Journal of Visualized Experiments

Quantification of protein interaction network dynamics using multiplexed co-immunoprecipitation

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60029R3
Full Title:	Quantification of protein interaction network dynamics using multiplexed co-immunoprecipitation
Keywords:	Pisces; protein interaction; immunoprecipitation; IP-FCM; Flow Cytometry; Proteomics; QMI
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Seattle, WA

TITLE:

Quantification of Protein Interaction Network Dynamics using Multiplexed Co-Immunoprecipitation

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KEYWORDS:

IP-FCM, proteomics, protein interaction, signaling, immunoprecipitation, QMI

SUMMARY:

Quantitative Multiplex Immunoprecipitation (QMI) uses flow cytometry for sensitive detection of differences in the abundance of targeted protein-protein interactions between two samples. QMI can be performed using a small amount of biomaterial, does not require genetically engineered tags, and can be adapted for any previously defined protein interaction network.

ABSTRACT:

Dynamic protein-protein interactions control cellular behavior, from motility to DNA replication to signal transduction. However, monitoring dynamic interactions among multiple proteins in a protein interaction network is technically difficult. Here, we present a protocol for Quantitative Multiplex Immunoprecipitation (QMI), which allows quantitative assessment of fold changes in

protein interactions based on relative fluorescence measurements of Proteins in Shared Complexes detected by Exposed Surface epitopes (PiSCES). In QMI, protein complexes from cell lysates are immunoprecipitated onto microspheres, and then probed with a labeled antibody for a different protein in order to quantify the abundance of PiSCES. Immunoprecipitation antibodies are conjugated to different MagBead spectral regions, which allows a flow cytometer to differentiate multiple parallel immunoprecipitations and simultaneously quantify the amount of probe antibody associated with each. QMI does not require genetic tagging and can be performed using minimal biomaterial compared to other immunoprecipitation methods. QMI can be adapted for any defined group of interacting proteins, and has thus far been used to characterize signaling networks in T cells and neuronal glutamate synapses. Results have led to new hypothesis generation with potential diagnostic and therapeutic applications. This protocol includes instructions to perform QMI, from the initial antibody panel selection through to running assays and analyzing data. The initial assembly of a QMI assay involves screening antibodies to generate a panel, and empirically determining an appropriate lysis buffer. The subsequent reagent preparation includes covalently coupling immunoprecipitation antibodies to MagBeads, and biotinylating probe antibodies so they can be labeled by a streptavidin-conjugated fluorophore. To run the assay, lysate is mixed with MagBeads overnight, and then beads are divided and incubated with different probe antibodies, and then a fluorophore label, and read by flow cytometry. Two statistical tests are performed to identify PiSCES that differ significantly between experimental conditions, and results are visualized using heatmaps or node-edge diagrams.

INTRODUCTION:

Dynamic protein-protein interactions constitute the molecular signaling cascades and motile structures that are the functional basis of most cellular physiology[1]. These processes are often depicted as linear signaling pathways that switch between steady states based on single inputs, but experimental and modeling data clearly show that they function as integrated networks[2-4]. In the case of G proteins, different receptors often have the ability to activate the same G protein, and a single receptor can also activate more than one type of G protein[5,6]. In order for the relatively small number of G protein classes to specifically modulate a vast array of cellular functions such as synaptic transmission, hormone regulation, and cell migration, cells must both integrate and differentiate these signals[4,5]. Evidence has shown that this signal specificity, for G proteins as well as others, is primarily derived on the basis of finely tuned protein-protein interactions and their temporal dynamics[1,3-7]. Because signaling networks are comprised of dynamic protein complexes with multiple inputs, outputs, and feedback loops, a single perturbation has the opportunity to alter the overall homeostatic balance of a cell's physiology[4,7]. It is now widely agreed that signaling should be examined from a network perspective in order to better understand how the integration of multiple inputs controls discrete cellular functions in health and disease[7-13]. In light of this, Quantitative Multiplex Immunoprecipitation (QMI) was developed to gather medium-throughput, quantitative data about fold changes in dynamic protein interaction networks.

QMI is an antibody-based assay in which cell lysate is incubated with a panel of immunoprecipitation antibodies that are covalently coupled to magnetic beads containing

distinct ratios of fluorescent dyes. Having specific antibodies coupled to distinct magnetic bead classes allows for simultaneous co-immunoprecipitation of multiple target proteins from the same lysate. Following immunoprecipitation (IP), magnetic beads are incubated with a second, fluorophore-conjugated probe antibody (or biotinylated antibody in conjunction with fluorophore-conjugated streptavidin). Co-associations between the proteins recognized by each IP antibody-probe antibody pair, or PiSCES (proteins in shared complexes detected by exposed surface epitopes), are then detected by flow cytometry and can be quantitatively compared between different sample conditions[14]. Illustrations in **Figure 1** show the steps involved in running a QMI assay, including a diagram of magnetic beads with immunoprecipitated protein complexes labeled by fluorescently conjugated probe antibodies (**Figure 1C**).

The sensitivity of QMI depends on the protein concentration of the lysate relative to the number of magnetic beads used for immunoprecipitation, and achieving a resolution to detect 10% fold changes requires only a small amount of starting material compared to other co-IP methods[14,15]. For example, the amount of starting material used in QMI is similar to that required for a sandwich Enzyme-Linked ImmunoSorbent Assay (ELISA), but multiple interactions are detected in a single QMI assay. QMI assays using 20 IPs and 20 probe targets have been performed using 1-5 x 10^5 primary T cells isolated from a 4 mm skin biopsy, P2 synaptosomal preparations from a 3 mm coronal section of mouse prefrontal cortex, or 3 x 10^6 cultured mouse primary cortical neurons[14,16,17]. This sensitivity makes QMI useful for analysis of cells or tissue with limited availability, such as clinical samples.

QMI can be adapted for any previously defined protein interaction network (provided that antibodies are available), and to date has been developed to analyze the T cell antigen receptor (TCR) signalosome and a subset of proteins at glutamatergic synapses in neurons[17,18]. In studies of T cell receptor signaling, QMI was first used to identify stimulation-induced changes in PiSCES, and then to distinguish autoimmune patients from a control group, detect endogenous autoimmune signaling, and finally to generate a hypothesis involving an unbalanced disease-associated subnetwork of interactions[14]. More recently, the same QMI panel was used to determine that thymocyte selection is determined by quantitative rather than qualitative differences in TCR-associated protein signaling[19]. In neurons, QMI was used to describe input-specific rearrangement of a protein interaction network for distinct types of input signals in a manner which supports newly emerging models of synaptic plasticity[17]. Additionally, this synaptic QMI panel was used to identify differences in seven mouse models of autism, cluster the models into subgroups based on their PiSCES biosignatures, and accurately hypothesize a shared molecular deficit that was previously unrecognized in one of the models[16]. A similar approach could be used to screen for other subgroups that might respond to different drug treatments, or assign drugs to specific responsive subgroups. QMI has potential applications in diagnostics, patient sub-typing, and drug development, in addition to basic science.

To assemble a QMI antibody panel, initial antibody screening and selection protocols are described in Section 1, below. Once antibody panels are identified, protocols for conjugation of the selected antibodies to magnetic beads for IP, and for biotinylation of the selected probe antibodies, are described in Section 2. The protocol for running the QMI assay on cell or tissue

lysates is described in Section 3. Finally, since a single experiment can generate ~5 x 10^5 individual datapoints, instructions and computer codes to assist in data processing, analysis, and visualization are provided in Section 4. An overview of the workflow described in sections 2-4 is shown in **Figure 1**.

PROTOCOL:

1. Assay design

1.1. Candidate antibody preparation

1.1.1. For each protein of interest, choose 3 to 5 antibodies to screen. When possible, use monoclonal antibodies that recognize different epitopes. Also include one non-specific control antibody.

1.1.2. To remove Tris, perform buffer exchange by adding the antibody to a 30 kDa spin filter, spinning down to its minimum volume, adding 500 µL of phosphate-buffered saline (PBS), and repeating 3 times. To remove carrier proteins, perform antibody purification according to the manufacturer's protocol (see **Table of Materials** for specific purification recommendation).

NOTE: This is done because carrier proteins and buffers with free amine groups (such as Tris) will react with COOH groups and quench the subsequent bead coupling and biotinylation reactions. Ensure that all antibodies are purified (no carrier proteins) and in a buffer free of primary amines (i.e., no Tris).

1.1.3. Couple each antibody to carboxylate modified latex (CML) beads as described by Davis and Schrum[20]. To conserve the antibody, scale down bead coupling reactions by up to 1/5 (i.e., 3.6 x 10^6 beads with 10 µL of 0.2-1 mg/mL antibody).

1.1.4. Estimate bead numbers using a hemocytometer (typically ~10^8/mL) and store at 4 °C. Beads have been stored for over a year and used successfully in QMI assays, but shelf life or expiration dates have not been formally established. NaN_3 in the B/S buffer prevents bacterial growth.

1.1.5. Biotinylate a portion of each antibody (see section 2.2 below). Store at 4 °C.

1.1.6. Confirm effective CML bead coupling and accurate counting by staining 1 x 10^5 beads with a PE-conjugated antibody reactive to the species in which the antibody was raised and reading on a flow cytometer.

1.1.7. Confirm antibody biotinylation by dot blot using streptavidin-HRP.

1.1.8. Once the lab has generated reagents that are known to be effective, use those reagents as positive controls in confirmation reactions in steps 1.1.5 and 1.1.6.

1.2. Antibody screening by IP-FCM (immunoprecipitation detected by flow cytometry)

1.2.1. Decide on an appropriate screening lysate. For this and all other pre-QMI screening steps (anything included in this section 1: Assay Design), do not use biosamples with limited availability. Instead, choose a comparable control material such as wildtype mouse tissue, cell lines, or normal human donor tissue that is not a limiting resource.

NOTE: Choosing a lysis buffer for this assay is not trivial, and is discussed in section 1.5: Detergent Selection, as well as in the penultimate paragraph of the Discussion section. Standard lysis buffers have a base of 150 mM NaCl, 50 mM Tris (pH 7.4), 10 mM sodium fluoride, 2 mM sodium orthovanadate, and protease and phosphatase inhibitor cocktails. Detergents compatible with QMI include 1% NP-40, 1% Digitonin, 0.1-1% Triton X-100, and 0.5-1% deoxycholate[14,16-19,21].

1.2.2. Calculate the total volume of lysate to be used for each IP, using 10 µL for each IP-probe combination to be screened. If screening X probe antibodies and using one IgG control, each IP will use (X+1) * (10 µL) * (1.1 for pipetting error); X+1 is to account for the required IgG probe control. For example, in a 3x3 antibody screen, each IP should use 44 ul. Remember to include an IgG IP control (see example screening setup in **Figure 2**).

1.2.3. Calculate the CML bead number to be used. If you are screening X probe antibodies, use [(X+1) * 5 x 10^4 beads]. Ideally, 5,000 beads per well will result in >2,000 beads per well being read by the flow cytometer. For example, in a 3 x 3 antibody screen, each IP should use 20,000 beads, which is approximately 0.66 µL of prepared CML bead stock from step 1.1.3 (beads should first be quantified using a hemocytometer to ensure accuracy).

1.2.4. Incubate the volume of lysate from step 1.2.2 with the volume of each CML bead to be screened from step 1.2.3, overnight at 4 °C with rotation to prevent beads from settling. Typically, perform incubations in the first column of a 96-well PCR plate, and cap with PCR tube strip caps.

1.2.5. Spin down CML beads at 3,200 x *g* for 1 min and remove lysate by a single, rapid flicking of the plate over the sink. A tiny white pellet should be visible at the bottom of each well both before and after flicking.

1.2.6. Resuspend CML beads in a volume of FlyP buffer to equal 20 µL for each bead-probe pair; for X probe antibodies, use (X+1) * (20 µL) * (1.1 for pipetting error), similar to step 1.2.2. For a 3 x 3 screen, resuspend in 88 µL of FlyP buffer. FlyP buffer is 100 mM NaCl, 50 mM Tris pH 7.4, 1% BSA, 0.01% NaN_3.

1.2.7. Distribute each IP across (X+1) wells of a 96-well PCR plate, where X is the number of probe antibodies being screened, using 20 µL/well. **Figure 2A** shows an example screening setup.

1.2.8. Wash 2 additional times using 200 µL of FlyP buffer per well. Spin the plate as in step 1.2.5 and flick the plate to remove wash buffer after each wash. Pellets will be extremely small, but should be visible after each wash.

1.2.9. For each biotinylated antibody to be screened, calculate the total volume as (Y+1) * 1.1 * 50 µL, where Y is the number of IP antibodies being screened. Dilute antibody to a working concentration in this volume of FlyP buffer, typically starting with 1:100 dilution of a 0.5 mg/mL stock.

1.2.10. Distribute each diluted antibody down each column of the 96 well plate, and ensure CML beads are resuspended.

1.2.11. Incubate at 4 °C for 1 h, either with rotation or pipetting at 15 min intervals to ensure CML beads remain in suspension.

