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Weak protein-protein interactions revealed by immiscible filtration assisted by surface tension (IFAST)

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Abstract

Biological mechanisms are often mediated by transient interactions between multiple proteins. The isolation of intact protein complexes is essential to understanding biochemical processes and an important prerequisite for identifying new drug targets and biomarkers. However, low-affinity interactions are often difficult to detect. Here, we use a newly described method called immiscible filtration assisted by surface tension (IFAST) to isolate proteins under defined binding conditions. This method, that gives a near-instantaneous isolation, enables significantly higher recovery of transient complexes as compared to current wash-based protocols, which require re-equilibration at each of several wash steps, resulting in protein loss. The method moves proteins, or protein complexes, captured on a solid phase through one or more immiscible phase barriers that efficiently exclude the passage of non-specific material in a single operation. We use a previously described polyol-responsive monoclonal antibody (PR-mAb) to investigate the potential of this new method to study protein-binding. In addition, difficult-to-isolate complexes involving the biologically and clinically important Wnt signaling pathway were isolated. We anticipate that this simple, rapid method to isolate intact, transient complexes will enable the discoveries of new signaling pathways, biomarkers, and drug targets.

Keywords

Immunoprecipitation; polyol-responsive; green fluorescent protein; lowdensity lipoprotein receptor; glycogen synthase kinase-3beta; exclusion-based sample preparation

Introduction

The identification of new protein-protein interactions will expand our understanding of cellular processes, unlock new drug targets, and clarify mechanisms of disease progression. Affinity purification methods, such as co-immunoprecipitation (co-IP), are commonly used

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to isolate protein complexes by employing an antibody to selectively capture one protein and identify other protein binding partners associated with the complex via mass spectrometry, Western blotting, ELISA, or other methods. Conventional co-IP techniques selectively capture protein complexes from sample lysate using a substrate such as a column of packed affinity beads or a paramagnetic particle (PMP). Once a protein complex is captured, the substrate is typically washed 3 to 6 times to separate the target complex from unbound, non-specific proteins in the lysate.

Protein-protein interactions that are weak (high dissociation constant, K_D) or brief (short half-life of the complex) tend to dissociate during washing, thus eluding detection (Fig. 1). Many critical biological processes are mediated through very transient interactions (e.g., binding of an enzyme to a substrate, transcription factors binding to transcriptional machinery). As each wash buffer is added to the immobilized complex, some proteins will dissociate as the complex re-equilibrates, resulting in loss of the complex. For labile complexes, the cumulative loss may be substantial, potentially preventing detection. While washing is essential to ensure the purity of a target protein complex, the time and manipulation required during this stage of the co-IP process can adversely affect the recovery of the intact complex. Although several researchers have raised this issue [1,2], simple solutions to this problem have been elusive. Techniques involving energy transfer between binding partners, such as bioluminescence resonance energy transfer (BRET) and fluorescence resonance energy transfer (FRET), have been developed and used successfully, but require pre-labeling of the potential binding partners with sensor molecules. This requirement mandates *a priori* knowledge of the potential interactors and production of labeled or fusion proteins that may not behave in a native manner. Therefore, a technology that enhances the ability to isolate and identify endogenous interactions would be of great value across the life sciences.

The advent of paramagnetic particle (PMP) techniques has vastly improved the speed of recovery of co-IP complexes. However, there is still considerable manipulation and time required to perform these experiments in a conventional procedure with multiple wash steps. Thus the binding partner can be lost during these manipulations.

In this paper, we describe a technique that can be used to identify and to study weakly bound protein complexes by replacing the wash steps of a conventional co-IP, using a PMP protocol, with an exclusion-based sample preparation (ESP) technology: Immiscible filtration assisted by surface tension (IFAST). This technique replaces entire washing protocols with a nearly instantaneous purification, thus eliminating washing-related dissociation of labile complexes. The IFAST technology is one of a class of ESP isolation methods that use exclusion principles pioneered by our lab [3–9]; and others [10–14] for the isolation of nucleic acids, whole cells, and single proteins with PMP. In these previous studies, immiscible phase filtration was used to expedite and streamline the isolation process. In this report, we show that the gentle, rapid IFAST technique dramatically improves the yield (and thus the detection) of weakly bound proteins and intact protein complexes.

