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Multiplex IP-FCM (immunoprecipitation-flow cytometry): Principles and guidelines for assessing physiologic protein–protein interactions in multiprotein complexes

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ABSTRACT

There is significant interest in the development of methods with the potential to increase access to 'the interactome' for both experimental and clinical applications. Immunoprecipitation detected by flow cytometry (IP-FCM) is a robust, biochemical method that can be used for measuring physiologic protein–protein interactions (PPI) in multiprotein complexes (MPC) with high sensitivity. Because it is based on antibody-mediated capture of protein complexes onto microspheres, IP-FCM is potentially compatible with a multiplex platform that could allow simultaneous assessment of many physiologic PPI. Here, we consider the principles of ambient analyte conditions (AAC) and inter-bead independence, and provide a template set of experiments showing how to convert singleplex IP-FCM to multiplex IP-FCM, including assays to confirm the validity of the experimental conditions for data acquisition. We conclude that singleplex IP-FCM can be successfully upgraded to multiplex format, and propose that the unique strengths of multiplex IP-FCM make it a method that is likely to facilitate the acquisition of new PPI data from primary cell sources.

1. Introduction

Biological processes are initiated and carried out by biochemical interactions between molecular components, and the summation of all such possible interactions is collectively termed "the interactome" [1]. As a field of study, Interactomics represents an exciting frontier in which progress is currently limited by both assay and analytical tools, to a degree beyond that which applies to Genomics or Proteomics [2]. Whereas these latter fields focus mostly on the identity and expression level of molecular species, the output information of these sciences is the input information for Interactomics. A complete Interactomic profile, which does not yet exist, would measure all possible combinations of interactions between molecules as reported by other 'omics' methods, and add exponential matrix-level interaction complexity that is even more data intensive than its Genomics/Proteomics parent sciences. To progress in this direction, there is great interest in the generation of assay and analytical tools that improve the accessibility of the interactome to experimentation, diagnosis, pharmacology, and medicine.

We and others have described immunoprecipitation detected by flow cytometry (IP-FCM) as a useful method for assessing the physiologic protein–protein interactions (PPI) within multiprotein complexes (MPC) [3–12]. IP-FCM represents a candidate approach to PPI analysis relying on the use of immunoprecipitation (IP) antibodies (Ab) coupled to polystyrene latex microspheres to immunoprecipitate proteins (primary analytes) from cell lysates; subsequently, fluorochrome-conjugated Abs probe interacting proteins (secondary analytes) for identification and quantification of proteins present in shared complexes. Strengths of the IP-FCM method include: (i) it allows a robust, quantitative or semi-quantitative biochemical assessment of native PPI with up to attomole sensitivity; (ii) no genetic engineering, epitope tagging, or radioactive labeling is required, allowing application to samples from wild-type subjects and clinical patients; (iii) it is compatible with multi-well plate-based high-throughput formatting; (iv) it is effective for assessing transmembrane as well as cytosolic/secreted proteins. Additionally, because both commercial and academic organizations are attempting to generate monoclonal Abs specific

Abbreviations: AAC, ambient analyte conditions; Ab, antibody; AP-MS, affinity-purification mass pectrometry; CML, carboxylate-modified latex; FCM, flow cytometry; FRET, Forster's resonance energy transfer; FSC, forward light scatter; IP, immunoprecipitation; IP-FCM, immunoprecipitation detected by flow cytometry; LTD, laboratory developed test; MPC, multiprotein complex; PE, phycoerythrin; PPI, protein–protein interaction; S-HS, sulfo-*N*-hydroxysulfosuccinimide; SSC, side light scatter; TCR, T cell antigen receptor.

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for all open reading frames of the human genome [13], we predict that IP-FCM could eventually be used to provide access to a significant portion of the interactome.

Converting IP-FCM from single- to multiplex format will help achieve this goal, allowing measurement of many PPI simultaneously. In technological terms, similar bead-based multiplex assays are already available from commercial and academic sources as robust laboratory developed tests (LDT), which typically measure the expression level of 5–30 analytes per sample [14,15]. However, there are two assay conditions that are often assumed in common multiplex bead assays, which are absolutely critical for multiplex bead-based PPI experimentation and must be directly monitored: (i) inter-bead independence, and (ii) analyte non-depletion. To illustrate by contrast, in a multiplex bead array for cytokines, an analyte such as IL-2 is measured on one bead type only, and is not expected to be present on beads assessing other cytokines; however, in a multiplex PPI experiment, a hypothetical analyte "A" might be expressed in complexes with several different proteins, and could thus be co-immunoprecipitated on several bead types within the multiplex set. Inter-bead independence is achieved when measurement of analyte "A" on one bead does not affect its measurement on another bead, allowing all beads to provide accurate data. One common way to fail to meet this criterion is if protein capture on the multiplex bead set causes analyte depletion from the sample, such that the assay itself decreases the analyte(s) that must be measured, erroneously lowering detection levels. In other words, "ambient analyte" conditions (AAC) must be met for all analytes involved in multiplex IP-FCM experimentation. Recent careful work by Joos [16,17], Kelso [18] and their colleagues have highlighted this idea with microsphere-based assays, reminding the field of early work by Ekins [19,20] who enunciated the concept of AAC.

