ONE-STEP ISOLATION OF TRANSITORY PROTEIN COMPLEXES WITH IFAST

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ABSTRACT

The ability to identify interactions between proteins is critical to understanding cellular processes. However, many of the complexes are transitory in nature, with half-lives of seconds or less. While important, these complexes are difficult to isolate as washing-based co-immunoprecipitation often results in inadvertent dissociation. Here, we present a technology for isolating complexes in a nearly instantaneous manner. Complexes are bound to paramagnetic particles (PMPs) and drawn through a barrier of immiscible liquid, thus purifying the complex from the bulk sample in a single step. Using model system, we have demonstrated the benefit of this method over traditional protocols.

KEYWORD

Isolation, Immunoprecipitation, Microfluidics, Multi-Phase, Protein Complexes

INTRODUCTION

Cellular pathways are mediated through the interactions between biomolecules, including proteins and nucleic acids. While some of these complexes are stable, many critical interactions are rapid or transitory in nature. The ability to manipulate these transitory protein complexes (e.g. enzymatic reactions, transcriptional factor activation) is critical for understanding cellular behavior and discovering drug targets. Unfortunately, current co-immunoprecipitation (co-IP) techniques to isolate intact complexes are inadequate. Typical IP protocols capture target complexes on a solid resin, then repeatedly wash the resin to remove unbound material and contaminants. This type of "dilutive" isolation exposes the complex to several wash buffers containing no protein, thus pushing the complex equilibrium toward dissociation (Figure 1). Dissociation is further promoted by the long protocol times (t ≈ 10 minutes). ***Current co-IP protocols promote dissociation of transitory or weakly bound complexes.***

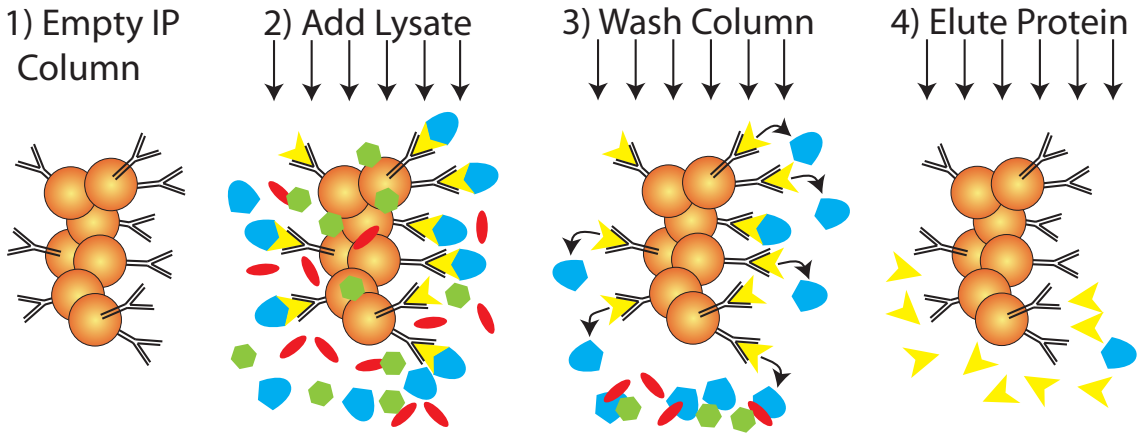

Figure 1 – Conventional co-IP protocol. An empty IP column with antibody (1) is loaded with lysate (2) and the complex of interest is captured. Unbound protein is washed away, but washing dissociates target complex binding partner (the protein not bound via antibody) (3), reducing yield in eluent (4).

EXPERIMENT

Here, we present a microfluidic co-IP process that leverages Immiscible Filtration Assisted by Surface Tension (IFAST) (previously reported for other applications [1, 2]) to isolate protein complexes in a nearly instantaneous manner, such that intact transitory protein complexes can be isolated. In this process, protein complexes are bound to paramagnetic particles (PMPs) via antibodies. Next, the PMP-bound complex is pulled across an oil barrier to effectively isolate it from the remainder of the sample. As the PMP/protein aggregate crosses the interface between the lysate and oil, the hydrophilic PMPs collapse into a tightly packed cluster that excludes all aqueous lysate except for a small interstitial volume (V ≈ 10 nL). This cluster, which forms in the instant that the PMPs cross the interface, contains all the complex protein and is then drawn into another aqueous solution for analysis (Figure 2). Because the process is nearly instantaneous and the sample is never diluted, complex equilibrium is never disturbed and dissociation is mitigated. Furthermore, microfluidics-derived physics (i.e., the dominance of surface tension over gravity) are employed to configure the device in a single plane, such that arrayed, high-throughput operation is enabled.