Materials and Methods

IFAST Device Fabrication

IFAST devices were fabricated from polydimethylsiloxane (PDMS; Sylgard 184, Dow Corning) using soft lithography, then pressed onto glass bottoms (No. 1 cover glass, Fisher) as described in [15]. The initial IFAST configuration consisted of three wells (volume/well = 8.5 μ l) connected by two trapezoidal microfluidic channels (Fig. 2A and 2B). The shape of the microfluidic conduit was chosen in order to establish a region of minimal surface energy,

termed a “virtual wall” [12,13]. During device filling, liquid will flow from the well area into the microchannel, but stop at the narrowest part of the microchannel rather than flow into the next well due to the consequent increase in surface energy. This phenomenon enables the serial filling of the interconnected wells since each liquid is sequestered within its own region by virtual walls (Fig. 2A). Alternative configurations containing an input well with larger volume (200 μ l) and/or additional oil barriers in series (total of 2 or 3) were also fabricated in a similar manner (Fig. 2C–E)

Protein Expression and Preparation of Lysates

The plasmid construct containing green fluorescent (GFP) with a C-terminal epitope tag consisting of the amino acids PEEKLLRAIFGEKAS (etGFP) and the expression of soluble protein by growth at 26°C in *E. coli* in the presence of an over-expressed *GroEL* and *GroES* system has been described [16]. Because the epitope tag was derived from the β -subunit of RNA polymerase, the bacterial lysate was adjusted to 300 mM NaCl and polyethyleneimine was added to a final concentration of 0.3%. The resulting precipitate was removed by centrifugation (7000 $\times$ g, 5 min). This treatment removed the nucleic acids and the RNA polymerase as well as some other anionic proteins. To this lysate was added an amount of His6-tagged red fluorescent protein (RFP) that had been produced in *E. coli* and purified on a Ni-NTA column (Qiagen). In this mixture, the initial concentration of RFP was 20 times higher than the concentration of etGFP. In this paper, the reference to “bacterial lysate” refers to this processed protein mixture.

Preparation of PMP for etGFP Experiments

PR-mAb 8RB13, which reacts with the etGFP, has been described [15]. A solution containing 15 mg/ml Protein G-conjugated PMPs (Dynabeads Protein G, Invitrogen) and 0.031 mg/ml mAb 8RB13 in phosphate-buffered saline (PBS) containing 0.01% Tween 20 (PBST) was prepared and incubated for 30 minutes at room temperature to allow mAb attachment to the PMPs. The beads were then washed twice with 100 μ l of PBST.

IFAST Operation and Characterization

mAb-labeled PMPs were re-suspended in PBS (15 mg/ml PMP concentration) and 2% (by volume) bacterial lysate was added. At this dilution, the concentrations of the etGFP and RFP were approximately 12 μ g/ml and 240 μ g/ml, respectively. Following a 10-minute incubation of the bacterial lysate with mAb-PMPs at room temperature with rotation, the etGFP was purified using both conventional PMP-based purification and IFAST.

Conventional PMP-based purification was done according to the manufacturer’s protocol (Invitrogen Immunoprecipitation Kit). Briefly, a magnetic stand (DynaMag-2, Invitrogen) was utilized to aggregate PMPs from 100 μ l of PMP/bacterial lysate solution onto the side of a 1.5-ml microcentrifuge tube. After removal of the supernatant, 200 μ l of wash buffer (Invitrogen IP kit) was added and the PMP aggregate was resuspended via agitation with a micropipette. This wash process was repeated for a total of four washes before the protein was eluted with elution buffer (50 mM Tris-HCl and 0.1 mM EDTA (pH 7.9) containing 750 mM ammonium sulfate (AS) and 40% propylene glycol).