Using the T cell antigen receptor (TCR) as a biomedically relevant model of a stable, membrane resident MPC (Fig. 1 and Ref. [21]), we demonstrate how to convert singleplex to multiplex IP-FCM. First, we illustrate the basic methods involved in microsphere coupling, assay design, and implementation. Second, we demonstrate how to determine lysate and assay conditions that are predicted to fulfill inter-bead independence and AAC criteria under multiplex conditions. Third, independence of each bead class in the multiplex array is confirmed. Fourth, AAC is confirmed under multiplex conditions. Thus, the current work provides a strategy to first predict and subsequently confirm valid multiplex PPI assay conditions for multiplex IP-FCM, using a work-flow intended to serve as a pattern that could be followed for other collections of physiologic PPI or MPC of interest.

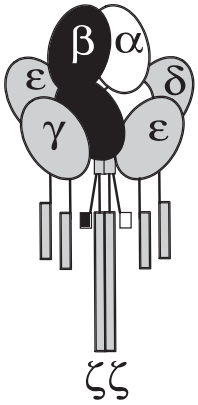

Fig. 1. The T cell antigen receptor (TCR) complex serves as a model to study PPI in a stable, membrane-resident multiprotein complex (MPC). Each subunit depicted is part of the constitutively associated TCR complex. Extracellular domains are depicted as ovals, transmembrane domains as vertical lines, and intracellular domains as rectangles. α, TCRα; β, TCRβ; ε, CD3ε; δ, CD3δ; γ, CD3γ; ζ, CD3ζ.

2. Materials and methods

2.1. Materials

The materials previously described for singleplex IP-FCM [6–8] were used here, with some additions. The following Abs were purchased from BD in purified and/or phycoerythrin (PE)-conjugated format: H57 (Anti-mouse TCRβ), B20.1 (Anti-mouse TCR Vα2), 30-H12 (Anti-mouse Thy1.2). Abs purified from hybridoma supernatant included: 2C11 (Anti-mouse CD3ε, binds both CD3εγ and εδ heterodimers), 17A2 (Anti-mouse CD3εγ), 7D6 (Anti-mouse CD3εγ). White carboxylate-modified polystyrene latex (CML) beads were purchased from Interfacial Dynamics Corp. (now part of Invitrogen). MagPlex-C Microspheres were purchased from Luminex. Sulfo-*N*-hydroxysulfosuccinimide (S-NHS) was purchased from Thermo Scientific. Cell lysates were obtained from TOT-1 cells, a spontaneous murine T cell lymphoma harvested from an OT-1 TCR transgenic mouse in our laboratory.

Magnetic IP Beads were washed using the BioPlex Pro II wash station and BioPlex Pro 96-well flat-bottom plates, with TopSeal-A seals from Perkin Elmer. IP beads were analyzed using Luminex X-map technology available in the BioPlex200 system from BioRad. The BioPlex200 system was calibrated with the BioPlex Calibration Kit and validated with the BioPlex validation kit. Multiplex IP-FCM data was analyzed using the BioPlex Manager 6.0 software from BioRad.

2.2. Methods

2.2.1. Singleplex IP-FCM

The technical protocol for IP-FCM has been described previously in singleplex format [6–8], which is outlined in general in Fig. 2A–D. Briefly, IP beads were generated by conjugating monoclonal Abs to CML microspheres (or "beads") using the zero-carbon-length crosslinker EDAC and the optional ingredient S-NHS (5 mg/mL, Pierce). Although it does not increase the maximum capture capacity of IP beads, inclusion of S-NHS in the coupling procedure can allow enhanced Ab coupling for low-concentrations of Ab (Supplemental Fig. 1).

To capture physiologic TCR complexes, IP-beads were incubated with TOT-1 cell lysate and washed to remove excess lysate (Fig. 2C) as previously described [6,7]. Next, IP beads were stained with a detection Ab specific for the primary or secondary analyte (Fig. 2D). Finally, IP beads were analyzed by flow cytometry (FCM).

2.2.2. Multiplex IP-FCM

Beads internally labeled with specific, unique fluorescent dye combinations (termed bead 'addresses' or 'classes'), were obtained from Luminex. Each bead class was conjugated to a unique Ab specific for a subunit of the TCR complex to create a multiplex set (schematically depicted in Fig. 2E). These IP beads were combined (Fig. 2F) into one tube and distributed into multiple wells of a plate. Multiplex IP was performed (Fig. 2G) with TOT-1 cell lysate, and afterwards excess lysate was washed away. IP beads and co-precipitated analytes were stained with detection Abs, specific for potentially co-associated analytes, using a single probe Ab per well (Fig. 2H). Finally, data from different IP-probe combinations performed in parallel wells were compared. Multiplex IP beads together with co-associated analytes and probes were analyzed using the Bio-Plex 200 flow cytometry system.

2.2.3. Singleplex IP-FCM Analysis

Fig. 3A–C presents singleplex IP-FCM data collected on a BD FACSCalibur cytometer and analyzed using FlowJo software. Forward light scatter (FSC) and side light scatter (SSC) were used to