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Multiparameter screen optimizes immunoprecipitation

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ABSTRACT

Immunoprecipitation (IP) coupled with mass spectrometry effectively maps protein–protein interactions when genome-wide, affinity-tagged cell collections are used. Such studies have recorded significant portions of the compositions of physiological protein complexes, providing draft 'interactomes'; yet many constituents of protein complexes still remain uncharted. This gap exists partly because high-throughput approaches cannot optimize each IP. A key challenge for IP optimization is stabilizing *in vivo* interactions during the transfer from cells to test tubes; failure to do so leads to the loss of genuine interactions during the IP and subsequent failure to detect. Our high-content screening method explores the relationship between *in vitro* chemical conditions and IP outcomes, enabling rapid empirical optimization of conditions for capturing target macromolecular assemblies.

METHOD SUMMARY

There is presently no way to know, *a priori*, the optimal experimental conditions to use to immunoprecipitate a target protein complex: different interactors and subpopulations respond diversely to experimental conditions, and these behaviors must be discovered empirically. We present a high-content screen to rapidly optimize immunoprecipitations.

KEYWORDS:

affinity proteomics ● complexomics ● interactomics ● macromolecular assemblies

The study of macromolecular interactions within cells is one of the current major challenges in systems biology, which has spawned the research field of interactomics [1]. Generally, proteins associate together in multicomponent assemblies that are the effectors of cell biology. Gaining understanding of the compositions of macromolecular assemblies and their inter-relationships facilitates deeper interrogation (and also exploitation) of those assemblies and the biological processes they enable. (Co-)immunoprecipitation (IP; also affinity isolation, purification, capture etc.), coupled with protein mass spectrometry (MS), is one of the most popular and successful techniques for analyzing compositions of multicomponent assemblies formed by proteins. A key benefit of IP is that it allows the capture of target macromolecules and their assemblies directly from endogenous biological sources. Used as an analytical technique, one can 'go fishing' for uncharacterized interactions by IP, for example, if one has a target in mind and a suitable antibody. Used as a preparative technique, IP may circumvent the need for recombinant expression and exogenous reconstitution of the target multicomponent assembly, while also ensuring native protein processing, assembly and post-translational modifications. To obtain physiologically relevant information using IP, the captured macromolecules must be maintained in a native-like state once transferred from the living cell into the test tube. For this, because of the physicochemical diversity of multicomponent assemblies, IP must be optimized on a case-by-case basis, requiring the exploration of many possible experimental conditions. For the same reason, there is rarely one 'right way' or 'perfect solution' in which to conduct an IP. Rather, different capture conditions will reveal (to a greater or lesser degree) different *bona fide* associations and provide accordingly different perspectives on the constituents of multicomponent complexes that may form with the target protein *in vivo*. Taking such information all together, a more comprehensive and holistic view of protein interaction networks can be obtained.

We still lack a comprehensive 'first draft' map of a human interactome, including contextual, cell type-specific and disease-specific interactions, despite the completion of numerous large-scale, high-throughput efforts [2–6]. Such studies have advanced the field of interactomics immeasurably but, on account of their focus on the breadth of target proteins explored rather than on IP optimization, have also left innumerable gaps to be filled by boutique studies. Increasing focus on improving endogenous macromolecule capture technique and sample preparation will further accelerate our progress toward complete knowledge [1,7,8]. Moreover, while many techniques can detect (directly or indirectly) protein interactions [9–11], few can maintain intact interactions while transferring them from the cell to the *in vitro* environment – and so retrieve parts of the cell in a form useful for further downstream research, expediting and improving, for example, biochemical, enzymological and structural studies.