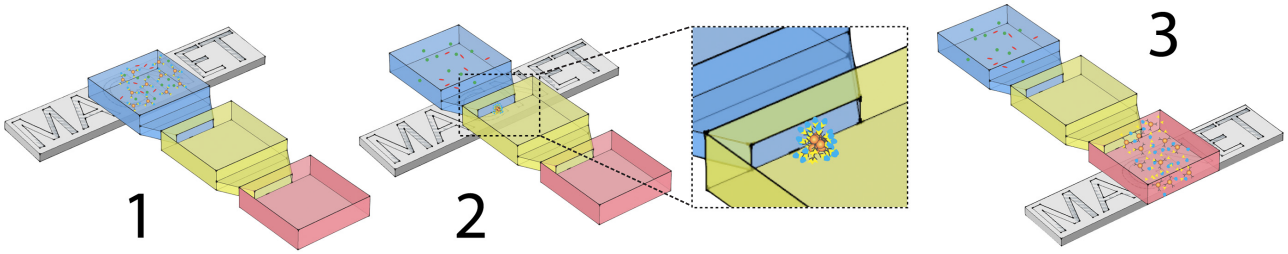

Figure 2 – IFAST co-IP protocol. An IFAST device is loaded with lysate and PMPs that capture complex of interest (1). A magnet is used to draw PMP-bound complex across oil barrier (2). The hydrophilic PMPs cluster to exclude aqueous carryover (inset) with efficiency equal to several conventional washes. The target complex is eluted in the IFAST output; significantly more binding partner is recovered since isolation is non-dilutive.

To quantify the ability of the IFAST to remove background contaminants, fluorescein dye was spiked into samples that were loaded into IFAST devices and operated. Following operation, the quantity of fluorescein dye that had been carried through the oil barrier was measured using a fluorescent scanner (Typhoon Trio, GE). It was determined that a single pass through an IFAST oil barrier inadvertently transferred ~1% of the background dye to the output of the device (Figure 3A). To obtain higher purity, PMPs were drawn through two oil barriers in series with a "wash" positioned between the two barriers (Figure 3B). Using this 2 barrier IFAST, ~99.8% of the background dye was excluded. As a comparison, "conventional" washes were performed on the fluorescein samples in which the PMPs were drawn to the side of the tube, such that the supernatant could be removed and replaced with fresh buffer. It was determined that a pass through a single IFAST oil barrier was equivalent to ~2 washes while passage through a 2 barrier device resulted in the removal of background associated with ~4 conventional washes.