For IFAST purification, 8.5 μ l of bacterial lysate, 8.5 μ l oil (FC40 Fluorinert Oil, 3M Corporation or olive oil, Unilever), and 8.5 μ l elution buffer (as described in previous paragraph) were added to the appropriate wells (Figure 2A) using a micropipettor or other liquid handler. Olive oil was used in initial experiments, but was later exchanged for FC40 oil, which, as a fluorocarbon, is immiscible with more liquids. It is also manufactured in a more controlled manner than olive oil. A magnetic bar (BX041, K&J Magnetics) was then placed under the input well and used to draw the PMP aggregate through the oil and into the

elution buffer at a rate of approximately 1–2 mm/sec (total traverse time $\approx$ 3–4 sec) (See Supplementary Video 1). Once in the elution buffer, PMPs were given 2 minutes for elution before the eluent was collected via pipette for analysis. In some experiments, samples were purified using two or three oil barriers in series with intermediate wells containing 8.5 μ l wash buffer in an attempt to generate higher purity. As the PMP/analyte aggregate passed through each volume of wash buffer, they were briefly ($t \approx$ 3 sec) mixed to facilitate release of carryover from the PMP/analyte aggregate.

Varying Binding Conditions

To demonstrate the ability of the IFAST to isolate weakly bound protein, lysate containing epitope-tagged etGFP protein (1% by volume, approximately 12 μ g/ml etGFP) was mixed with mAb-labeled PMPs in a variety of solutions containing 20% propylene glycol and 0 to 250 mM AS and incubated for 30 minutes at room temperature to allow protein binding. Previous work [16] demonstrated that the strength of the mAb 8RB13/epitope tag interaction could be weakened by increasing AS concentration, such that weakly bound complexes could be artificially generated in a predictable and repeatable manner. IP of etGFP was performed using both IFAST and washing-based protocols as previously described, except that the washing and binding solutions were replaced by AS buffers (50 mM Tris-HCl and 0.1 mM EDTA, pH 7.9) containing 0 to 250 mM AS and 20% propylene glycol. As before, elution was performed in a solution containing 750 mM AS and 40% propylene glycol and the etGFP recovered was quantified.

Quantification of etGFP and RFP

In order to quantify the etGFP, ($\lambda_{\text{excitation}} = 490$ nm and $\lambda_{\text{emission}} = 509$ nm) and RFP ($\lambda_{\text{excitation}} = 563$ nm and $\lambda_{\text{emission}} = 582$ nm), in the various steps in the IP (bacterial lysates, depleted lysates, washing steps (where applicable), and eluted materials), solutions were loaded into well plates (384- or 1536-well) and imaged using a fluorescent scanner (Typhoon Trio, GE) and quantified with ImageQuant software. Well plates were used to prevent evaporation during the scanning process, which took approximately 15 minutes, as the IFAST devices are not sealed. A two-tailed, unpaired Student's t-test was used to determine significance. Representative IFAST samples were fluorescently imaged during purification (Fotodyne Luminary).

Measurement of Carryover

Calculation of interstitial carryover of material, using fluorescein dye, in the PMP aggregates is presented in the Supplemental Material.

SDS-PAGE

Samples were loaded onto SDS-PAGE gels (NuPAGE 4–12% Bis-Tris Gel, Invitrogen), electrophoretically separated for 50 minutes at 200 V, and imaged using silver stain (SilverQuest Silver Staining Kit, Invitrogen).

Application to Wnt Pathway

Wild-type mouse embryonic fibroblast (MEF) cells were lysed using a protocol described previously [17]. Briefly, approximately 400 μ g of lysate protein was mixed with 5 μ l of PMPs coated with 1.2 μ g of anti-LRP5 antibody (Cell Signaling) overnight at 4°C. Lysate / PMP mixtures were loaded into the input wells of IFAST devices with 200 μ l input volumes and two oil barriers in series. Wash buffer (1 $\times$ PBS, 0.01% Tween-20) was loaded between the two oil barriers to provide a brief (<1 second) wash. The output well of the device was also loaded with wash buffer. Isolated protein complexes were mixed with SDS-PAGE sample buffer, and 2 μ l of purified protein complexes or 15 μ g of unbound protein were

separated by SDS-PAGE and Western blots were prepared. All antibodies for probing the Western blots were purchased from Cell Signaling, except for the antibody to vinculin, which was purchased from Chemicon. Western blots were imaged using a BioRad Imager and quantified using Image J software by measuring the intensity of each band three times by comparing the intensity of the band to the intensity of the background in the lane