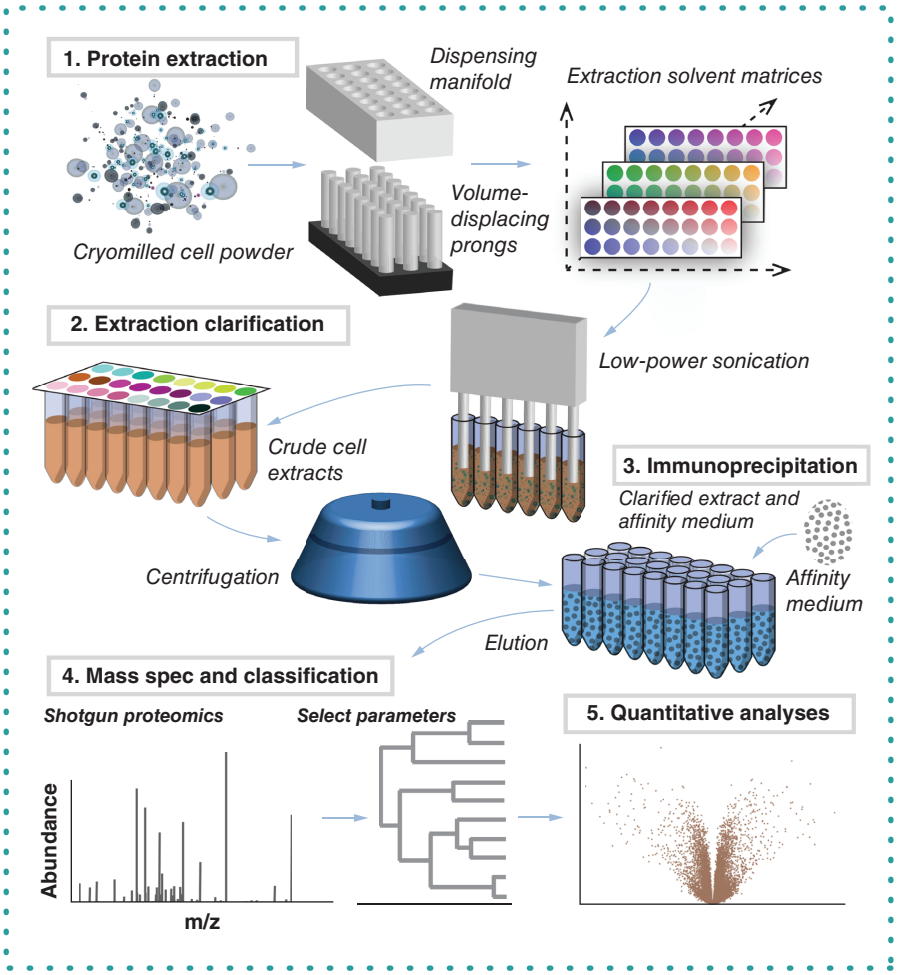


Figure 1. Summary of the screening approach. Cryomilled cell powders are distributed using a dispensing manifold and lysed with different extraction solutions (step 1); then samples are sonicated to disperse and homogenize the extracts, followed by centrifugal clarification (step 2); the clarified extracts are subjected to IP (step 3); finally, protein eluates are analyzed using MS (steps 4 and 5).
Reproduced from [27].

Features necessary for the next decade of interactome studies include both those that accelerate the detection and discrimination of physiological protein interactions (with other proteins as well as with all other macromolecule classes) and those that yield the intact endogenous macromolecules for detailed functional and structural analyses *in vitro*. The latter case is exemplified by the growing demand for samples suitable for the rapidly evolving native MS [12], crosslinking MS [13] and cryoelectron microscopy [8,14] methods that are revolutionizing structural biology. IP has the potential to satisfy both of these criteria if properly executed. Because maintaining physiological interactions *in vitro* while also limiting artifacts is extremely difficult [15–25], the following considerations are crucially important in a well-optimized IP:

- The interactions to be studied must be maintained throughout the capture (preventing false negatives);
- The target protein and its interactors should not form spurious interactions (preventing false positives);
- The antibody used must, first, be able to bind its target in the context of associations the target forms with other proteins in the cell (preventing false negatives) and second, exhibit low off-target binding (preventing false positives).

We developed an approach for parallelized IP–MS, depicted in Figure 1, that constitutes a high-content screen of the behaviors of macromolecular interactions *in vitro* [26–28]. Results from the screen first demonstrate the scenario described above – apparent interactors are susceptible to change, given the experimental conditions – and accordingly, second reveal interactors that go missed in unoptimized studies. This approach shares logic with crystallographic screening [29], where empirical sampling of many different crystallization solutions is necessary to identify suitably optimized conditions that support protein crystal formation and growth; likewise, solution conditions needed to stabilize multipartner macromolecules *in vitro* are often not known in advance. Indeed, the macromolecular compositions associated with the target protein are usually not known in full, in advance, given our sparse knowledge of interac-

tomes [30,31]; so predicting the necessary IP conditions is further complicated by this. We have found that multiparameter screening frequently returns new *bona fide* hits, offering new biological insights and research opportunities, even when applied to long-studied targets (elaborated further in the 'Results & discussion' section). Here we describe procedures relevant for the screening method and discuss numerous practical and theoretical concerns; we have previously reviewed IP-based affinity proteomics in general [32–34].