1.2.12. Wash 3x in 200 µL of FlyP buffer, for each wash centrifuge and remove lysate as in step 1.2.5.

1.2.13. Resuspend all CML beads in 50 µL of 1:200 Streptavidin-PE in FlyP buffer.

1.2.14. Incubate at 4 °C in the dark for 30 min.

1.2.15. Wash 3x in 200 µL of FlyP buffer, for each wash centrifuge and remove lysate as in step 1.2.5.

1.2.16. Resuspend in 200 µL of FlyP buffer, and then run on a flow cytometer.

1.3. Choosing Antibodies to Include in Assay

1.3.1. Gate on size using FSC-H vs SSC-H, and eliminate doublets using FSC-H vs FSC-A.

1.3.2. Generate histograms of PE fluorescence intensity and overlay both IgG controls (IgG bead-test probe, test bead-IgG probe) onto tested pairs (**Figure 2**).

1.3.3. Look for a bead-probe pair that gives clear signal over noise (**Figure 2B**). Additionally, it is not ideal to use the same antibody for both bead and probe. Differential epitope recognition maximizes chances of observing interactions because some epitopes may be occluded in certain protein complexes. If there are no acceptable options, repeat screen with additional antibodies.

1.4. Confirmation of Antibody Specificity

1.4.1. To ensure antibody specificity for the intended targets, use a lysate sample in which the target has been knocked out; for example, a knockout mouse or an RNAi cell line. Alternatively,

use lysate from a target-negative cell line in which the target protein has been artificially expressed.

1.4.2. Perform IP-FCM as described in step 1.2, modifying to fit the experiment.

1.5 Detergent Selection

1.5.1. As detergents are critical in co-IP experiments, empirically test different variations to ensure that the assay has maximum likelihood of detecting changes. To start, choose a relatively small panel of interactions (4-8) that are known to change in a given condition and/or are of particular interest to your study.

1.5.2 Using the non-fluorescent, antibody-conjugated CML beads made for initial screens, perform IP-FCM as described in 1.2 using varied lysis buffer detergent conditions. Detergent screens can be performed with detergent as the only variable, or with different cell conditions for each detergent. Always use IgG controls for both beads and probes, since detergents occasionally produce unexpected background in some IP-Probe combinations.

1.5.3 Based on the MFIs from the screen, choose a detergent that optimizes the signal for the PiSCES of interest. It is likely that some compromises will need to be made[22].

2. Multiplex reagent preparation

2.1. Magnetic bead coupling

2.1.1. Using the magnetic bead region map, select bead regions to use in a pattern that minimizes risk of cross-detection. Magnetic bead typically smear up and to the right, so avoid bead regions that are diagonally adjacent. Beads from every other column of the bead diagram shown on the Luminex website are recommended (https://www.luminexcorp.com/magplex-microspheres/).

2.1.2. Prepare the carrier-free antibody at 0.1 mg/mL in PBS (as in 1.1.2) in 250 µL. Keep on ice for later use.

2.1.3. Vortex magnetic beads extensively, and then aliquot 250 µL into an amber microcentrifuge tube (to protect beads from photobleaching).

2.1.4. Magnetically separate magnetic beads for 60 s and remove the supernatant.

2.1.5. Add 250 µL of MES buffer (50 mM MES pH 6.0, 1 mM EDTA), vortex, magnetically separate for 60 s, and remove the supernatant. Repeat and resuspend magnetic beads in 200 µL of MES buffer.

2.1.6. Add 40 µL of MES to a 2 mg of single-use tube of Sulfo-NHS to make a 50 mg/mL stock.

2.1.7. Add 25 µL of freshly made Sulfo-NHS to the magnetic beads . Vortex.

2.1.8. Add 25 µL of 50 mg/mL freshly dissolved EDAC [1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide hydrochloride, also called EDC] in MES buffer. Vortex.

2.1.9. Cover and shake on a vortexer with a tube-holding attachment for 20 min at room temp, 1000 rpm.

2.1.10. Magnetically separate for 60 s and remove the supernatant.

2.1.11. Resuspend in 500 µL of PBS, vortex, magnetically separate for 60 s and remove the supernatant. Repeat.

2.1.12. Resuspend in 250 µL of antibody solution from step 2.1.2. Vortex.

2.1.13. Incubate 2 h at room temp with shaking on a vortexer at 1000 rpm.

2.1.14. Add 500 µL of PBS to the magnetic beads, vortex, magnetically separate for 60 s, and remove the supernatant.

2.1.15. Add 750 µL of Blocking/Storage (B/S) buffer (1% BSA in PBS pH 7.4, 0.01% NaN_3). Cover and incubate 30 min at room temp, 1000 rpm.

2.1.16. Magnetically separate for 60 s and remove the supernatant. Resuspend in 100 µL of B/S buffer.

2.1.17. Store at 4 °C. Beads have been stored for over a year and used successfully in QMI assays, but shelf life or expiration dates have not been formally established. NaN_3 in the B/S buffer should prevent bacterial growth.

2.1.18. Validate magnetic bead coupling by staining ~0.25 µL of coupled magnetic beads with a fluorescent anti-host species secondary and reading on a flow cytometer, as in step 1.1.5.

2.2. Biotinylation

2.2.1. Ensure that antibodies are in PBS with no carrier protein.

2.2.2. Calculate the total µg of antibody to be biotinylated (100-200 µg recommended for use in multiplex, 25-50 µg recommended for screening).

2.2.3. Prepare fresh 10 mM sulfo-NHS-biotin (can be done by adding 224 µL of ddH_2O to a 1 mg no-weigh tube).

2.2.4. Add 1 µL of 10 mM sulfo-NHS-biotin per 25 µg of antibody, vortex or pipette up and down to mix.

2.2.5. Incubate at room temp for 1 h.

2.2.6. Incubate at 4 °C for 1 h.

2.2.7. Use a 30 kDa spin filter to remove unbound biotin and stop the reaction. Add 500 µL of PBS and spin the column until the minimum volume is reached. Add 500 µL of additional PBS and repeat for 3 total buffer exchanges.

2.2.8. Estimate concentration by measuring the absorbance of 1-2 µL on a spectrophotometer, and then bring the antibody concentration to 0.5 mg/mL.

2.2.9. Store at 4 ˚C.

3. Quantitative multiplex immunoprecipitation

3.1. Plate layout

NOTE: This assay works best when performed using 96-well plates and 2-4 sample conditions.

3.1.1. Always run appropriate controls (i.e., stimulated v. unstimulated cells) on the same plate in order to detect changes between conditions. Distribute each sample horizontally across the plate, and use each column for a different probe antibody. A set of technical replicates for each probe should be run immediately after the first set. See **Figure 3** for an example.

3.1.2 Carefully document the plate layout to facilitate accurate plate loading and analysis.

3.2. Sample preparation & immunoprecipitation (Day 1)

3.2.1. Lyse tissue or cells in appropriate detergent with protease and phosphatase inhibitors and incubate on ice for 15 min. Take care to keep the lysate cold at all times.

NOTE: The exact quantity of stating biomaterial and lysate protein concentration must be empirically determined, and some examples of previously used samples are listed in the third paragraph of the introduction. In general, in the range of 200 µL of 2 mg/mL protein per sample has been successful in the past for 20 IP and 20 probe targets, but ideal inputs for each antibody panel and cell or tissue type must be determined empirically.

3.2.2. Spin down at 4 °C for 15 min at 16,000 x *g* to remove membranes and debris; keep supernatant as lysate.

3.2.3. Perform a BCA Assay or similar to determine protein concentrations, and then normalize protein concentration between samples. If using cells, begin with an equal number of cells per condition and normalization is optional.

3.2.4. Prepare a master magnetic bead mix that contains ~250 magnetic beads of each class per well in the assay. Adjust the bead numbers after data analysis so that in future assays an average of 110 beads of each class will be read per well.

NOTE: Example calculation: (New bead volume) = [(Run Average) / 110] * (previous bead volume). Bead volumes should be adjusted in this way about every 8 runs or as needed. Typically, 3-4 µL of each magnetic bead(prepared as above) are used for a 2-plate experiment.

3.2.5. Wash the magnetic bead mix 2x in FlyP buffer with magnetic separation, and then resuspend in FlyP buffer. For resuspension, use 10 µL per sample per plate. FlyP buffer is 100 mM NaCl, 50 mM Tris pH 7.4, 1% BSA, 0.01% NaN_3.

3.2.6. After thoroughly vortexing the magnetic bead mix, aliquot 10 µL into ice cold microcentrifuge tubes (one tube per sample). Add equal volumes of lysate (with normalized concentrations) to each tube for immunoprecipitation.

3.2.7. Aliquot the lysate-magnetic bead mixture into one tube for each plate being run; e.g. for a 2-plate experiment, split the lysate into two tubes. Place tubes on a rotator at 4 °C overnight for immunoprecipitation, covered to keep out light.

3.3. Running the assay (Day 2)

3.3.1. Start with the lysate-magnetic bead tubes for Plate #1. Use a magnetic bead rack to remove lysate from the magnetic beads, and reserve lysate for future analysis. Wash beads 2x in 500 µL of ice cold FlyP buffer. Keep tubes tubes always on ice or at 4 °C.

3.3.2. Calculate resuspension volume as (number of probes) * (2 technical replicates) * (25 µL per well) * (1.1 for pipetting error). Resuspend IPs in calculated volume of ice-cold FlyP buffer.

3.3.3. After thoroughly resuspending magnetic beads by gentle pipetting, distribute 25 µL per well across a flat-bottomed 96 well plate, on ice.

3.3.4. In a different 96-well plate, dilute biotinylated probe antibodies to 2x working concentration (working concentration is typically 1:100 or 1:200, empirically determined) in FlyP buffer so that their order matches the columns on the plate layout (see **Figure 3**). The final volume of probe antibodies at the working concentration will be 50 µL per well, so the volume of each 2x antibody prepared should be (25 µL) * (number of biological samples) * (2 technical replicates) * (1.1 for pipetting error).

3.3.5. Use a multichannel pipette to distribute 25 µL of each probe antibody dilution into the magnetic bead-containing assay plate.

3.3.6. Shake on a horizontal plate shaker to mix and resuspend the magnetic beads, and then incubate at 4 °C for 1 h, shaking at 450 rpm in the dark.

3.3.7. Wash 3x with FlyP buffer on a magnetic plate washer at 4 °C.

3.3.8. Resuspend the magnetic beads in 50 µL of 1:200 Streptavidin-PE.

3.3.9. Shake to mix and resuspend beads, and then incubate at 4 °C for 30 min, shaking at 450 rpm in the dark.

3.3.10. Wash 3x with FlyP buffer on a magnetic plate washer at 4 °C.

3.3.11. Resuspend in 125 µL of FlyP buffer.

3.3.12. Shake for 1 min at 900 rpm to thoroughly resuspend beads.

3.3.13. Run on refrigerated flow cytometer (see diagram in **Figure S1**). Use the "high RP1 target" setting in the flow cytometer software, and a stop condition of 1,000 beads per region (greatly overshooting the number that should be in any individual well to prevent the machine from stopping prematurely) and sample volume of 80 µL.

3.3.14. Pause the run half way through and resuspend the beads to prevent settling.

3.3.15. Export data files in the .xml format.

3.3.16. Repeat the process for the remaining plates, starting at step 3.3.1.

4. Data analysis

NOTE: The ANC code was designed to compare two conditions from N = 4 experiments, each with 2 technical replicates for each condition. For example, cells are stimulated four independent times, QMI is run on four different days on control (unstimulated) and stimulated cells, with technical replicates as above, and data analysis proceeds as described below.

4.1. Adaptive non-parametric with adjustable alpha cutoff (ANC)

4.1.1. Open MATLAB and set the active directory to a folder containing the ANC program components and the .xml files exported from the flow cytometer.

4.1.2. Fill in the "ANC input" file to reflect the details of the experimental design. The example file included in **Supplementary File** has been pre-filled to run the example data, also provided.

4.1.3. Run the program, which will write a .csv file into the active directory. The file reports PiSCES that are significantly different, at a false positive (alpha) level of 0.05, between Control and Experimental conditions, in all 4 experimental replicates, or at least 3/4 replicates.

4.1.4. Note 'ANC hits,' which are defined as PiSCES with significant differences in at least 3 experimental replicates, represented as 3/4∩4/4 in the file, for use in step 4.3.1.

4.2. Weighted correlation network analysis[23] (CNA)

4.2.1. Paste-transpose the column titles of the data file output by Matlab ending in "_MFI.CSV" into the first row of a new excel sheet. Add the columns "experiment" for experiment number, and "treatment", for experimental treatment, or any other variables to be analyzed. Save this file as "traits.csv".

4.2.4. Open R studio and set the working directory to a folder containing the "_MFI.CSV" and "TRAITS.CSV" files.

4.2.5. Run the R commands as indicated in the commented command file and the detailed in the instructions included with the files. The WCNA modules significantly correlated with each experimental trait are output as a graphic file, and the correlation of each interaction IP_i_$Probe_J$ with each module is output as a .csv file.

