal adjacent tissue or necrotic portions with no cellular activity (2,4,5). These problems, inherent in tissue based proteomics, may be by-passed by profiling with SeraFILE™ because within a given range, the amount of protein applied to the SeraFILE™ matrices does not appear to affect the proteomic profile generated. This would imply that normalizing to tissue weight or total soluble protein would not be a factor in the experimental outcome.
•SeraFILE™ may be used as part of a multiplexed clinical proteomic approach. Protein profiling, native activity profiling, and isoform discovery may enhance disease detection strategies.