Materials & methods

We have previously described 96-well and 24-well versions of our screening procedure [26,27]. Our 96-well approach proved most handy for working with yeast, where cell quantity was not a limiting factor. For studies with human cell lines, where material was typically much less abundant, we favor the 24-well procedure, presented here. Although this is less comprehensive in terms of parameter space explored, it allows judicious consumption of the available cell material. Here, we also describe adaptations for a 32-well version of the protocol for added bandwidth. All materials related to this study can be found in Supplementary File 1; the detailed procedures are in Supplementary File 2.

The described procedures use cryogenically milled cell powder and antibody-conjugated magnetic beads [32,33]. This protocol also uses multiprobe, microtip sonication: human cell extracts in certain solutions may produce viscosity and/or may initially yield nonhomogenous extracts containing aggregates. These attributes may be detrimental to the IP procedure, but brief microtip probe sonication can cut viscosity and disperses aggregates. The goal is to apply the minimum energy that produces relatively homogenous extracts in the shortest time. High-speed centrifugation is used to clarify cell extracts (e.g., 10 min at ~20,000 relative centrifugal force): benchtop microcentrifuges are available that accept up to 48 samples at once, and using an adjustable-spacer, multichannel pipette simplifies the transfer of solutions from the microplate to 1.5-ml microcentrifuge tubes and back after centrifugation.

Culturing, harvesting & cryomilling

Procedures for cell culture, harvesting and cryomilling have been described previously [33]. Cell culture methods (and other handling considerations) will vary according to the cell type being cultivated, and appropriate considerations should be made. Some cell types may be more or less fragile; therefore mechanical scraping versus proteolytic release from culturing dishes should be evaluated when necessary, as well as, for example, evaluating the maximum relative centrifugal force that can be applied during cell pelleting to remove media and washes. Prematurely breaking the cells during scraping or centrifugation will spill their contents into the medium or washing solution that will be discarded. The intactness of cells can be checked at different steps for further optimization using vital staining techniques [34]. The amount of residual solution that is frozen along with the cells prior to milling should be minimized but will be influenced by the handling procedures for each cell type. Once milled, the formerly internal cellular milieus are now exposed, in the form of frozen powder, for direct access by the extractant solutions, enabling multiparameter screening.

IP-based interactome screen

The detailed procedures are described in Supplementary File 2, which includes mentions of alternative strategies we have tested at different steps to help facilitate use by most laboratories; where indicated, additional information may be found in the 'Notes' section (Supplementary File 3) and in the Supplementary Video.

Liquid chromatography–tandem mass spectrometry

Proteomic analyses by LC–MS/MS can be successfully carried out using a range of procedures. The LC system and MS instrument settings will vary widely depending on the setup and operator. Here we summarize our own settings for context and reference. For analyzing bands or regions excised from SDS-PAGE gels, see Shevchenko *et al.* [35]. For shotgun proteomic analysis of whole IPs directly from the screen, we favor S-traps [36] (see Notes 17 and 18 in Supplementary File 3) for typical sample preparation using the manufacturer's 'micro high recovery' procedure (in version 2 at the time of writing, at https://protifi.com/pages/protocols) [37]. The dried-down samples should be resuspended in 25 µl at a final concentration of 5% (w/v) SDS, 8 M urea, 100 mM glycine, pH 7.55; for the dried-down sample described in Step 21 of Supplementary File 2 (40 µl initial volume), that entails adding 25 µl of 1.8% (w/v) SDS, 8M urea and 100 mM glycine, pH 7.55.