4.2.6. Note 'CNA hits,' which are defined as interactions with module membership (MM) > 0.7 and $p < 0.05$ for membership in a module that was identified as significantly correlated with the experimental variable of interest, for use in step 4.3.1.

4.3. Positive 'hits' & visualization

4.3.1. For each interaction in the "3/4∩4/4 hits" list in the ANC output file (from step 4.1.4), identify if that interaction is also a "CNA hit" by checking the CNA output file (see step 4.2.6). Create a new column that indicates if each ANC hit is also a CNA hit.

4.3.2. Calculate the average $\log_2$ fold change value for each ANC∩CNA hit by averaging the values given in the ANC output spreadsheet "_Hits.csv" from step 4.1.3. Convert values to $\log_2$ fold change before averaging. For interactions that were significant in only 3/4 replicates, delete the outlier value.

4.3.3. Make a spreadsheet with each ANC∩CNA hit listed as an IP in one column, a probe in the second column, and the fold change value in the third column. Use this spreadsheet to create a node-edge diagram in Cytoscape by importing the file as a network.

REPRESENTATIVE RESULTS:

Antibody Screening

Figure 2B shows the results of a screen for the protein Connexin36. Most IP_probe combinations produce no signal over IgG controls. IP with the monoclonal antibody 1E5 and probe with either 1E5 or the polyclonal antibody 6200 produces a rightward shift in the bead distribution compared to IgG controls. Here, IP 1E5 and probe 6200poly were selected to avoid using the same antibody as IP and probe, both to reduce the probability of a non-specific protein being recognized by two independent antibodies, and to increase the chance of detecting co-associations using different epitopes. It is best to choose an IP_probe combination with at least 1-2 log higher MFI compared to IgG controls, but occasionally pairs producing weaker MFIs that are consistently distinguishable from controls may be used if no alternatives are identified. **Figure 2C** shows a specificity validation experiment for the 1E5-6200poly combination. Lysate from 293 cells transfected with a Connexin36 plasmid produced a ~1.5-log rightward shift in the bead distribution, while untransfected cells overlapped with the IgG controls. When confirming the specificity of a pair, negative control lysate from a knockout animal or cell line without the target protein should have an MFI similar to the IgG controls.

Bead Coupling

A typical magnetic bead coupling quality control reaction will give an MFI 3-4 logs above background when stained with a secondary antibody conjugated to a fluorophore with a brightness index between 3 and 5 (such as PE or FITC). **Figure 4** shows a typical quality control reaction comparing the conjugation of a new magnetic bead compared to the older batch being replaced.

Data Analysis

In each experiment, ANC compares the fluorescence distributions of each magnetic bead class in each well (i.e. all possible IP_Probe combinations) between a user-defined control and experimental condition. It assigns a *p*-value to each combination that reflects the probability that the beads have been sampled from identical populations based on Kolomogrov-Shmirnov (K-S) statistics. The program then calculates the K-S *p*-value required to produce a false-positive rate of 0.05 by correcting for multiple comparisons and accounting for technical variability (differences between the technical replicates). IP_probe combinations (PiSCES) whose K-S test *p*-value falls below the calculated cut-off in all four experiments, or at least 3/4 experiments (3/4∩4/4) are identified. Since the *p*-value cut-offs differ depending on these different levels of stringency, occasionally PiSCES will be identified in 4/4 but not 3/4∩4/4, so separate lists are calculated. For detailed ANC equations, see (Smith et al. 2016).[14] Details about WCNA analysis and results are discussed thoroughly by Langfelder et al.[23]

Data Presentation

ANC and CNA[23] analyses are performed to identify PiSCES that both (1) show significant fold changes between experimental conditions in at least 3/4 of runs and (2) belong to a CNA module that is correlated with the experimental variable. These high-confidence PiSCES that are identified by two independent statistical approaches are referred to as ANC∩CNA PiSCES. These interactions can be visualized as a node-edge diagram using the open-source software Cytoscape

(**Figure 5a**) or as a heatmap by using the R code and analysis instructions included in the supplementary material (**Figure 5b**).

FIGURE AND TABLE LEGENDS:

Figure 1. Overview of Quantitative Multiplex Immunoprecipitation. (**a**) Previously screened antibodies are covalently coupled to different classes of magnetic beads in separate reactions. (**b**) Overnight, protein complexes are immunoprecipitated using a mixture of the antibody-coupled magnetic beads. (**c**) Co-immunoprecipitated proteins are labeled by a probe antibody and a fluorophore. (**d**) Magnetic beads and labeled protein complexes are run through a refrigerated flow cytometer to quantify relative amounts of proteins occurring in shared complexes. See **Figure S1** for schematic details of custom refrigeration. (**e**) The flow cytometer Manager Software separates MagBeads by class and (**f**) displays fluorescence histograms from each bead region. (**g**) Data are exported as .xml files and analyzed by two independent statistical approaches. Only PiSCES identified by both analyses are reported using heatmap and node-edge diagram visualizations.

Figure 2. Connexin 36 antibody screening using IP-FCM. (**a**) IP-FCM was performed on mouse brain lysate using a 4x4 panel of Connexin 36 (Cx36) CML beads and probes. Lysate was immunoprecipitated with each CML bead in a separate row of the plate. After washes, each bead was distributed across its row so that one probe antibody can be added per column. (**b**) Most antibody combinations show no signal (orange) over IgG background (gray, blue). The 1E5 IP with the 6200Poly probe shows acceptable positive signal. The 1E5 bead/probe and 6200Poly bead/probe pairs each show acceptable signal, but it is not ideal to use the same antibody for both bead and probe. Differential epitope recognition maximizes chances of observing interactions because some epitopes may be occluded in certain protein complexes. The 6200Poly bead with the 1E5 probe gives the strongest signal and was chosen to use in the multiplex assay pending specificity confirmation. (**c**) IP-FCM using the pair of Cx36 antibodies selected from screening was performed on the lysate of 293T cells transfected with Cx36 and non-transfected controls. There is clear signal from the Cx36-transfected cells, but the non-transfected cells are indistinguishable from IgG bead/probe controls.

Figure 3. Example plate layout. A 4-condition multiplex is set up in a 96-well plate. Samples 1-4 are loaded in consecutive rows (each biological sample represented here by a different color), and technical replicates are loaded in the same order in the following 4 rows. One probe is used per column.

Figure 4. A typical quality control reaction comparing the conjugation of a new MagBead compared to the older batch being replaced. The bead gives an MFI 2-4 logs above background, and the new batch has an MFI similar to that of the old batch.

Figure 5. QMI identifies synaptic PiSCES that change in magnitude following 5 minutes of NMDA stimulation in cultured cortical neurons. A QMI experiment compared NMDA stimulated vs. unstimulated (ACSF control) neurons. PiSCES that were identified by both ANC and CNA analyses

are presented. (**a**) In a node-edge diagram produced using the open source software Cytoscape, the nodes indicate the antibody targets (proteins) that were included as IPs and probes in the QMI panel. The edges represent ANC∩CNA PiSCES, with the color and thickness of the edge indicating the direction and magnitude of the fold-change between NMDA treatment and control. PiSCES that did not change between the NMDA and control conditions are not included in the figure. (**b**) A heatmap produced in R using the Heatmap.2 function represents the same ANC∩CNA PiSCES. ComBAT-normalized, $\log_2$ MFI values are normalized by row to account for data that spans ~3 logs, and the relative MFI for each experimental replicate is show to demonstrate the relative magnitude and consistency of each reported PiSCES. The data and code required to reproduce these figures are included in the **Supplementary File**.

Figure S1: Diagrams of custom refrigeration of the flow cytometry system. The flow cytometer's array reader must be kept at room temperature, but the lower portion (the microplate platform) must be refrigerated to maintain PiSCES during analysis. See the **Table of Materials** for model information about flow cytometer and sandwich prep refrigerator used. (**a**) The upper attachments and food storage bins were removed from a sandwich prep refrigerator. The microplate platform was placed on the metal supports meant to hold the plastic food storage bins. The plastic housing of the microplate platform was removed to make it fit. A custom plexiglass platform was built with measurements shown in (**b**) to cover the upper opening of the refrigerator. The plexiglass was insulated with ½" foam insulation cut to match the size of the plexiglass, and sealed the gap with insulating tape. A hole was then drilled through the plexiglass to allow the sample needle from the flow cytometer assay reader to access the microplate platform when extended. A black coupling device that was originally screwed into the top of the microplate platform was removed, and screwed back into the microplate platform from above the plexiglass, which aided in alignment. A door in the Plexiglass cover allows user access to the microplate carrier when it is extended out of the unit. Note that the flow cytometer software will alert the user that the plate carrier is too cold, but the user can override the warning and run cooled QMI experiments. (**c**) Photograph of the assembled system. (**d**) Detail of the front right corner, as drawn in (**a**), showing assembly of plexiglass cover and insulation. (**e**) Detail of the sample needle aligned above the holes. (**f**) Detail of the shaved-down section of insulation that allows airflow under the unit. (**g**) Detail of the open door showing the flow cytometer microplate platform below.

Supplementary File. Data and code required to reproduce these figures.

DISCUSSION:

The QMI assay requires substantial investment in antibody panel development, equipment and reagents, but once the assay is established, one can collect high-dimensional data observing protein interaction networks as they respond to experimentally-controlled stimuli. Technically, QMI requires careful pipetting and tracking of sample and antibody well locations. Carefully labeling the assay plates is useful, as is making a detailed template of well locations on paper, which is then saved for data analysis. The importance of keeping the beads and lysate cold at all times, including in the flow cytometer microplate carrier (see **Figure S1** for custom refrigeration

instructions) cannot be overstated. Protein interactions will rapidly dissociate at room temperature, and early attempts at using an unmodified, room temperature flow cytometer ended with the identification of many temperature-labile interactions, but not those that changed with the intended stimulation.

QMI is an antibody-based assay, so the initial selection of antibodies is critical. Monoclonal or recombinant antibodies should be used whenever possible to reduce variability in results. Polyclonals show lot-to-lot variation, but peptide-based polyclonals to a short epitope seem to be relatively stable over time. Drift can be minimized by buying large batches of antibodies; this also allows for custom-orders of carrier-free antibodies which precludes the need to purify antibodies using Melon Gel and spin columns, and the associated antibody loss.

It is also important to note that, because detecting a signal is reliant upon available epitopes, the lack of a signal does not necessarily indicate the lack of an interaction, a limitation that is common with other protein interaction methodologies.[14] Further, when a signal is detected, it is impossible to unambiguously state weather the protein interaction is direct (A interacts with B) or indirect (A interacts with X and Y, which then interact with B), which is why the observed interactions are referred to as PiSCES rather than protein-protein interactions (PPI), which may imply direct binding. A limitation of all antibody-based methods that should be kept in mind is that the addition of antibodies may disrupt or stabilize protein complexes. Another limitation of using flow cytometry rather than western blots is that size information to confirm antibody specificity is not available. To overcome this limitation, IgG controls are used in screening each antibody pair, and specificity is confirmed with knock-out or knock-in cell lines or animals before proceeding with QMI experiments (section 1.4).

IgG controls are not used in the QMI assay because each IgG produces a different level of background signal, making it impossible to know the correct background value to subtract. For example, if IP (X)_probe IgG gives an MFI of 100 and IP IgG_probe Y gives an MFI of 200, which background value should be subtracted from IP X_probe Y? Similarly, sometimes undetected interactions (e.g., IP X probe Z) will have a lower MFI than the nonspecific IgG interactions. To account for this limitation of not knowing the absolute MFI signal, PiSCES are not reported solely for being detected above an arbitrary background level. Instead, only PiSCES that change in response to a given stimulation are reported. While high MFI can be caused by nonspecific noise, this noise would not be expected to change with stimulation. In addition, a portion (10-20%) of condition-dependent interactions observed are generally confirmed by a second method, typically IP-western. This confirmation is analogous to confirming high-throughput RNA sequencing results with RT-PCR and is meant to increase confidence QMI results.

Expression effects influencing QMI results cannot be ruled out without additional tests, because QMI does not distinguish between increased absolute levels of a protein and increased homo-multimerization of a protein. To minimize uncertainty regarding expression, experiments can be performed using acute treatment conditions with short timescales that minimize potential changes in protein expression levels. Other methods are needed to rule out expression effects in chronic treatment conditions or primary patient samples.