Results

The IFAST Process and Device

In the IFAST process, the sample (e.g., bacterial lysate) is first mixed with antibodies immobilized on PMPs to capture a protein-of-interest as well as any interacting proteins. The sample is then loaded into an IFAST device containing three small chambers (Fig. 2A and 2B), the middle chamber was loaded with a hydrophobic, water immiscible, liquid (oil barrier). A magnet, applied from below, was used to draw the PMP-bound protein (or protein complex) through the immiscible-phase oil barrier and into an elution buffer to complete the isolation (Fig. 2A, and Supplemental video). The immiscible barrier effectively prevents transfer of unbound components into the elution buffer while allowing PMP-bound components to pass into the elution buffer, thereby accomplishing a significant purification. Furthermore, the flexibility of IFAST enables robust processing of multi-sample arrays (Fig. 2B and 2C), larger volume samples (Fig. 2D and 2E), as well as the addition of wash chambers between oil barriers (Fig. 2C–E).

Characterization of the IFAST platform using a PR-mAb

To examine the ability of the IFAST platform to isolate protein targets bound with varying affinities, we employed a previously described monoclonal antibody (mAb 8RB13), which is a “polyol-responsive” monoclonal antibody (PR-mAb) [16]. PR-mAbs are antibodies whose affinity can be decreased in the presence of a combination of nonchaotropic salt and a low molecular weight polyhydroxylated compound (polyol). These mAbs have been isolated for numerous target proteins [17]. Thus, the affinity of mAb 8RB13 can be decreased by increasing the concentrations of ammonium sulfate (AS) salt or propylene glycol (PG) in the binding / washing buffers. The change in affinity of this reaction under the different binding conditions has been difficult to determine, largely due to the viscosity of the polyol. However, estimated changes in the K_D of the mAb 8RB13 and the etGFP upon the addition of PG and AS were determined by IR-based microscale thermophoresis (MST), using a Monolith NT.115 instrument (Nanotemper Technologies, San Bruno, CA). This technique showed that in the presence of 20% PG, the EC_{50} was about 13 nM, but the addition of 50 mM AS to the 20% PG weakened the interaction to about 46 nM (data not shown). Using this mechanism, we can generate “strong” binding conditions (e.g., 0 mM AS, 20% propylene glycol) as well as “weak” binding conditions (e.g., greater or equal to 30 mM AS, 20% propylene glycol) with a single antibody, thus removing antibody-to-antibody variability from the characterization. In addition, the epitope for this mAb is known (PEEKLLRAIFGKAS) and had been genetically fused to GFP [16]. Thus, we were able to use the etGFP to enable a facile fluorescent readout of protein recovery under different binding conditions.

The PR-mAb was adsorbed onto a protein G-conjugated PMP and was used to bind etGFP in the bacterial lysate. The recovery of the etGFP was measured, using both the IFAST platform and conventional wash-step protocol. Fig. 3A illustrates the transfer of etGFP from the input well, through the oil barrier, and into the output well with minimal carryover of RFP. This purification took less than 5 seconds to draw the PMP/etGFP complex through the oil using only a handheld magnet, compared to ~5–10 minutes to clean the samples by repeated washes. The actual isolation occurs as the PMP/etGFP complex enters the oil (thus

trapping proteins in the PMP cluster, and excluding liquid containing contaminants), which only requires ~1 second. Additional detail concerning the methods of this characterization, as well as a video of the process, can be found in the Supplemental Information.

IFAST Under Strong Binding Conditions

To test the IFAST method under strong binding and washing conditions (0 mM AS, 0% propylene glycol), IFAST devices with one (Fig. 2B), two (Fig. 2C), and three (not shown) oil barriers were used. Although 92% of etGFP was recovered with a single barrier IFAST purification, 81% was recovered with four conventional washing steps; a difference that was not statistically significant ($p = 0.23$). Protein recovery declined somewhat when additional oil barriers were added in series in the IFAST device (82% was recovered with two oil barriers and 66% was recovered with three oil barriers).