The peptides eluted from S-traps are completely dried using a centrifugal vacuum concentrator and are then resuspended in 25 µl of a water:methanol:formic acid solution (94.9:5.0:0.1 parts by volume). From this suspension, 5 µl are loaded onto a 75 µm × 50 cm Acclaim™ PepMap™ RSLC nano Viper column filled with 2 µm C18 particles (Thermo Fisher Scientific, Bremen, Germany) via a Dionex UltiMate™ 3000 HPLC system interfaced with a Orbitrap Exploris™ 480 mass spectrometer (Thermo Fisher Scientific). Column temperature is set to 40°C. Using a flow rate of 300 nl/min, peptides are eluted in a gradient of increasing acetonitrile, where solvent A is 0.1% (v/v) formic acid in water and solvent B is 0.1% (v/v) formic acid in acetonitrile. Peptides are ionized by electrospray at 1.8–2.1 kV as they elute. The elution gradient length is 60 min, as follows: 3% B over 3 min; 3 to 50% B over 45 min; 2 min to 80% B; then wash at 80% B over 5 min, 80 to 3% B over 2 min and then the column is equilibrated with 3% B for 3 min. Full scans are acquired in profile mode at 120,000 resolution. The top 25 most intense ions with charge state +2–6 in each full scan are fragmented by higher energy collisional dissociation. Previously sequenced precursors are excluded for 20 s, within a mass tolerance of 10 p.p.m. Fragmentation spectra are acquired in centroid mode at 15,000 resolution. The normalized automated gain control target is set to 300% with a maximum injection

time of 50 ms. The normalized collision energy is set to 30%, using an isolation window of 1.4 m/z units. All replicates of the same kind are run one after the other, separated from the preceding and following IP conditions, or from the HeLa digest runs, by a blank run to clean the column of any carryover that might interfere in the analysis. The blank run is a 40-min LC method using a see-saw gradient consisting of three cycles of solvent B percentage going from 3 to 98% and then back to 3% (see Note 19 in Supplementary File 3).

Data processing

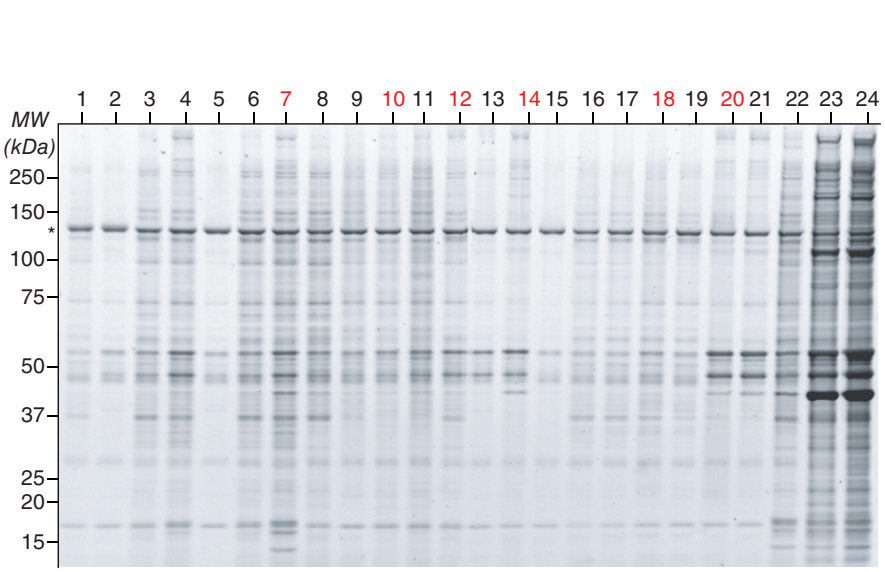
A full analysis pipeline has been described by Dou *et al.* [27]. Briefly, we typically process RAW data in MaxQuant with default settings (using version 2.1.4.0 as of the time of writing) and the following adjustments [38]. Variable modification: oxidation (M), acetyl (protein N-term) and phospho (STY); fixed modification: MMTS (C) (see Note 20 in Supplementary File 3); modifications included in protein quantification: oxidation (M); acetyl (protein N-term); match between runs (within groups of cognate experiments and controls): true. Second peptides: true. Separate label-free quantification (LFQ) in parameter groups: true. Require MS/MS for LFQ comparisons: true. Because we are typically analyzing the compositions of human protein complexes, we use a protein database composed of the Uniprot human proteome (reviewed entries; canonical and isoform), supplemented with additional sequences if necessary for the analysis. Proteins marked as 'contaminants' or 'reverse' by MaxQuant were removed, and protein LFQ intensities were $\log_2$-transformed. Any missing values of LFQ intensities were imputed to 0. Hierarchical clustering is performed on the $\log_2$-transformed LFQ intensities across 24 conditions. From this and from the cognate gel images (step 20 in Supplementary File 2), diverse conditions can be selected to use in a second round of screening with replicates. The goal is to maximize the proteomic diversity, while maintaining quality control and getting replicates to empower statistical inference. Six to eight conditions can be used in a second round of screening, in four replicates each, to produce quantitative comparisons of the compositions in each IP. A bioinformatic pipeline for this and for analyzing and visualizing the enrichment of known protein complexes can be accessed here: https://bitbucket.org/lacavalab/lfqscreening/src/master.