It is vital to select an appropriate lysis buffer for QMI. Too weak of a detergent can leave membranes intact and hold together proteins that are not in complex, while too strong a detergent can destroy protein complexes. Additional factors such as the presence of calcium or its chelators can dramatically affect PiSCES and should be carefully considered before screening antibodies to include in a QMI panel. For IP-western experiments, lysis conditions are usually optimized for each PiSCES on a case-by-case basis, but the best conditions for detecting a single PiSCES may not translate to other PiSCES in the same protein network[22]. Detergent selection presents a chicken-and-egg dilemma, in that a lysis buffer is needed to screen antibody candidates, but a panel of antibodies is needed to screen for an appropriate lysis buffer. While not a perfect solution, one can select a small panel of beads and probes that are of particular interest and/or have known associations or dissociations in response to a stimulus, and testing their behavior under different lysis conditions on the CML beads initially used for screening antibodies (step 1.1.3). An ideal detergent should allow for both reliable detection of PiSCES and recapitulation of known physiological protein behavior (association/dissociation) with a given stimulus. If there is any concern that a detergent does not fully solubilize membranes, a negative control antibody can be added that would only give signal if two proteins were linked by membrane[24]. When appropriate lysis buffers are selected, changes in even weak interactions—such as those between a kinase and substrate—can be reliably detected (e.g. TCR-LCK)[14].

Previous work using QMI in neurons and T cells has both carefully confirmed previous findings in order to increase confidence in the validity of QMI results, and generated new hypotheses that led to discoveries about signal transduction and disease pathways. In the future, QMI can be adapted to other protein interaction networks and expanded up to 500 proteins with the current microsphere classes available. Using QMI to study how networks of multi-protein complexes change in response to stimuli as they control cellular processes has the potential to yield important insights into both health and disease.

ACKNOWLEDGMENTS:

The authors wish to acknowledge Tessa Davis for important contributions to QMI assay development, and current and former members of the Smith and Schrum labs for technical guidance and intellectual input. This work was funded by NIMH grants R01 MH113545 and R00 MH 102244.

DISCLOSURES:

The authors have no conflicts of interest to disclose.

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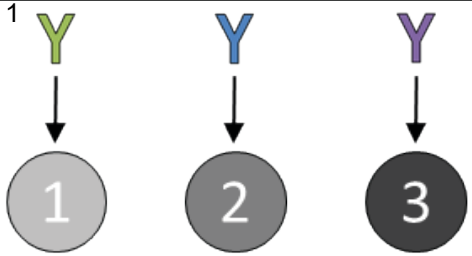
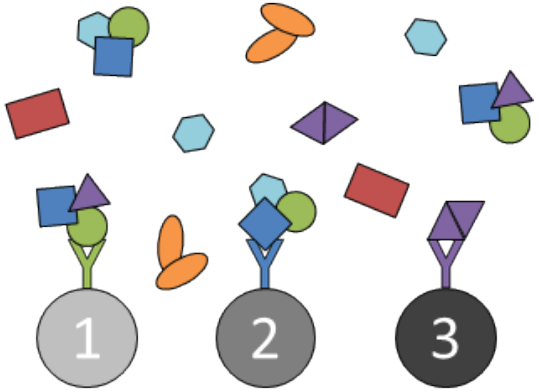
Figure 1

A



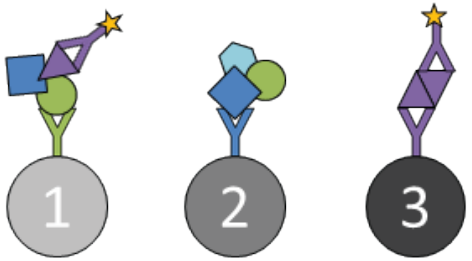
Covalently couple antibodies to Magplex beads

B



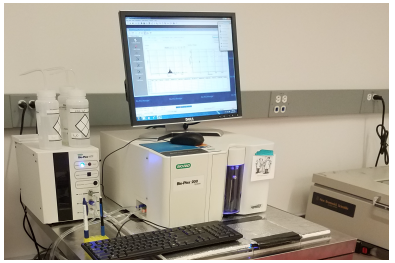
Incubate with lysate

C



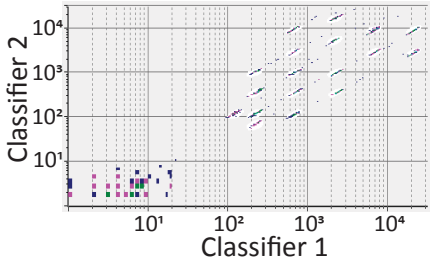
Wash out unbound proteins and probe for remaining complexes

D



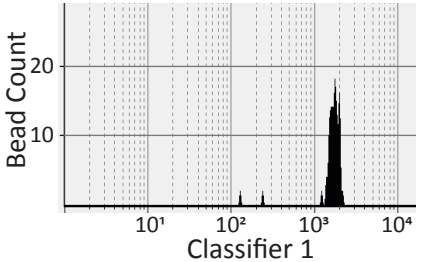
Read bead fluorescence using custom-refrigerated Bioplex200 flow cytometer

E



Cytometer sorts Magplex beads by color...

F



...and quantifies fluorescence of each Magplex bead

G

Export .xml files from Bioplex200

Identify PiSCES that meet ANC criteria

Identify PiSCES that meet CNA criteria

Merge ANC and CNA hits

Visualize data using heatmaps and node-edge diagrams

Analyze data using statistical packages included in supplimentary materials.