In order to address specificity and the liquid carry-over, we incorporated a 200-fold excess of RFP into the starting material, which was also measured in the output wells of the IFAST devices and at the conclusion of the conventional wash-step protocol. While four conventional wash steps were found to reduce RFP concentration to 0.79% of the original amount, a single oil barrier was able to reduce RFP concentration to 1.8% of the original amount, and two oil barriers produced an output with only 0.08% of the initial RFP ($p = 0.003$ compared to conventional washing). RFP was undetectable following traverse of three oil barriers (Fig. 3B). Samples of the unbound material and the eluted material from both the IFAST and the conventional wash-step protocol were run on an SDS-PAGE gel and silver stained (Fig. 3C). This gel confirmed that the bulk of the bacterial proteins present in the initial lysate had been removed. These experiments indicate that two oil barriers will achieve similar recovery of etGFP in a strongly bound setting while also obtaining higher purity as measured by the reduction of the nonspecific RFP than the conventional wash-step protocol. The time required to perform the IFAST was at least 60 times faster (5 seconds) than the conventional wash-step protocol (5 minutes).

IFAST Under Weak Binding Conditions

Because PR-mAbs are “tunable,” we were able to use this system to weaken the binding by increasing the amounts of AS added to the 20% propylene glycol binding and washing buffers. It was found that a single barrier IFAST recovered significantly more etGFP than the conventional wash-step protocol, particularly for AS concentrations ranging from 1 to 30 mM. In the conventional wash-step protocol, complex dissociation during washing (total time = 5 to 10 minutes) was substantial and large quantities of etGFP were lost in the wash buffer (Fig 4). However, when binding occurred under AS concentration in excess of 30 mM, the majority of the etGFP was not recovered with either method, although significantly more etGFP was recovered with IFAST ($p = 0.001$). The inability to recover the majority of the etGFP with the IFAST at higher AS concentration is due to the unfavorable binding conditions during the 30-minute binding reaction and the equilibrium reached during this time left the majority of the mAb sites unoccupied. Therefore, the majority of the unrecovered etGFP was not “lost”, but rather “unbound” at the beginning of the purification process.

To further investigate this binding behavior, protein interactions were captured in strong binding conditions (0 mM AS, 0% PG), then exposed to weaker binding conditions for specific time periods using the IFAST. This experiment would reduce the “unbound” protein in the system and enable more detailed analysis of the protein that is “lost” during washing and purification. Specifically, strongly bound mAb / etGFP complexes were drawn through one oil barrier and into a buffer where mAb / etGFP affinity is reduced (1, 30, or 250 mM AS, each with 20% PG). After exposing the complex to this intermediate buffer for a

specific amount of time (0.1, 1, 10, or 100 minutes), the remaining intact complex was drawn through a second oil barrier into the strongly eluting buffer (750 mM AS, 40% PG) where the remainder of the complex dissociated (Fig. 5A). At the end of the IFAST traverse, the solutions from the aqueous wells, as well as the conventional 4 wash-step protocol were removed to a new 3-well IFAST device and imaged (Fig. 5B). In this manner, the etGFP in the complex that dissociated during the controlled exposure to the intermediate buffer relative to the etGFP in the complex that remained intact (until it was dissociated in the IFAST elution well) could be measured as a function of intermediate buffer strength and time. As a comparison, strongly bound complex was conventionally washed four times with each of the intermediate buffers, such that the dissociation with a single, controlled equilibrium perturbation in the IFAST could be compared to multiple perturbations (conventional step washing). The quantified results are presented in (Fig. 5C).

Some etGFP dissociation was observed for each of the reduced affinity reactions and dissociation was found to increase with increasing dissociation time and increasing AS concentration, as expected. As before, the control protein, RFP, remained in the input well, confirming the specificity of the isolation. For the cases where the controlled dissociation was short (0.1 minutes), IFAST recovered significantly more etGFP ($p = 0.02$) for all tests except the least dissociative condition, with AS concentration of 1 mM. (Fig. 5B and 5C). This result further demonstrates that IFAST can isolate weakly-bound complexes better than a corresponding conventional wash-based protocol. It should be noted that the 10-minute IFAST dissociations typically recovered more protein than the corresponding washed-based protocol, even though the latter protocol was performed in approximately 5 minutes. This enhanced dissociation was driven by exposure to *multiple* wash buffers during isolation, where each individual buffer change promoted dissociation during equilibration of the protein complex with the surrounding buffer.