Results & discussion

Oftentimes, differences in interactors obtained under different IP conditions are immediately apparent by visual inspection upon screening; see, for example, the work by Winczura *et al.* (in particular their Figure 1A) [28] and multiple examples from Hakhverdyan *et al.* [26]. In other cases, statistical inferences will be needed to reveal differences that are not obvious by visual inspection, to tease out fine-grained detail. Figure 2 shows an example of the prescreening of the nuclear cap-binding complex using 24 different extraction solutions (reproduced from the paper by Dou *et al.* [27]). Details of the 24 extraction solutions can be found in Supplementary file 4. To select a range of conditions that cover the breadth and depth of candidate interactors of the target protein, the SDS-PAGE profiles (i.e., from step 20 in Supplementary File 2) and hierarchically clustered MS data (described in 'Data processing', above) were considered together [26,27]. Gel lanes 22–24 exhibit telltale signs of high nonspecific background; in contrast, conditions 1, 2, 5 and 13 exhibited relatively few bands. The gel bands provide a simple characteristic profile of interactors to distinguish the effects of each IP condition. Meanwhile, MS provides more in-depth information, with the interactors identified and semi-quantified across the screening conditions. These MS data can therefore also be used to cluster interactors according to, for example, gene ontology or pathway enrichment, enabling selection of conditions by enrichment of a target biological compartment or function. The visual inspection of the gel and the clustered MS data led us to select six conditions (7, 10, 12, 14, 18 and 20) for additional screening with replicates. These six conditions were then employed in IPs using both the tagged target protein cell line and its cognate control cell line (in this case, affinity tag-only cells), each with four replicates, to identify *bona fide* interactors by label-free quantitative MS and statistical inference [27]. Published results from our screens and conditions used to obtain them can be retrieved at http://copurification.org/ [26,39].

Beyond the solution conditions

Several other factors can influence IP-based results, such as incubation time, antibody characteristics and bead type. When starting an IP using magnetic beads, it is essential to devote attention to the particle characteristics, including their size and surface chemistry, among other physicochemical properties. We have shown that, for example, when working with Dynabeads® in an otherwise well-optimized IP procedure, most of the nonspecific protein interactions are likely to originate from the target protein itself and, after that, from antibody paratopes present in excess of their cognate epitopes [33]; this is not true for all stationary phases [40], and further engineering of bead surfaces as well as extraction solutions could enhance control of IP-induced false positives [41,42]. Sources of false positives will vary with experimental conditions, so methods that readout on post-lysis binding events are of high value: the Isotopic Differentiation of Interactions as Random or Targeted (I-DIRT) method is among the best and most practical tools to control for false positives in IP–MS experiments [16,43,44]. We intend to integrate this method into the multiwell IP screening procedure in the next version. Recently, we have started to also explore the use of magnetic separation systems that enable working under controlled conditions of constant magnetic force, as this is expected to improve reproducibility and facile scaling-up to large volumes without unexpected changes in capture efficiency [45–47]. While magnetic separations are subject to lower shearing than, for example, column-based separations, many variables are at play and may contribute to different enrichment outcomes with changes in bead size, magnetism, surface coating and the solution physical chemistry [48–50]. The behaviors of magnetic beads in different solutions and at different volumes in different vessels can be monitored using a Sepmag® device (Sepmag Technologies, Barcelona, Spain), allowing experiments at different scales to be harmonized.