Figure 2

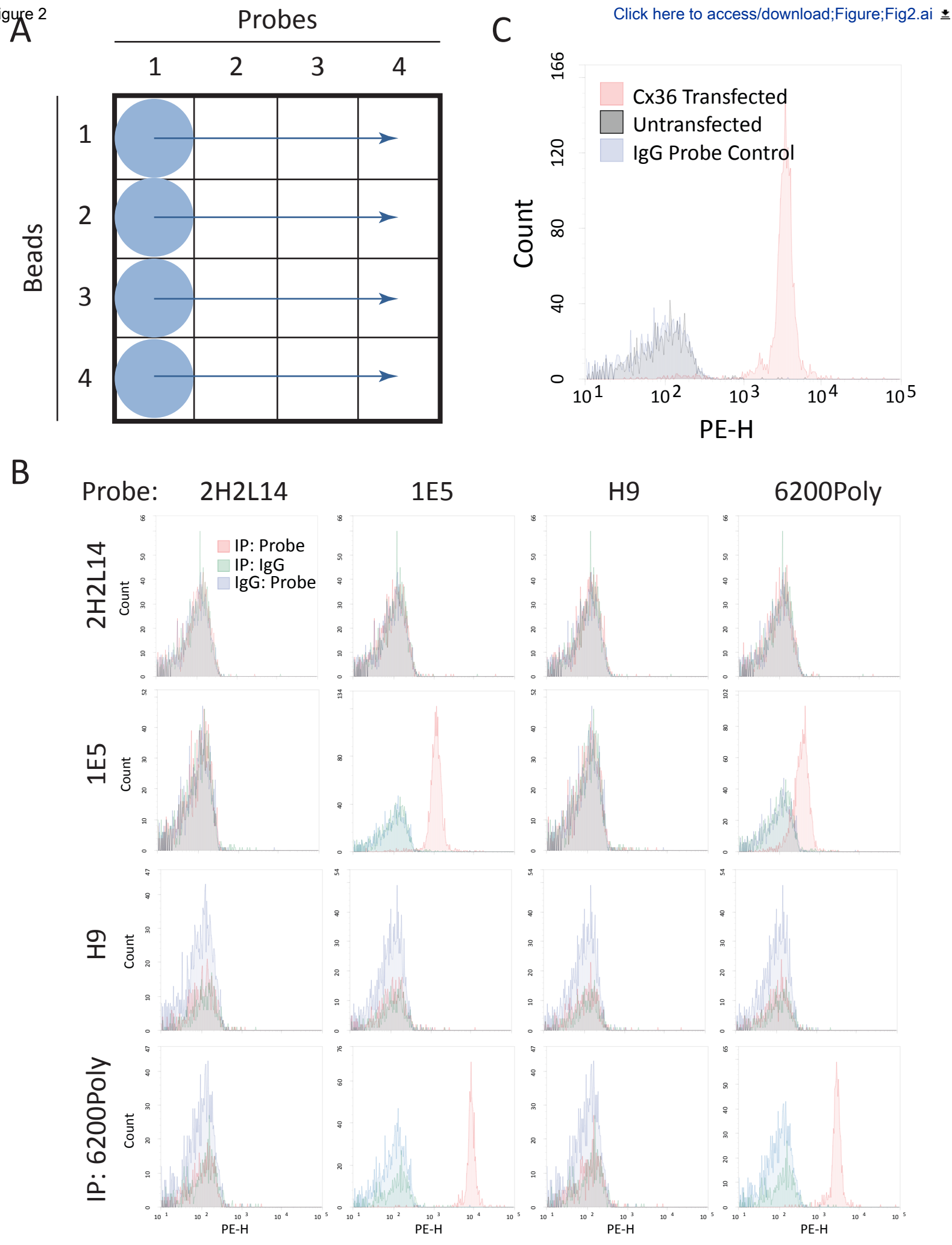


Figure 3

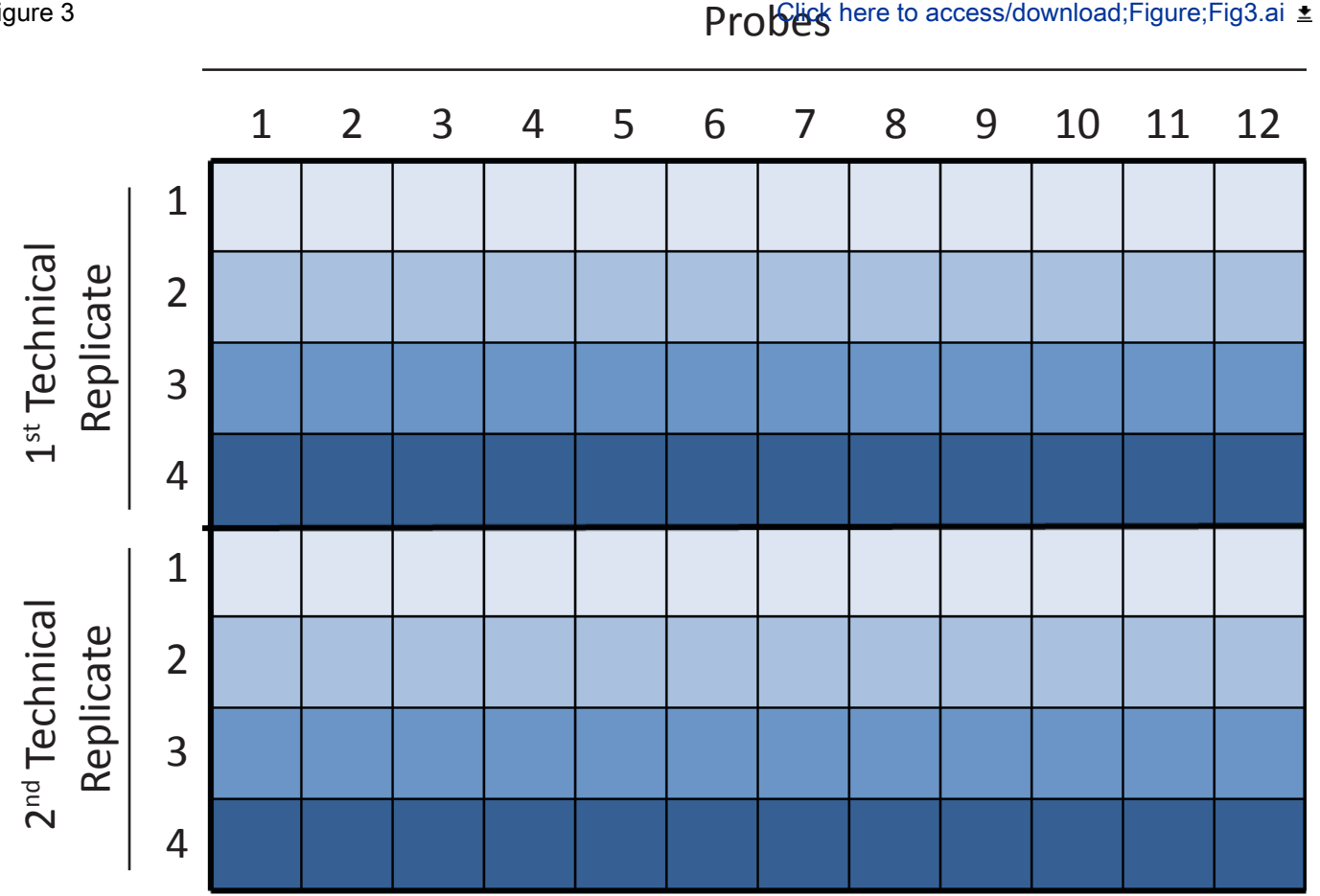


Figure 4

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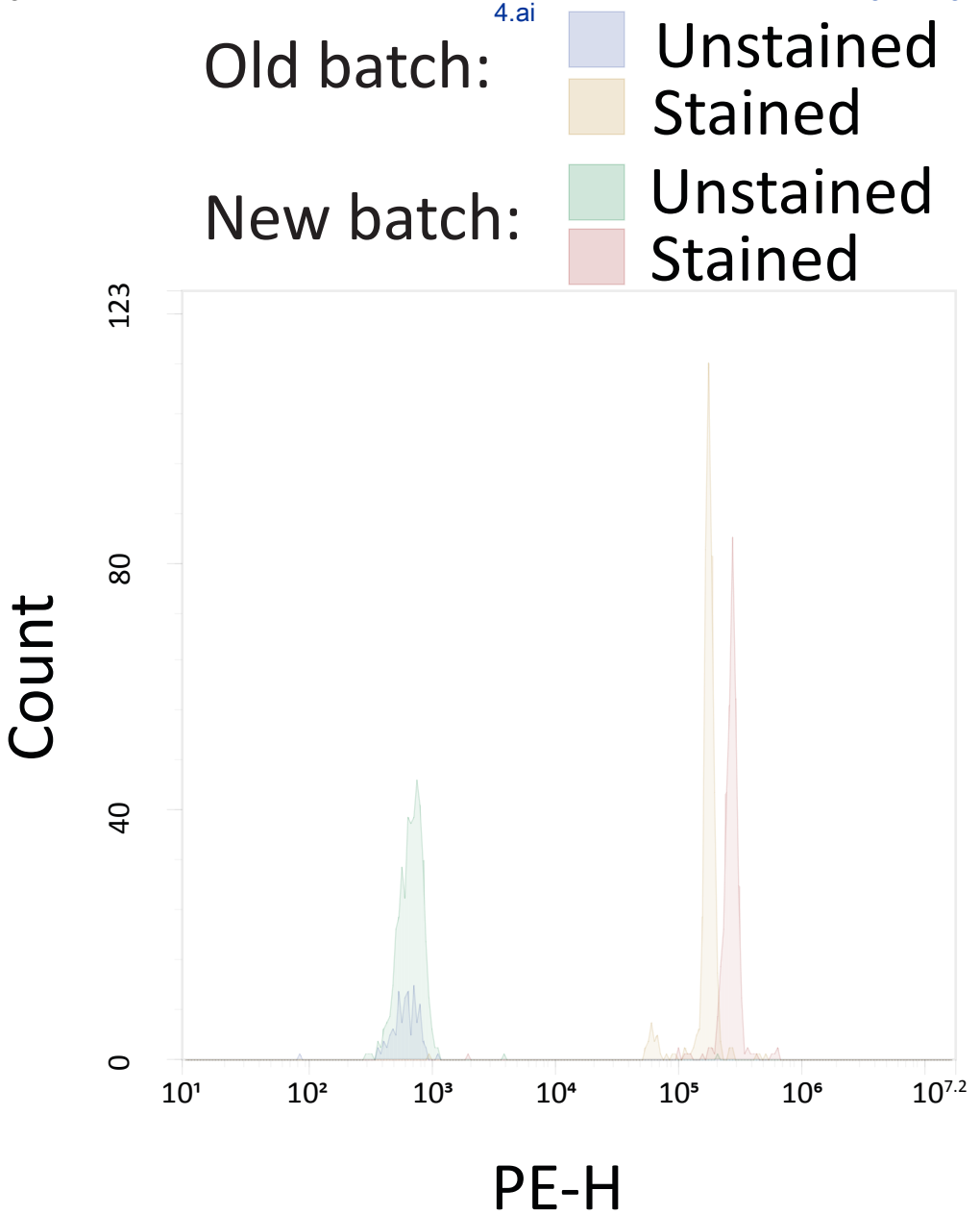

Figure 5

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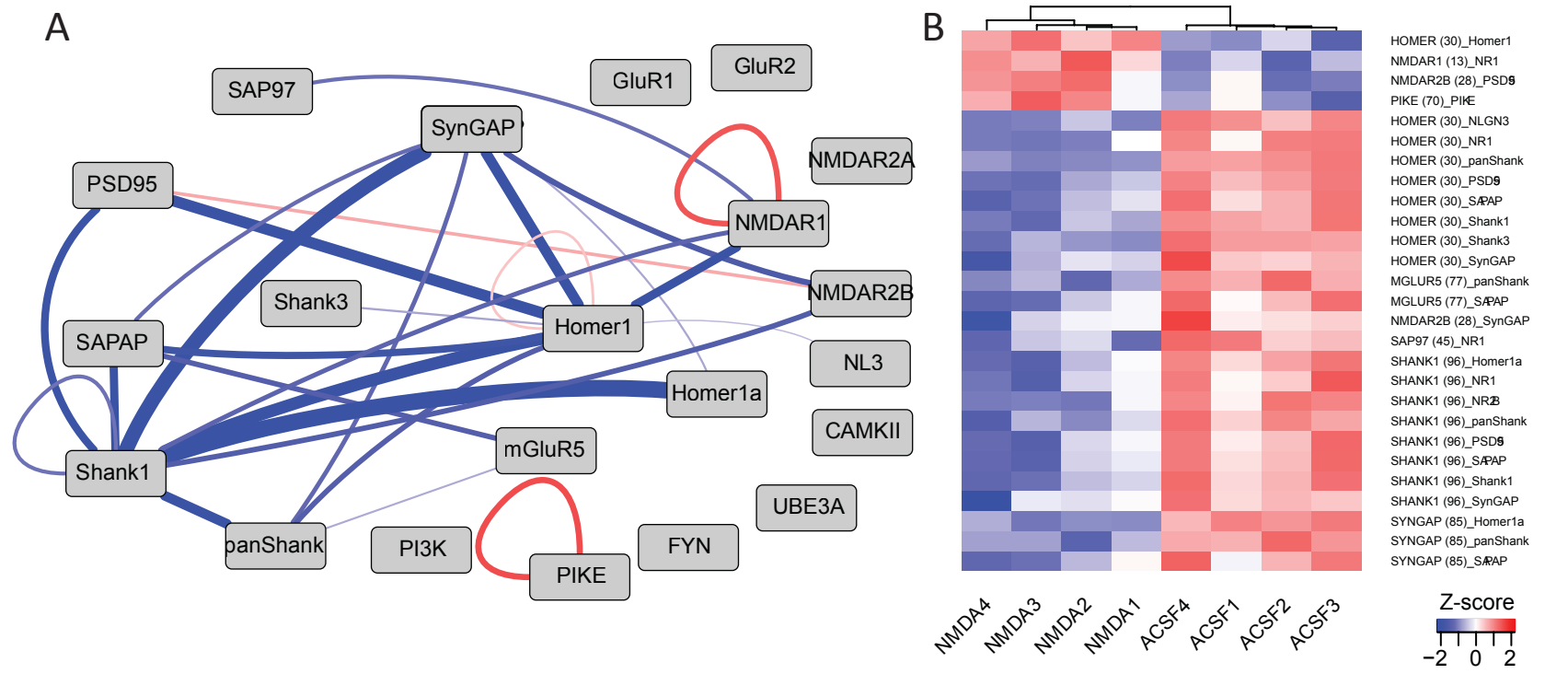

Table of Materials

Click here to access/download;Table of Materials;QMI JoVE_Materials.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
96-well flat bottomed plates	Bio Rad	171025001	
96-well PCR plates	VWR	82006-704	
Bioplex 200 System with HTF	Bio Rad	171000205	modiefied to keep partially refrigerated, see Figure S1 for c
Bio-Plex Pro Wash Station	Bio Rad	30034376	
BSA	Sigma		
CML beads	Invitrogen	C37481	
EDTA	Sigma	E6758	
EZ-Link Sulfo-NHS-Biotin	Thermo Scientific	A39256	
MagPlex Microspheres	Luminex	MC12xxx-01	xxx is the 3 digit bead region
Melon Gel IgG Spin Purification Kit	Thermo Scientific	45206	used for antibody purification
MES	Sigma	M3671	
Microplate film, non-sterile	USA Scientific	2920-0000	
Phosphotase inhibitor cocktail #2	Sigma	P5726	
Protease inhibitor cocktail	Sigma	P8340	
Sandwich Prep Refrigerator	Norlake	SMP 36 15	for custom refrigeration of Bioplex 200
Sodium fluoride	Sigma	201154	
Sodium orthovanadate	Sigma	450243	
Streptavidin-PE	BioLegend	405204	
Sulfo NHS	Thermo Scientific	A39269	
Tris	Fisher Scientific	BP152	

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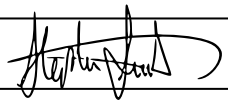
Signature:	
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Response to Reviews:

We thank the reviewers for their thoughtful and constructive critiques. We have responded to each comment below in blue text; changes in the manuscript are shown highlighted in green.

Specific Comments:

Reviewer #1:

Major Concerns:

1) Please describe more accurately what you mean with mesoscale. Is this referring to the number of interactions studied? The intensity of changes able to be detected? The time frame?

We agree that the term 'mesoscale' lacked clarity as to what it referred to, so we have changed the title to be more descriptive:

Quantification of protein interaction network dynamics using multiplexed co-immunoprecipitation.

We have also removed 'mesoscale' from the summary, which now reads:

Quantitative Multiplex Immunoprecipitation (QMI) uses flow cytometry for sensitive detection of differences in the abundance of targeted protein-protein interactions between two samples. QMI can be performed using a small amount of biomaterial, does not require genetically engineered tags, and can be adapted for any previously defined protein interaction network.

2) Figure 1 is not referenced in the main text. Please eliminate the figure or reference it where appropriate.

We thank the reviewer for catching our oversight and have now referenced Figure 1 in Lines 90, 92, and 126.

3) Figure 1B is not adding any value to the manuscript. According to the figure legend, it is supposed to indicate that the flow cytometer is refrigerated, but it is hard to say looking at the picture. The cytometer used by the authors according to the material list (Bioplex 200 System with HTF) does not offer a refrigerated option but it is indicated that it was "modified to keep partially refrigerated". There are no details in the manuscript about the modifications realized. Since the authors highlight (rightfully) that temperature affects protein complexes and keeping samples refrigerated is key to obtain reliable results, this is a major deficiency in the manuscript that should be explained so other groups can reproduce these results. Please explain how the machine was modified.

We agree that it is important to include more detail about refrigeration. These details are too extensive to include in this figure, so we have edited the legend of Figure 1 to refer to the newly-added supplemental Figure S1, which provides detailed instructions on how to modify the cytometer.

(b) Microspheres are run through a refrigerated flow cytometer to quantify relative amounts of proteins occurring in shared complexes. See Fig S1 for schematic details of custom refrigeration.

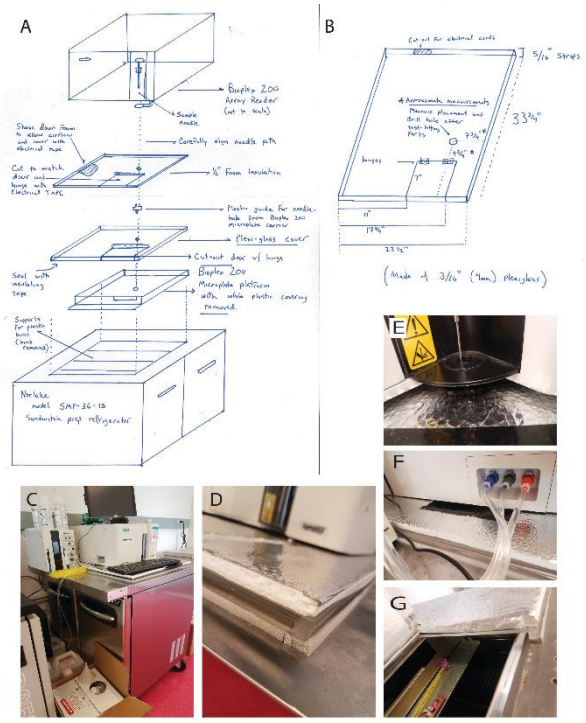

Figure S1: Diagrams of custom refrigeration of the Bioplex 200 system. The Bioplex200 array reader (the flow cytometer) must be kept at room temperature, but the lower portion (the microplate platform) must be refrigerated to maintain PiSCES during analysis. (a) We purchased a Norlake model SMP-36-15 Sandwich Prep Refrigerator and removed the upper attachments and food storage bins. We placed the Bioplex200 microplate platform on the metal supports meant to hold the plastic food storage bins. We had to remove the plastic housing of the microplate platform to make it fit. We then custom-built a plexiglass platform with measurements shown in (b) to cover the upper opening of the refrigerator. We insulated the plexiglass with ½" foam insulation cut to match the size of the plexiglass, and sealed the gap with insulating tape. We then drilled a hole through the plexiglass to allow the sample needle from the Bioplex200 Assay Reader to access the microplate platform when extended. A black coupling device that was originally screwed into the top of the microplate platform was removed, and screwed back into the microplate platform from above the plexiglass, which aided in alignment. A door in the plexiglass cover allows user access to the microplate carrier when it is extended out of the unit. Note that the Bioplex Manager software will alert the user that the plate carrier is too cold, but the user can override the warning and run cooled QMI experiments. (c) Photograph of the assembled system. (d) Detail of the front right corner, as drawn in (a), showing assembly of plexiglass cover and insulation. (e) Detail of the needle aligned above the holes. (f) Detail of the shaved-down section of insulation that allows airflow under the unit. (g) Detail of the open door showing the Bioplex microplate platform below.

