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Weak protein–protein interactions revealed by immiscible filtration assisted by surface tension

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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, which gives a near-instantaneous isolation, enables significantly higher recovery of transient complexes compared to current wash-based protocols, which require reequilibration 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 nonspecific material in a single operation. We use a previously described polyol-responsive monoclonal antibody 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.

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 coimmunoprecipitation (co-IP), are commonly used 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 three to six times to separate the target complex from unbound, nonspecific 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 reequilibrates, 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 and fluorescence resonance energy transfer, have been developed and used successfully, but require prelabeling 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.

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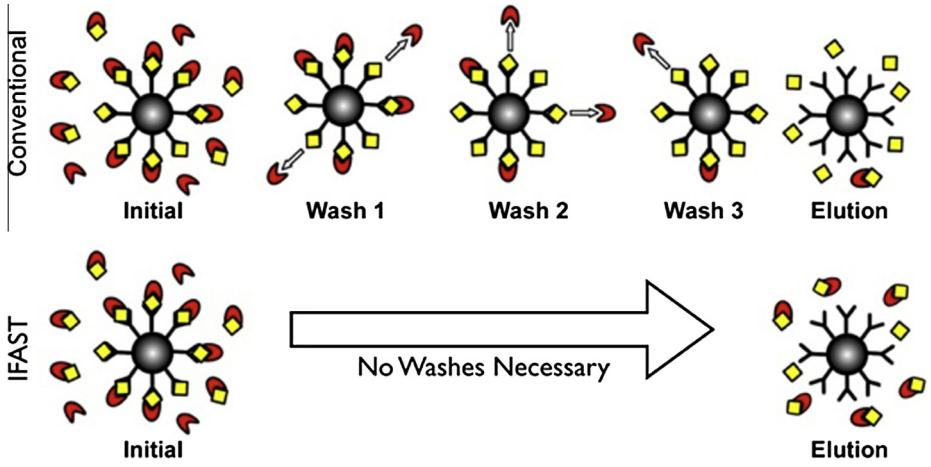

Fig.1. Comparison of IFAST and conventional co-IP. Transient or weak binding partners (red symbol) are often dissociated from their PMP-captured partners (yellow symbol) during washing, resulting in minimal recovery of intact complex. In contrast, IFAST purification does not disturb complex equilibrium, resulting in significantly more recovered intact complex. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The advent of 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 (Sylgard 184; Dow Corning) using soft lithography and 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 μl) connected by two trapezoidal microfluidic channels (Fig. 2A and B). The shape of the microfluidic conduit was chosen 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 owing 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 μl) and/or additional oil barriers in series (total of two or three) were also fabricated in a similar manner (Fig. 2C–E)

Protein expression and preparation of lysates

The plasmid construct containing green fluorescent protein (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 *Escherichia coli* in the presence of an overexpressed *GroEL* and *GroES* system have 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*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

The "polyol-responsive" monoclonal antibody (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 min at room temperature to allow mAb attachment to the PMPs. The beads were then washed twice with 100 μl of PBST.

IFAST operation and characterization

MAb-labeled PMPs were resuspended 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 and 240 μg/ml, respectively. Following a 10-min 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 μl of PMP/bacterial lysate solution onto