Application to Wnt Pathway

While the PR-mAb and etGFP interaction is useful as an experimental system to quantify weak binding protein interactions, it is not compelling from a biological or clinical standpoint. In order to demonstrate the ability of IFAST to isolate clinically relevant, transient protein complexes, we next focused on the biologically important, canonical Wnt signaling pathway. This pathway is involved in embryonic development and adult tissue homeostasis, and its misregulation has been linked to tumor formation in various tissues, including intestinal and breast cancer [18]. Using IFAST, we have isolated endogenous levels of the low-density lipoprotein receptor (LDLR)-related protein (LRP5) and LRP6, one of the functional interactors of LRP5 [17]. Here we compare the recovery of LRP5 and LRP5-interacting proteins by using IFAST and the conventional wash step protocol (total washing time = 5 to 10 minutes). To provide a control for non-specific pull-through of immunoprecipitates, PMPs coated with non-immune rabbit IgG were analyzed in parallel. To detect these interactions, Western blots were probed with antibodies to LRP5, LRP6, and the effector kinase, glycogen synthase kinase-3 β (GSK3 β). These potential binding partners were deduced from genetic interactions discovered by our group [17] and by others [19–21]. Indeed the interaction of the intracellular domain of LRP5 with GSK3 β has been confirmed *in vitro* using overexpressed proteins, but never using endogenous levels of either protein [22]. Detection of trans-membrane proteins not known to interact with LRP5 (insulin receptor beta; IR β and epithelial growth factor receptor; EGFR) in MEFs, and a general non-interacting control (vinculin) were included to evaluate reaction specificity.

The results (Fig. 6) showed that LRP5 was successfully isolated using both the IFAST and conventional co-IP methods, but the efficiency of extraction was higher using IFAST (120% of conventional immunoprecipitation). The rabbit IgG and known non-interactor controls

were negative in both methods. Significantly more of the associated proteins (LRP6 and GSK3 β) were pulled through with the primary LRP5 protein target using IFAST. The yield of LRP6 was up to 150% of standard co-IP, and over 700% more GSK3 β was pulled through by IFAST. Indeed, GSK3 β was almost undetectable by the conventional technique. This result illustrates the potential of IFAST to isolate intact physiologically relevant, rapidly dissociating endogenous protein complexes from a biologically important pathway.

Discussion

The IFAST platform enables the capture of transient protein-protein interactions, a class of interactions with growing implications in disease. Using an epitope-tagged fluorescent protein with tunable affinity to the antibody, we were able to demonstrate that the IFAST platform could isolate a similar level of strongly bound protein and a significantly higher level of weakly bound protein when compared with conventional wash-based protocols. Target protein purity was improved beyond that observed with the conventional wash-step protocol by adding a second (or optionally, a third) oil barrier in series, while maintaining a superior level of target recovery. As liquid carryover in the PMP aggregate interstitial space is a potential mechanism for contamination, purity can be further improved by utilizing a second oil barrier in series or by optimizing the quantity of PMPs to match the expected antigen levels without an overabundance of PMPs.

Once optimized, the IFAST system was employed to isolate several different endogenous complexes involving the Wnt signaling pathway. Of particular importance is the ability of the IFAST to isolate transient complexes that have been predicted by genetic studies but are dissociated during conventional protocols (e.g., LRP5-LRP6, LRP5-GSK3 β). This demonstrated ability to detect very transient and / or low affinity interactions will have broad implications across life science research, potentially enabling the elucidation of new signaling pathways, biomarkers, and drug targets.

IFAST overcomes several problems associated with conventional wash-step protocol. First, the residual liquid present after traversing the oil phase is extremely low in the IFAST system; thus carryover is low, allowing fewer wash steps. (A thorough examination of the characteristics of this space within the context of the oil barrier traverse is given in Supplemental Information.) Second, because the elution occurs in a separate chamber from the IP, non-specific adsorption of material to the IP reaction tube is not released during elution. Perhaps most importantly, the IFAST allows the rapid isolation of complexes that would likely have dissociated in the multiple wash-step protocol. However, non-specific binding remains an issue that similarly affects performance of both the IFAST and conventional PMP protocols, as the same antigen-binding matrix (mAb-conjugated PMPs) is employed in each technique.