3) There is some confusion about which beads the authors are referring to thorough the manuscript. E.g. in section 1.2 (antibody screening by IP-FCM) it is not clear which beads are being used. Are these CML or magnetic beads? Are these a third kind of beads? This is aggravated by the fact that nomenclature is not consistent through the manuscript. E.g. the following terms are used to reference the same beads: Luminex beads, magnetic beads, Magplex beads. Please use consistent nomenclature and try to avoid the generic term "beads" when possible.

We thank the reviewer for noting this and have standardized and clarified our nomenclature. We now specify bead type whenever we refer to beads. The generic term 'beads' is now only used after bead type is specified within the same step or sentence, as in the examples below.

Excerpt from abstract: To run the assay, lysate is mixed with Magplex beads overnight, then the beads are divided and incubated with different probe antibodies, then a fluorophore label, and read by flow cytometry.

1.2.3. Incubate the volume of lysate from 1.2.1 with the volume of each CML bead to be screened from 1.1.3, overnight at 4 °C with rotation to prevent beads from settling.

4) Step 2.1.14: It is indicated to keep the antibody solution for troubleshooting, but this is not referenced again in the manuscript and it is not explained which troubleshooting the authors are referring to. Please eliminate this sentence or explain.

We have removed this step because we do not believe it is necessary.

5) Step 4.1.2.: Please, clarify how to fill the "ANC input" file and what information is requested here.

The ANC input file requires the user to input details about Magplex bead class, IP and probe antibody identity, and details of the plate layout. These details are provided in the "Analysis Instructions" document included with the ANC program in the supplemental material, and instructions on how to fill each individual field are commented out in the Matlab program. In addition, the "ANC_input.m" file is pre-populated with the values necessary to run the ANC analysis program using the example data files provided.

6) Step 4.1.3.: Define what "Hit" means more accurately at this point. There is an explanation in line 490 that I would move here and expand.

We agree that more detail should be given at this point to clarify analysis methods, so in response to this suggestion we have added steps to describe what the program outputs as "hits" and why.

4.1.3. Run the program.

4.1.4. In each experiment, ANC compares the fluorescence distributions of each Magplex bead class in each well (i.e. all possible IP_Probe combinations) between user-defined control and experimental conditions. It assigns a p-value to each combination that reflects the probability that the beads have been sampled from identical populations based on Kolomogrov-Shmirnov (K-S) statistics. The program then calculates the K-S p-value required to produce a false-positive rate of 0.05 by correcting for multiple comparisons and accounting for technical variability (differences between the technical replicates). IP_probe combinations (PiSCES) whose K-S test p-value falls below the calculated cut-off in all four experiments, or at least 3/4 experiments (3/4∩4/4) are identified. Since the p-value cut-offs differ depending on these different levels of stringency, occasionally PiSCES will be identified in 4/4 but not 3/4∩4/4, which is why the separate lists are calculated. For detailed ANC equations, see (Smith et al 2016)

4.1.5 The program writes a .csv file into the active directory. The file reports PiSCES that are significantly different, at a false positive (alpha) level of 0.05, between Control and Experimental conditions, in all 4 experimental replicates, or least 3 replicates. We typically use only the "at least 3 replicates" criteria, represented as 3/4∩4/4 in the file.

7) Figure 3: what does the gradient in color means? Does it represent different conditions? Please clarify in the figure legend.

We have clarified that in Figure 3 each color represents a different condition.

Figure 3. Example plate layout. A 4-condition multiplex is set up in a 96-well plate. Samples are loaded in consecutive rows (each biological sample represented here by a different color), and technical replicates are loaded in the same order in the following rows. One probe is used per column.

8) Figure 5: need more information to understand the image. (E.g. What does the thickness and colors of the lines mean? According to the text only significant PPI are included here, so why some boxes have lines while others do not?)

We have edited the legend for Figure 5 substantially in order to provide clear information about data visualization and interpretation. It now reads:

> **Figure 5. QMI identifies synaptic PiSCES that change in magnitude following 5 minutes of NMDA stimulation in cultured cortical neurons.** A QMI experiment compared NMDA stimulated vs. unstimulated (ACSF control) neurons. PiSCES that were identified by both ANC and CNA analyses are presented. (a) In a node-edge diagram produced using the open source software Cytoscape, the nodes indicate the antibody targets (proteins) that were included as IPs and probes in the QMI panel. The edges represent ANC∩CNA PiSCES, with the color and thickness of the edge indicating the direction and magnitude of the fold-change between NMDA treatment and control. PiSCES that did not change between the NMDA and control conditions are not included in the figure. (b) A heatmap produced in R using the Heatmap.2 function represents the same ANC∩CNA PiSCES. ComBAT-normalized, $\log_2$ MFI values are normalized by row to account for data that spans ~3 logs, and the relative MFI for each experimental replicate is show to demonstrate the relative magnitude and consistency of each reported PiSCES. The data and code required to reproduce these figures are included in the supplementary material.

Minor Concerns:

1) The term PiSCES is used in the introduction but never used again in the protocol or the discussion. It looks like it is replaced by PPI later in the paper, but the definition of PiSCES does not look to be the same than PPI. Please clarify this.

We thank the reviewer for catching this inconsistency and have now changed all references of QMI measurements to "PiSCES" to indicate that we measure proteins in shared complexes, but not necessarily direct interactions that the term "PPI" implies.

2) Are there any flow cytometry apparatus with refrigeration capacity available in the market compatible with this method? If there are, I would recommend the authors to indicate some options.

Unfortunately, there are no refrigerated flow cytometers currently on the market, so we have added Figure S1 to give instructions for custom refrigeration of the Bioplex 200 system. (see reviewer 1, response 3)

3) Line 187: Wrong step referenced (1.3.2.). Please reference correct step.

We corrected this line to reference step 1.1.3.

4) Line 188: Is this 96-well plate flat bottom? Will you see a pellet then?

We have changed the text to specify that a PCR plate is used in step 1.2.3; in the subsequent centrifugation step we added that a pellet should be visible before and after removing lysate. The pellet is made up of beads and should not be present in a suspension (step 1.2.3), but should be present after spin down (step 1.2.4). We feel that this addition, as well as the instruction in 1.2.3 to rotate the plate in order to prevent beads from settling, provide enough clarity for when there should and should not be a visible pellet.

> 1.2.3. Incubate the volume of lysate from 1.2.1 with the volume of each CML bead to be screened from 1.1.3, overnight at 4 °C with rotation to prevent beads from settling. Typically, incubations are performed in the first column of a 96-well PCR plate, and capped with PCR tube strip caps.

1.2.4. Spin down CML beads at 3200 x g for 1 min and remove lysate by a single, rapid flicking of the plate over the sink. A tiny white pellet should be visible at the bottom of each well both before and after flicking.

5) Lines 197 and 198: 96-well PCR plate is missing from the material list. Figure 2 is referenced in the same step but it is not clear what the connection between figure and text is.

We have added a 96-well PCR plate to the materials list, and have clarified the contents of Figure 2 and related the figure to the step as follows:

1.2.6. Distribute each IP across (X+1) wells of a 96-well PCR plate, where X is the number of probe antibodies being screened, using 20 µL/well. Figure 2A shows an example screening setup.

6) Line 200: Referenced step does not exist.

We have corrected the referenced step to 1.2.4.

7) Lines 214 and 220: Add details about the washing conditions (e.g. centrifugation conditions) or reference a previous step.

We have changed both steps to reference the previous wash, shown below:

1.2.11. Wash 3x in 200 µL FlyP buffer for each wash centrifuge and remove lysate as in 1.2.4.

8) Line 283: What does EDAC means?

We added the full chemical name of EDAC.

2.1.8. Add 25 µL of 50mg/mL freshly dissolved EDAC [1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide hydrochloride, also called EDC] in MES buffer. Vortex.

9) Line 300: Please indicate what "wash" means in this context.

This usage was redundant, so we removed it.

10) Step 3.3.4.: Please add the final volume needed of the antibody solutions.

We have changed the step to include the final volume and prevent confusion about which concentration and volume should be prepared.

3.3.4. In a different 96-well plate, dilute biotinylated probe antibodies to 2X working concentration (typically 1:100 or 1:200, empirically determined) in FlyP buffer so that their order matches the columns on the plate layout (see figure 3). Final volume of probe antibodies at the working concentration will be 50 µL per well, so the volume of each 2X antibody prepared should be (25 µL) * (number of biological samples) * (2 technical replicates) * (1.1 for pipetting error)

11) Step 3.3.8.: Please add streptavidin-PE information to material list.

We added streptavidin-PE to the materials list.

12) Line 527: Correct symbol typo between ANC and CNA.

We corrected the symbol typo between ANC and CNA.

13) Line 536: The acronym PPI is used before describing it.

We removed the acronym PPI, and replaced it with PiSCES, which we made sure to define earlier in the text.

Reviewer #2:

The presented QMI method is an interesting approach to study the composition of protein complexes in cell lysates. However, in this manuscript the advantages and disadvantages of this method are not clearly enough stated and discussed. Additionally, the presented data are not conclusive and well described. Also, they do not sufficiently support the claimed advantages of the QMI method. Therefore, in its current form the manuscript is not suitable for publication.

Major Concerns:

1) The authors state that an advantage of this technique over other immunoprecipitation-based methods is that minimal amounts of biomaterials are needed to perform experiments (e.g. line 53). A direct comparison of the method with other PPI detection assays, however, is missing. How would QMI outperform for instance an ELISA in terms of the amount of samples required for analysis?

We thank the reviewer for bringing this concern—we had already included specific biomaterial inputs that we've used in the past in lines 98-101:

> QMI assays using 20 IPs and 20 probe targets have been performed using 1-5 x 10^5 primary T cells isolated from a 4 mm skin biopsy, P2 synaptosomal preparations from a 3 mm coronal section of mouse prefrontal cortex, or 3 x 10^6 cultured mouse primary cortical neurons[14,16,17].

and in response to the reviewer's request we have added a comparison with ELISA (below), which we believe is the most similar assay. Reviewer 3, response 3 contains a description of additional changes that were made regarding biomaterial specifications.

> Line 96: For example, the amount of starting material used in QMI is similar to what's required for an Enzyme-Linked ImmunoSorbent Assay (ELISA), but multiple interactions are detected in a single QMI assay.

2) Other advantages of the QMI technique should be stated more clearly. For instance, in lines 86-89 it is mentioned that a FRET-based assay is not easily scaled up for network analysis which is not the case. It is true, however, that FRET cannot be directly applied for the detection of PPIs in biological samples and is mainly used for the detection fo binary interactions with genetic methods. The strengths of the QMI assay should be emphasized differently, e.g. similar amounts of sample needed as for ELISA, but multiple proteins detectable at the same time.

We thank the reviewer for this critique and suggestion, and in response we have more carefully emphasized the strengths of QMI while trying to avoid pointing out limitations of other techniques. The mention of FRET has been removed completely.

3) How do you rule out expression effects? How do you make sure that not detecting a certain protein in a network is not due to fluctuations in protein expression? Do you first quantify the expression of the tested network proteins with RNA-seq or Western blotting?

This is a great concern to bring up: Expression effects are an issue, because in QMI we cannot distinguish between increased absolute levels of a protein vs. increased homo-multimerization of a protein. For example, IP_X probe X could increase because the levels of X increase, or because a stimulus has caused X to multimerize such that for each molecule of X that we IP, more probe antibody is bound. We generally try to assure that changes we observe by QMI are not due to differences in protein levels by designing stimulations that do not allow enough time for protein levels to change. Changes in protein expression generally take several minutes, and are typically measured after 1/2 an hour or more. We emphasize timepoints on the order of 5 minutes or less to make it less likely that protein expression levels will change in such a short period of time. However, this does remain a valid limitation of the assay. We now discuss this limitation beginning in Line 638:

> Expression effects influencing QMI results cannot be ruled out without additional tests, because QMI does not distinguish between increased absolute levels of a protein and increased homo-multimerization of a protein. Additionally, QMI attempts to take a sample of the protein complexes in a lysate rather than fully depleting it (as is typical for other IP methods)[23]. This means that even if expression levels of a protein are reduced in a sample, that change might not be detected unless it is a dramatic difference, as in knockout conditions[16]. Differences in the composition of protein complexes affected by a change in expression, however, would be detected by QMI. To minimize uncertainty regarding expression, experiments can be performed using acute treatment conditions with limited timescales that minimize potential changes in protein expression levels. Other methods are needed to rule out expression effects in chronic treatment conditions or primary patient samples.

4) In line 117 it is mentioned that any protein interaction network can by studied by QMI. Later, in line 135, it was clarified that any previously defined protein interaction network can be studied by QMI. This particular formulation is essential as only these protein networks can be addressed due to the limitations mentioned in line 541: it is better to use monoclonal or recombinant antibodies. Additionally, you should check for antibody cross-reactivity.

In response to this critique, we have made sure to state early on in the manuscript that QMI can work for any defined protein network with appropriate antibodies available.

> Line 104: QMI can be adapted for any previously defined protein interaction network (provided that antibodies are available).