#	Solution composition
7	20 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM deoxy big CHAP
10	20 mM HEPES, pH 7.4, 300 mM NaCl, 0.5 mM sarcosyl
12	20 mM HEPES, pH 7.4, 150 mM NaCl, 0.5% (v/v) Triton X-100
14	20 mM HEPES, pH 7.4, 50 mM $MgCl_2$, 0.5% (v/v) Triton X-100
18	125 mM NH_4 acetate, pH 7, 100 mM NaCl, 1% (v/v) Triton X-100
20	20 mM Tris, pH 8, 125 mM Na_3 citrate, 1% (v/v) Triton X-100

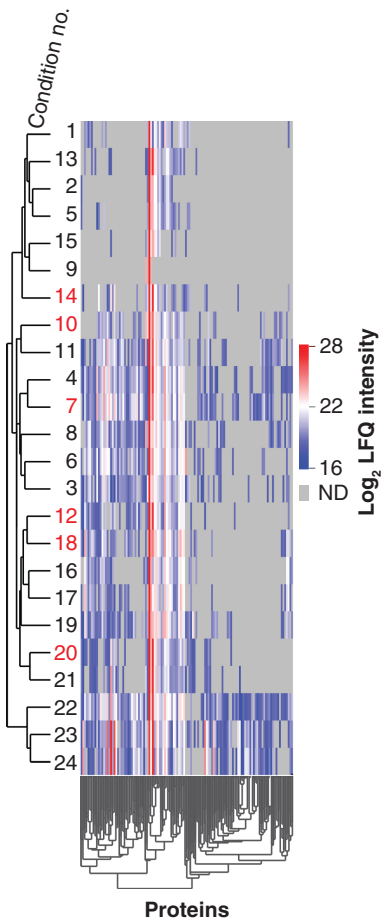


Figure 2. Example immunoprecipitation–mass spectrometry prescreen. SDS-PAGE/Sypro Ruby stain of NCBP1-LAP co-immunoprecipitations with 24 different extraction solutions (left) and hierarchical clustering of the cognate MS data using log_2 LFQ intensity (right). Not detected proteins are indicated in gray. Six conditions were selected for subsequent quantitative screening: red numbers above lanes and table below the gel.
LFQ: Label-free quantification; ND: Not detected.
Reproduced from [27].

Biological validation

As outlined above, there are various ways to limit false positive results in IP–MS-based interactome studies, but some approaches offer better true positive signal discrimination than others; generally speaking, results from interaction screens that pass statistical tests for enrichment with the target protein should be treated as hypotheses that such an interaction exists *in vivo* (and may have functional consequences). The better the method, the more reliable the hypotheses; even with highly reliable methods, follow-up validation of the biological roles of the detected interactions is key to provide meaning and context for them. Fluorescence colocalization is an example approach that can show proximity between two proteins, corroborating an *in vivo* interaction inferred from IP–MS analyses [51]. That is, the co-IP demonstrates a physical interaction (direct or indirect) and the cofluorescence demonstrates that, in the cell, these objects can be found in sufficient proximity that their signals are visualized together (supporting the conclusion of physical interaction). Other modes of validating physical interactions *in vivo* may require even closer proximity, which is more consistent with a direct interaction: for example, protein complementation assays (e.g., bimolecular fluorescence complementation) and Förster resonance energy transfer, among others [52–54]. Although many approaches can demonstrate when proteins that co-immunoprecipitate are proximal or interact within cells, they do not typically reveal anything about the functional significance of the interactions. To provide a concrete example: a complementary study by Dou *et al.* biologically validated findings from the interactome screen described here [27,55]. This was done, for example, first using fluorescence colocalization methods to corroborate *in vivo* proximity between putative interactors; and second by assaying the degree of similarity between cellular phenotypes resulting from perturbations to putative interactors of known and as-yet-unknown function. Proteins that function together tend to do so through physical proximity [56–58]. Perturbations that affect one member of a protein complex, and produce an assayable phenotype, may be (partially or completely) replicated by perturbing a different member of the same complex: constituent proteins tend to work together to give rise to the function of the complex.