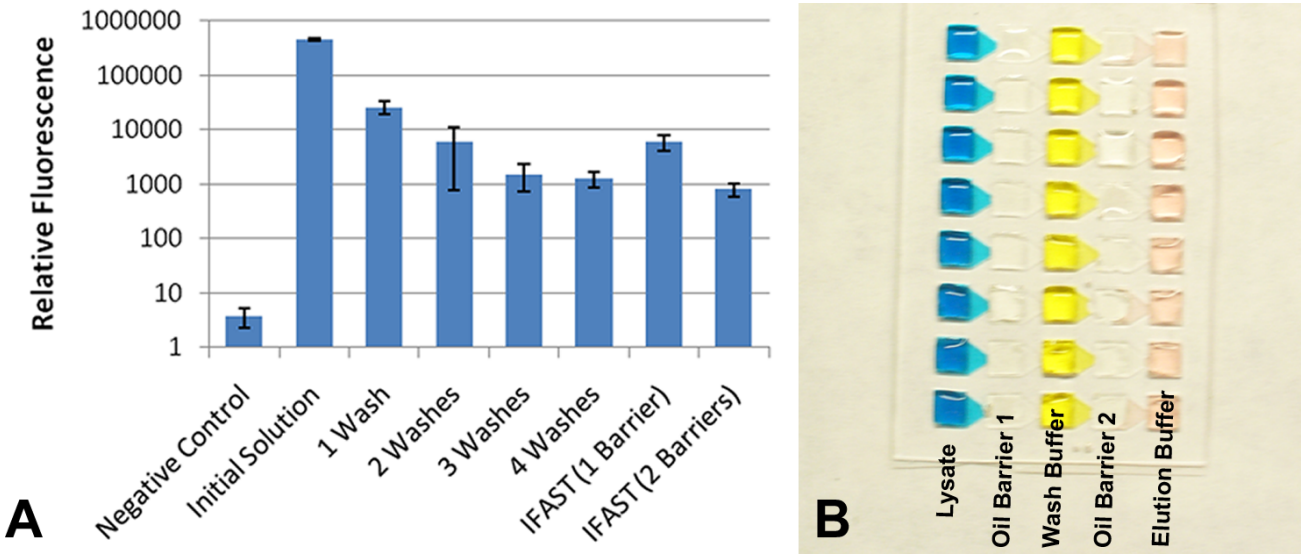

Figure 3 – A) Fluorescein dye was spiked into samples that were purified with either conventional washing or with IFAST. The level of the fluorescein remaining after purification is indicative of the level of "background" that will be present following the purification protocol. It was found that a single oil barrier removed 98.7% of background while two oil barriers removed 99.8% of background. B) An array of 8 IFAST devices that each have two oil barriers with a "wash" buffer between the barriers.

To characterize our system, we used a previously reported antibody / fluorescent epitope system [3] that has a "tunable" affinity. In strong binding conditions (no salt), little difference was seen between the IFAST method and a conventional, washing-based co-IP protocol. However, in weak binding conditions (high salt), IFAST was able to recover significantly more fluorescent epitope than the conventional protocol (Figure 4A). In addition, we were able to isolate biologically relevant complexes that are "weak but known" (i.e., interactions that are suspected based on other data, but difficult to isolate using conventional co-IP). One such interaction is difficult-to-isolate SUMO ligase involved in the NFkB pathway [4] (NEMO-PIASy) (Figure 4B). By comparison, attempts to isolate this complex using conventional washing-based co-IP resulted in no detectable complex. Two strong complexes (NEMO-IKKα and NEMO-IKKβ) are also shown in Figure 4B to demonstrate that similar results are obtained by conventional and IFAST processes for very stable complexes. Lastly, we demonstrated an on-chip fluorescent readout to fully leverage the streamlined nature of the IFAST technology (Figure 4C). In this experiment, complexes were tagged with fluorescent antibodies and fluorescence was measured directly in the IFAST device output following isolation.

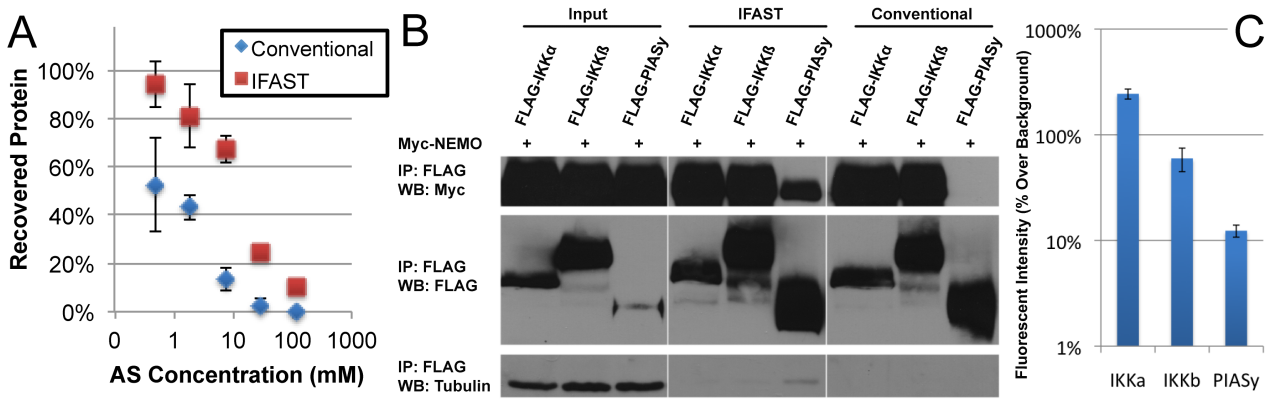

Figure 4 – A) As binding affinity is reduced (by increasing AS concentration) in a tunable fluorescent epitope system [3], IFAST recovers increasingly more target protein than conventional washing-based protocol, illustrating utility of IFAST for isolating weakly bound complexes. B) Co-IP of two strong complexes (NEMO-IKKα and NEMO-IKKβ) showed little difference between IFAST and conventional protocol. Co-IP of weak complex (NEMO-PIASy) showed significant advantage for IFAST. A NEMO mutant (R62A) does not complex with PIASy, again showing specificity of IFAST. C) NEMO was fluorescently labeled while binding partners (IKKα, IKKβ, PIASy) were attached to PMP via antibodies. Intact complex was quantified by fluorescent measurement directly in IFAST.

In summary, we developed an IFAST-based co-IP technology to isolate transitory protein complexes, thus enabling the study of biologically important interactions that were difficult or impossible to isolate with conventional co-IP protocols.

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