Additionally, we had addressed cross-reactivity in section 1.4 (below) and shown representative results for this screen in Figure 2C:

> 1.4. Confirmation of Antibody Specificity
>
> 1.4.1. To ensure antibody specificity for the intended targets, use a lysate sample in which the target has been knocked out; for example, a knockout mouse or an RNAi cell line. Alternatively, use lysate from a target-negative cell line in which the target protein has been artificially expressed.
>
> 1.4.2. Perform IP-FCM as described in 1.2, modifying to fit the experiment.

In response this critique we have more explicitly covered antibody specificity in the discussion as well.

> Line 627: Another limitation of using flow cytometry rather than western blots is that size information to confirm antibody specificity is not available. To overcome this limitation, IgG controls are used in screening each antibody pair, and specificity is confirmed with knock-out or knock-in cell lines or animals before proceeding with QMI experiments (section 1.4).

5) In line 137 the statement was made that QMI is useful to study protein networks in their native state. However, the fact has to be considered that due to cell lysis and the loss of transient/weak interactions only a near-native state is reached. A protein-protein interaction or a protein complex that was precipitated from a lysate is not necessarily in a native state.

The reviewer correctly points out that QMI is a post-lysis assay, and like any other post-lysis analysis of protein interactions, one can only measure protein interactions that survive the lysis process. The use of detergents is a critical factor in determining which protein interactions survive lysis, and is extensively discussed in the manuscript, and in our pre-print paper, currently in review elsewhere (Lautz et al, 2019 BioARXIV). Both of these caveats, shared by any assay that measures post-lysis protein interactions, are discussed in the manuscript.

6) What is the composition of your basis lysis buffer? It is critical for the application of this method that lysis buffers and detergents are defined What about weaker protein-protein interactions? Have you tested for cell lysis conditions that enable the detection of such interactions (e.g., a kinase and a substrate)?

We thank the reviewer for catching this omission and have added our basic lysis buffer recipe to Step 1.5.2:

Standard lysis buffer includes 150 mM NaCl, 50 mM Tris (pH 7.4), 10 mM sodium fluoride (Sigma, 201154), 2 mM sodium orthovanadate (Sigma, 450243), and protease/phosphatase inhibitor cocktails (Sigma, P8340/P5726).

We have also added references to previous publications that document the detection of weaker protein interactions as suggested (below). See reviewer 3, response 4 for expanded discussion of the lysis buffer in the protocol.

Line 670: When appropriate lysis buffers are selected, changes in even weak interactions—such as those between a kinase and substrate—can be reliably detected (e.g. TCR-LCK)[14].

7) It was stated that this method can detect changes in protein complex compositions. However, a convincing proof-of-concept experiment to support this statement is missing. For instance, it would be important to demonstrate that a disruption of a protein-protein interaction with a compound can be quantified with this method.

Since this manuscript focuses on detailed methods for performing QMI, we feel that providing a proof-of-concept experiment is beyond the scope of this manuscript. However, we provide references to 7 years worth of publication that have each demonstrated QMI's ability to detect changes in protein composition in several experimental systems. See excerpts from our References section below:

10 Schrum, A. G. & Gil, D. Robustness and Specificity in Signal Transduction via Physiologic Protein Interaction Networks. Clin Exp Pharmacol. 2 (3), S3.001, doi:10.4172/2161-1459.S3-001, (2012).

14 Smith, S. E. et al. Multiplex matrix network analysis of protein complexes in the human TCR signalosome. Sci Signal. 9 (439), rs7, doi:10.1126/scisignal.aad7279, (2016).

15 Schrum, A. G. et al. High-sensitivity detection and quantitative analysis of native protein-protein interactions and multiprotein complexes by flow cytometry. Sci STKE. 2007 (389), pl2, doi:10.1126/stke.3892007pl2, (2007).

16 Brown, E. A. et al. Clustering the autisms using glutamate synapse protein interaction networks from cortical and hippocampal tissue of seven mouse models. Molecular Autism. 9 (1), 48, doi:doi:10.1186/s13229-018-0229-1, (2018).

17 Lautz, J. D., Brown, E. A., Williams VanSchoiack, A. A. & Smith, S. E. P. Synaptic activity induces input-specific rearrangements in a targeted synaptic protein interaction network. J Neurochem. 146 (5), 540-559, doi:10.1111/jnc.14466, (2018).

18 Smith, S. E. et al. Signalling protein complexes isolated from primary human skin-resident T cells can be analysed by Multiplex IP-FCM. Exp Dermatol. 23 (4), 272-273, doi:10.1111/exd.12362, (2014).

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21 Smith, S. E. et al. IP-FCM measures physiologic protein-protein interactions modulated by signal transduction and small-molecule drug inhibition. PLoS One. 7 (9), e45722, doi:10.1371/journal.pone.0045722, (2012).

22 Schrum, A. G., Gil, D., Turka, L. A. & Palmer, E. Physical and functional bivalency observed among TCR/CD3 complexes isolated from primary T cells. J Immunol. 187 (2), 870-878, doi:10.4049/jimmunol.1100538, (2011).

8) In lines 231-222 you comment on the selection of antibodies. How many runs of antibody testing with QMI can be performed with the biosample until it is depleted?

We do not screen antibodies on limited biosamples; rather we use wildtype mouse tissue, cell lines, or normal human donor tissue that is not a limiting resource. Then, once the panel is generated and validated, we use more limited resources. We thank the reviewer for pointing out the lack of clarity and have added a step in the manuscript to reflect this:

1.2.1. Decide on an appropriate screening lysate. For this and all other pre-QMI screening steps (anything included in this section 1: Assay Design), biosamples with limited availability should not be used. Instead, choose a comparable control material such as wildtype mouse tissue, cell lines, or normal human donor tissue that is not a limiting resource.

9) In section 1.4 it was suggested to test for antibody specificity ideally with samples from a knock-out mouse or cell line. In many cases no such models are available or there is no certainty that you see the same antibody specificity in your biological sample. Do you alternatively analyze your samples with Western blots and test different antibody concentrations?

We thank the reviewer for this well-founded concern. Unfortunately, western blotting is not a useful way to confirm antibody specificity for IP-flow cytometry because denatured proteins are used for western blots, while proteins in their native conformations are used for IP-FCM; antibody recognition and specificity are often not conserved between techniques. We have clarified this in the Discussion, line 642:

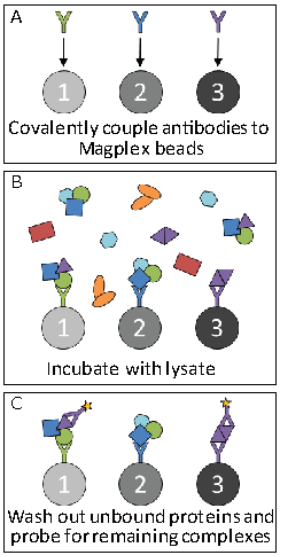
Another limitation of using flow cytometry rather than western blots is that size information to confirm antibody specificity is not available. To overcome this limitation, IgG controls are used in screening each antibody pair, and specificity is confirmed with knock-out or knock-in cell lines or animals before proceeding with QMI experiments (section 1.4).

Further, we had written that RNAi or artificial expression in target-negative cell lines could be used to confirm specificity. These may be more accessible ways for labs to confirm specificity, but overall it is an unavoidable challenge.

1.4.1. To ensure antibody specificity for the intended targets, use a lysate sample in which the target has been knocked out; for example, a knockout mouse or an RNAi cell line. Alternatively, use lysate from a target-negative cell line in which the target protein has been artificially expressed.

If unexpected, non-specific noise is present in a biological sample of interest; using an appropriate control should be sufficient to keep this noise from appearing as a 'hit' since QMI is only to be used for measuring changes in PiSCES, and there should not be statistically significant differences in noise levels between comparable samples.

10) In Figure 1A a scheme illustrating the different steps of the QMI assay is missing. In general, it does not become immediately clear from the text how the technique works. Are beads linked with different antibodies or are different beads mixed prior to immunoprecipitation in the same well? The current description of the method in my view is confusing.



Each bead class is first linked with a different antibody, and then mixed immediately prior to immunoprecipitation. We have added more detail in Figure 1A-C (right) to better briefly illustrate the steps of QMI.

11) In Figure 2B it is not clear where the detection threshold lays. Furthermore, the formulation in lines 510-511 "probe shows some positive signal" is not scientific. In what range related to the background is the signal you measure? In line 512, it was stated that using the same antibody for both bead and probe is not ideal. Why? Is this not a necessary control to confirm the presence of the target protein in the input?

We have added the following text to the Antibody Screening section of Representative results, which includes the MFI range difference to look for.

Figure 2B shows the results of a screen for the protein Connexin36. Most IP_probe combinations produce no signal over IgG controls. IP with the monoclonal antibody 1E5 and probe with either 1E5 or the polyclonal antibody 6200 produces a rightward shift in the bead distribution compared to IgG controls. Here, we selected IP 1E5 probe 6200poly, because we avoid using the same antibody as IP and probe, both to reduce the probability of a non-specific protein being recognized by two independent antibodies, and to increase the chance of detecting co-associations using different epitopes. It is best to choose an IP_probe combination with at least 1-2 log higher MFI compared to IgG controls, but occasionally pairs producing weaker MFIs that are consistently distinguishable from controls may be used if no alternatives are identified.

Line 512 references Figure 2, which shows a screen of different antibody clones that each recognize the protein Connexin36. Using one Connexin36 antibody clone for immunoprecipitation and another as a probe, we are indeed able to detect this target protein. However, as stated previously, QMI detects changes in protein interactions, not their absolute presence or absence due to the challenges defining background noise, now addressed in the Discussion beginning at line 639:

To account for this limitation of not knowing the absolute MFI signal, we do not report PiSCES that we detect above a arbitrary background level. Instead, we report PiSCES that change in response to a given stimulation. While high MFI can be caused by nonspecific noise, this noise would not be expected to change with stimulation, while actual PiSCES will. In addition, we typically confirm a portion (10-20%) of activity-dependent interactions that we observe by a second method, typically IP-western. This confirmation is analogous to confirming high-throughput RNA sequencing results with RT-PCR and is meant to increase confidence QMI results.

In the Representative Results section referenced by the reviewer, we have expanded the description of why different antibodies should be used:

Here, we selected IP 1E5 probe 6200poly, because we avoid using the same antibody as IP and probe, both to reduce the probability of a non-specific protein being recognized by two independent antibodies, and to increase the chance of detecting co-associations using different epitopes.

Similarly, we have added the following sentence to the legend for Figure 2.

Differential epitope recognition maximizes the chances of observing interactions because some epitopes may be occluded in certain protein complexes.

12) A general remark concerning all presented figures: They are all of low graphic quality which makes it difficult to understand the message; the axes labelling must be improved.

We apologize for the low graphic quality of the figures; we believe this is an artifact of the PDFs given for review, and we have double-checked that the graphics in our submission materials are up to JoVE's standards.

13) In the discussion, in lines 548-552, two points are made. On the one hand epitope availability is mentioned as a limitation "that is common with other protein interaction methodologies". This is true only for antibody-based techniques. On the other hand, you comment on the issue of not distinguishing between direct or indirect interactions. Based on this fact, it would be useful to mention early on that with the QMI method protein complex compositions rather than direct interactions are detected.

We thank the reviewer for pointing out that we did not make the nature of QMI-detectable protein interactions clear until later in the manuscript. In response to this critique and others, we have updated the manuscript to consistently use the more accurate term Protein Interactions in Shared Complexes detected by Exposed Surface epitopes (PiSCES), which clarifies from the beginning of the manuscript that the protein interactions measured by QMI are those in shared complexes.

14) A summary of the results presented in Figure 5 is missing in the text. What is the outcome of this experiment? Does this experiment represent a proof of concept study? If yes, it should be clearly emphasized.

Figure 5 is discussed in the Representative Results section (below). It is used as an example of output visualization after data analysis, which we believe is clear because it is referenced under the heading "Data Presentation."

Data Presentation. ANC and CNA analyses are performed to identify PiSCES that both (1) show significant fold changes between experimental conditions in at least 3/4 of runs and (2) belong to a CNA module that is correlated with the experimental variable. We refer to these high-confidence PiSCES that are identified two independent statistical approaches as ANC□CNA PiSCES. These interactions can be visualized as a node-edge diagram using the open-source software Cytoscape (Figure 5a) or as a heatmap by using the R code and analysis instructions included in the supplementary material (Figure 5b).

15) Even though mass spectrometry is mentioned as an alternative approach (line 90), it was not made clear why mass spectrometry approaches to study protein-protein interaction complexes are less sensitive compared to QMI.

We thank the reviewer for this critique, and in response we have more carefully emphasized the strengths of QMI while trying to avoid pointing out limitations of other techniques. The mention of mass spectrometry has been removed completely.

16) A clear limitation of QMI is that the applied antibodies might disrupt or stabilize protein complexes. This may introduce a bias, which should be discussed in this paper.