In the work described in this paper, target protein recovery was quantified by fluorescence readings or semi-quantitative Western blot. However, it has recently been shown that IFAST-recovered proteins can also be identified by mass spectroscopy [24]. In this study, while known fibronectin-interacting proteins were identified, MS also identified 24 unknown proteins that potentially interact with fibronectin, demonstrating the ability of IFAST to identify truly unknown interactors without the need for specific antibodies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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D. J. B. holds equity in Bellbrook Labs, LLC, Ratio, Inc., and Salus Discovery LLC. S. M. B., L. N. S., and R. R. B. hold equity in Salus Discovery LLC. R. R. B. and N. E. T. hold equity in NeoClone, LLC, which markets mAb 8RB13.

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Fig. 1. Comparison of IFAST and conventional co-IP

Transient or weak binding partners (red symbol) are often dissociated from their PMP-captured partners (yellow symbol) during washing, resulting in minimal recovery of intact complex. In contrast, IFAST purification does not disturb complex equilibrium, resulting in significantly more recovered intact complex.

Fig. 2. IFAST device operation and configurations

A) Aqueous sample solution is first added to input well (blue) and elution buffer is added to the output well (red) of the three-well device. The microfluidic constrictions act as virtual walls, preventing the solution from filling into the middle well. Oil is then added to the middle well (yellow) to establish the immiscible barrier. During operation, a magnet is used to draw PMP-bound antigens through the oil barrier and into the output well, where they are collected via pipette. Arrays of IFAST devices on a single chip can be operated in parallel with a long bar magnet. IFAST devices have been fabricated with a single oil barrier (B) or two oil barriers in series (C) with a wash well between them (dye green). IFAST devices

have also been fabricated with larger input wells to accommodate 200–300 μ l samples with two (D) and three (E) oil barriers in series.

Fig. 3. Characterization of IFAST performance

Experiments were performed under strong binding conditions (0 mM AS and 20% propylene glycol) A) Fluorescent images of IFAST purification of etGFP (top row) and RFP (middle row) from a bacterial lysate. The bottom row (PMP) illustrates the position of the PMPs during each stage in the processing. B) Quantification of etGFP and RFP recovered from IFAST under strong binding conditions and traversed through one, two and three oil barriers (See Fig 2) or recovered from a 4 step wash protocol. C) Isolation of etGFP from a bacterial lysate containing a mixture of etGFP and RFP as illustrated by SDS-PAGE followed by silver staining.

Fig. 4. Measuring binding under varying ammonium sulfate concentrations

IFAST recovers more etGFP compared with conventional wash-based protocols when the binding strength of the mAb and etGFP is weakened by the addition of AS ($n = 4$ and error bars represent standard deviation).

Fig. 5. Measuring dissociation in IFAST

Experiments were performed under varying binding and washing conditions. A) Schematic of the controlled dissociation experiment. etGFP is bound to PMPs and loaded into an IFAST device (step 1). PMPs are drawn into an intermediate well containing varying concentrations of AS and 20% PG and allowed to dissociate for 0.1, 1, 10, or 100 minutes (step 2). Following dissociation, PMPs are drawn into the final elution well (step 3), thus isolating any remaining intact complex. B) Material from the input, wash, and output wells was transferred to a 3-well IFAST device and fluorescently scanned, showing the recovery of etGFP for the various IFAST and wash step conditions. RFP remained in the input, confirming the specificity of the etGFP capture by mAb 8RB13. C) Quantification of etGFP

that was recovered in under each condition. Percent etGFP was expressed as the amount of etGFP in the output well compared to the total amount of etGFP that was bound by the mAb 8RB13/PMP (derived by totaling the etGFP in the wash well and the elution well).

Fig. 6. Isolation of transient complexes with IFAST

IFAST purification of anti-LRP5 co-IPs preserves transient binding partners LRP6 and GSK3 β . A significantly higher proportion of LRP5 is purified by IFAST relative to a standard co-IP consisting of four washes. The yield of the LRP6 and GSK3 β is much higher than for standard immunoprecipitation protocols. Other proteins (vinculin, IR β , and EGFR) do not interact and serve as controls. PMPs coated with IgG also serve as controls in this experiment. “Unbound” refers to protein remaining in the input well of the IFAST devices after operation and “Bound” refers to material recovered in the output of the device. Two identical IFAST trials (#1 and #2) are shown for the LRP5 IP. All reactions were performed using aliquots of MEF cell lysate from the same cell preparation. Blots have been cropped; original blots can be found in Supplementary Figure 3.