We thank the reviewer for this note and have added it to our discussion:

Line 619: A limitation of all antibody-based methods that should be kept in mind is that the addition of antibodies may disrupt or stabilize protein complexes.

Reviewer #3:

Major Concerns:

1) The manuscript's tittle is overly complicated and does not inform of the content. In particular, the use of term 'at mesoscale', normally referring to 100nm to 10µm cellular substructures, is rather misleading as the mesoscale aspect of the method is in no way highlighted in the manuscript.

We have changed the title (below) to be more informative and removed the term 'mesoscale.'

Quantification of protein interaction network dynamics using multiplexed co-immunoprecipitation.

2) Even though the methods is useful and should be made accessible for the scientific community, I found the current flow of both the protocol itself and the abstract rather difficult to follow. This is mostly because of lack clear distinction between the antibody-testing step and the actual QMI measurement of protein interaction. In fact, the purpose of this first antibody-testing section is only properly explained at the very end of the manuscript, in the discussion. The authors should consider revising the order of the manuscript or endeavour to make the aim of each step clearer, to make it easier to follow.

We appreciate the reviewer's feedback that the flow of both the protocol and abstract were difficult to follow, and especially their guidance as to why. In response to this critique we have made changes to the abstract which include the following overview of what to expect in the protocol, placed after we describe the basic principles of a QMI assay and its applications and advantages:

This protocol includes instructions to perform QMI from initial antibody panel selection through to running assays and analyzing data. Initial assembly of a QMI assay involves screening antibodies to generate a panel, and empirically determining an appropriate lysis buffer. The subsequent reagent preparation includes covalently coupling immunoprecipitation antibodies to Magplex beads, and biotinylating probe antibodies so they can be labeled by a streptavidin-conjugated fluorophore. To run the assay, lysate is mixed with Magplex beads overnight, then the beads are divided and incubated with different probe antibodies, then a fluorophore label, and read by flow cytometry. Two statistical tests are performed to identify PiSCES that differ significantly between experimental conditions, and results are visualized using heatmaps or node-edge diagrams.

Similarly, we have added a paragraph at the end of the introduction to orient readers for what to expect in the protocol section:

To assemble a QMI antibody panel, initial antibody screening and selection protocols are described in Section 1, below. Once antibody panels are identified, protocols for conjugation of the selected antibodies to Magplex beads for immunoprecipitation (IP), and for biotinylation of the selected probe antibodies, are described in Section 2. The protocol for running the QMI assay on cell or tissue lysates is described in Section 3. Finally, since a single experiment can generate ~5 x 10^5 individual datapoints, instructions and computer codes to assist in data processing, analysis, and visualization are provided in Section 4.

We believe that the order of the protocol: (1) Assay Design, (2) Multiplex Reagent Preparation, (3) Quantitative Multiplex Immunoprecipitation, and (4) Data Analysis should be left as is to reflect the order in which these steps are performed.

3) Moreover, a better idea of the minimal requirement of biological material necessary for the successful execution of the QMI protocol would be critical for scientist to make a decision whether the approach could be useful in their research. It would also allow to better compare the method with regular co-IP. The authors claim that the comparison with regard to input requirement is favourable for QMI (line 52), but it is impossible to judge this statement without any information on actual inputs used. Authors could refer to their own data here, and state the lowest cell numbers/protein concentration per a single well/IP that produced a clear signal.

Specific protein concentrations and cell numbers needed will vary depending on starting material, proteins of interest (since protein abundance varies over several orders of magnitude, even in a simple cell type such as yeast), and antibody affinity. We hope that our examples of biomaterials used in publications (detailed in lines 96-101) will be sufficient to give users a starting point, but input requirements must be empirically determined by users. To clarify this we have added a suggested starting point for users in Step 3.2.1., but stressed the need for empirical determination.

3.2.1. Lyse tissue or cells in appropriate detergent with protease and phosphatase inhibitors and incubate on ice for 15 minutes. Take care to keep the lysate cold at all times. The exact quantity of starting biomaterial and lysate protein concentration must be empirically determined, and some examples of previously used samples are listed in the third paragraph of the introduction. In general, 200 µL of 2 mg/mL protein have been successful in the past for 20 IP and 20 probe targets, but ideal inputs for each antibody panel and cell or tissue type must be determined empirically.

4) Similarly, to make the protocol more accessible for new users in the field, some examples of lysis buffer that could serve as starting conditions for optimisation should be provided. This is important as the authors themselves state that appropriate lysis buffer is critical (line 554). It would therefore be extremely useful to give composition of lysis buffers that were successfully used in QMI experiments as well as provide a list of detergents compatible with the assay.

We have added our standard lysis buffer composition and detergents known to be compatible with QMI in the following step:

1.5.2 Using the non-fluorescent, antibody-conjugated CML beads made for initial screens, perform IP-FCM as described in 1.2 using varied lysis buffer detergent conditions. Standard lysis buffer includes 150 mM NaCl, 50 mM Tris (pH 7.4), 10 mM sodium fluoride (Sigma, 201154), 2 mM sodium orthovanadate (Sigma, 450243), and protease/phosphatase inhibitor cocktails (Sigma, P8340/P5726). Detergents compatible with QMI include 1% NP-40, 1% Digitonin, 0.1-1% Triton X-100, and 0.5-1% deoxycholate. Detergent screens can be performed with detergent as the only variable, or with different cell conditions for each detergent. Always use IgG controls for both beads and probes, since detergents occasionally produce unexpected background in some IP-Probe combinations.

5) In general, more in-depth discussion of the differences in lysis buffer and input requirements between classical IP and QMI would be essential for the fellow scientists to be able to judge the applicability and potential advantages of using QMI over other methods in their research.

Lysis buffers compatible with standard IP are also compatible with QMI. The complication arises from the need to optimize the detection of multiple interactions in a single lysis buffer, where each interaction has different optimal conditions (as opposed to optimizing for one interaction in a traditional IP-western experiment). We understand that this presents an overall problem to the field, and have recently uploaded a manuscript to BioRXiv detailing our lab's attempts to deal with this issue {Lautz, 2019, Activity-dependent changes in synaptic protein complex composition are consistent in different detergents despite differential solubility}. Based on these data we feel confident, however, that detergents are not altering the changes detected by QMI, merely its aptitude for detecting them.

Another major difference between bead-based IP and IP-western is that in the latter, one is trying to pull out as much protein from as a sample as possible, while in the former, one is trying to sample protein without changing the overall protein concentration of the lysate to achieve quantitation (see Bida Methods 2012). Some of the discussion we have included about lysis buffer and input requirements is written below:

Line 94: The sensitivity of QMI depends on the protein concentration of the lysate relative to the number of Magplex beads used for immunoprecipitation, and achieving a resolution to detect 10% fold changes requires only a small amount of starting material compared to other co-IP methods[14,15]. For example, the amount of starting material used in QMI is similar to what's required for a sandwich Enzyme-Linked ImmunoSorbent Assay (ELISA), but multiple interactions are detected in a single QMI assay. QMI assays using 20 IPs and 20 probe targets have been performed using 1-5 x 10^5 primary T cells isolated from a 4 mm skin biopsy, P2 synaptosomal preparations from a 3 mm coronal section of mouse prefrontal cortex, or 3 x 10^6 cultured mouse primary cortical neurons[14,16,17].

Line 658: It is vital to select an appropriate lysis buffer for QMI. Too weak of a detergent can leave membranes intact and hold together proteins that aren't in complex, while too strong of a detergent can destroy protein complexes. Additional factors such as the presence of calcium or its chelators can dramatically affect PiSCES and should be carefully considered before screening antibodies to include in a QMI panel. For IP-western experiments, lysis conditions are usually optimized for each PiSCES on a case-by-case basis, but the best conditions for detecting a single PiSCES may not translate to other PiSCES in the same protein network[22]. Detergent selection presents a chicken-and-egg dilemma, in that a lysis buffer is needed to screen antibody candidates, but a panel of antibodies is needed to screen for an appropriate lysis buffer. While not a perfect solution, we suggest selecting a small panel of beads and probes that are of particular interest and/or have known associations or dissociations in response to a stimulus, and testing their behavior under different lysis conditions on the CML beads initially used for screening antibodies (step 1.1.3). An ideal detergent should allow for both reliable detection of PiSCES and recapitulation of known physiological protein behavior (association/dissociation) with a given stimulus. If there is any concern that a detergent does not fully solubilize membranes, a negative control antibody can be added that would only give signal if two proteins were linked by membrane.[24] When appropriate lysis buffers are selected, changes in even weak interactions—such as those between a kinase and substrate—can be reliably detected (e.g. TCR-LCK)[14].

The above responses 3 and 4 to this reviewer incude additions to the text which are also relevant here.

6) The authors underline the important of the samples being kept cool at all times. In particular, they emphasize that the flow cytometer needs to be refrigerated, which they achieved by custom adaptation. Some description of how it was achieved/advice would be very useful for researchers, who want to reproduce the setup in their own laboratories.

We thank the reviewer for pointing out the need for a description of our custom refrigeration, and in response we have added Figure S1, which contains detailed instructions and diagrams (see reviewer 1, response 3).

7) In addition, even a brief description of the antibody coupling to CML beads, instead of simply referring to another paper (line 158) would vastly improve the protocol.

There are two reasons we choose not include these steps in our (already lengthy) paper. 1) The referenced protocol is on JoVE, so a reader accessing this paper can easily access that one as well. 2) The CML bead coupling is very similar to Magplex bead coupling and would be repetitive. However, it also contains several critical, sensitive steps that the Davis protocol carefully addresses, but that we would have difficulty addressing in a brief description. Therefore we believe that referencing the protocol is a better approach.

Minor Concerns:

1) Lines 161 and 308: please state how long are the respective beads stable at 4 deg C.

We have added the following information at the appropriate steps:

Beads have been stored for over a year and used successfully in QMI assays, but shelf life or expiration dates have not been formally established. NaN_3 in the B/S buffer should prevent bacterial growth.

2) Line 176: "Calculate your IP volume". Not immediately clear if this refers to single IP or the total volume. Please clarify

We have clarified the calculation needed for this step.

1.2.1. Calculate the total volume of lysate to be used for each IP, using 10 µL for each IP-probe combination to be screened. If you are screening X probe antibodies and using one IgG control, each IP will use (X+1) * (10 µL) * (1.1 for pipetting error); X+1 is to account for the required IgG probe control. For example, in a 3x3 antibody screen, each IP should use 44ul.

3) Line 184: How was 0.66 µl of bead stock calculated? Is the concentration assumed to be 108/ml? Please clarify.

We have clarified that 0.66 µL is given as an estimate based on what typically results from our CML bead coupling, but indicated that users should be quantifying their own beads before use.

1.2.2. Calculate the your CML bead number to be used. If you are screening X probe antibodies, use [(X+1) * $5x10^4$ beads]. Ideally, 5,000 beads per well will result in >2,000 beads per well being read by the flow cytometer. For example, in a 3x3 antibody screen, each IP should use 20,000 beads, which is approximately 0.66 µL of prepared CML bead stock from 1.1.3 (beads should first be quantified using a hemocytometer to ensure accuracy).

4) Line 198: Figure 2A? Please specify the panel.

We have specified that this refers to panel 2A.

5) Lines 204-206: Not clear how or why this calculation is made. Could you please clarify? Also, please provide the dilution range.

The step has been modified as follows for clarity:

1.2.8. For each biotinylated antibody to be screened, calculate the total volume as (Y+1) * 1.1 * 50 µL, where Y is the number of IP antibodies being screened and 50 µL is the final volume per well. Dilute antibody to a working concentration in this volume of FlyP buffer, typically using a 1:100 dilution of a 0.5mg/ml stock.

6) Line 229: Figure 2B? Please specify the panel.

We have specified that this refers to Figure 2B.

7) Line 232: Figure 2C? Please specify the panel.

We have specified that this refers to Figure 2B.

8) Line 389: 1:100 - does this refer to the 2x or the final dilution? Please specify.

We have clarified this in the step:

3.3.4. In a different 96-well plate, dilute biotinylated probe antibodies to 2X working concentration (working concentration is typically 1:100 or 1:200, empirically determined)

9) Lines 510, 511 and 513: IE-5H5 is not labelled in the figure. Please correct the labelling or provide the explanation in the legend.

We have corrected to 1E5 in the legend.

10) Line 511: UW label is not present in the figure 2 at all. Please correct the labelling.

We have corrected this to 6200Poly in the legend.

11) Lines 527-530: Figure 5 legend is very short and not informative. Please provide more details, including the meaning of the colour and linewidth of the edges for panel A.

We have changed the legend for Figure 5 to be more descriptive, shown in reviewer 1, response 8.

12) Figure 5: Some of the nodes in panel A are different and are partially missing the enclosing line. Is this meaningful? If yes, please explain, otherwise please correct.

This difference was an error and has now been corrected.

13) Materials: Please provide the source of Streptavidin-PE.

We have added Streptavidin-PE to the materials list.

Supplemental File Figure S1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Fig S1 diagrams of refrigeration setup-01.tif