Conclusion

Protein–protein interaction networks are complex and dynamic and lead to a spectrum of stable and transient macromolecular assemblies in the cell. In IP, the extraction solution plays crucial but poorly defined roles in dictating the stability of macromolecular assemblies and affects the quality of co-immunoprecipitates obtained: once extracted from cells, it is complicated and challenging to maintain multiprotein assemblies, *in vitro*, in states that authentically reveal their *in vivo* compositions and forms. This is compounded by the fact that the full gamut of interactors of a target protein is rarely (if ever) known in advance and cannot therefore be anticipated and tuned for, *a priori*. As a result, IP is an idiosyncratic experimental endeavor: there is no single or small set of extraction solutions and conditions that could work well for every target protein complex (or even all the complexes formed by a single target protein); when using IP to study the macromolecular assemblies formed by a protein, the extraction solution(s) should be extensively optimized by the investigator, and we have previously discussed many of the necessary considerations in fine detail [26,27,32,59,60]. Here we have described recent tweaks and updates to our interactome analysis optimization screen for IP [26,27]: we have increased the bandwidth available for routine mammalian IP from several to 24 or 32 conditions per run, through the design of a modified cell powder distribution manifold and an 8-/16-microtip ultrasonication probe.

Future perspective

With the advent of off-the-shelf *in situ* proximity labeling [61], we anticipate combining this form of interactome analysis of target proteins with IP–MS-based screening, in conjunction with I-DIRT for true positive signal discrimination. By identifying *in vitro* procedures that maximally preserve proximal *in vivo* interactions and induce minimal post-lysis rearrangements, we could potentially speculate the physicochemical mechanisms by which different procedures mimic, emulate or reproduce key features of presently uncharted subcellular milieus. In the near term, we imagine that combining wet lab empiricism with computational chemistry [62,63] and 'AlphaFolding' [64,65] may lead to more comprehensive theory, and/or machine learning models, that will enable predictions of superior formulations of *in vitro* conditions to maximally extract, preserve, yield and manipulate target protein complexes.

Executive summary

Background

- Complete and accurate profiling of macromolecular interactions within cells is challenging.
- We report modifications to a high-content screen that optimizes immunoprecipitations (IPs) for comprehensively capturing and characterizing the constituents of target macromolecular assemblies.

Experimental

- Cultured cells are cryomilled; the cell powder is carried forward to the protein interactions screen.
- Semi-quantitative prescreening (by IP–LC–MS/MS) reveals the proteomic diversity obtained within the initial experimental conditions explored; selected conditions that recapitulate most of the proteomic diversity of the prescreen, while removing likely noise, can then be chosen.
- Typically, six to eight selected conditions are used in a subsequent quantitative screen (including a condition-matched control screen to reduce false positive identifications) with four replicates per condition (by IP–LC–MS/MS); these data are used for label-free quantitative MS and statistical inference of *bona fide* interactors.

Results & discussion

- The candidate interactors vary under different IP conditions (i.e., the experimental conditions affect the relative retrieval of signal and noise).
- Several other factors, including incubation time, antibody characteristics and bead type, can also influence IP results.
- Affinity tag-only cells were used as controls here, but other approaches can also be employed to eliminate false-positive signals (e.g., Isotopic Differentiation of Interactions as Random or Targeted).

Conclusion

- We modified a screen to increase the bandwidth available for IP-based interactome analyses in cell lines from 24 to 32 IPs at once. This required the production of a new cell powder-dispensing manifold and a customized 16-tip microprobe sonication device.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.2144/btn-2023-0051. To view the supplementary video, please visit https://data.mendeley.com/datasets/62pzmpk68b/1.

Author contributions

Conceptualization: J LaCava. Methodology: S Xie, L Saba, H Jiang, M Oghbaie, J LaCava. Software: M Oghbaie, O Bringas. Investigation: S Xie, L Saba, H Jiang, L DiStefano. Resources: V Sherman. Writing (original draft): S Xie, L Saba, H Jiang, J LaCava. Writing (review and editing): S Xie, J LaCava. Supervision: J LaCava. Project administration: J LaCava. Funding acquisition: J LaCava.

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Competing interests disclosure

The authors have no competing interests or relevant affiliations with any organization or entity with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

Writing disclosure

No writing assistance was utilized in the production of this manuscript.

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• **A technique, based on isotopic labeling, that distinguishes false-positive interactors from genuine interactors in immunoprecipitation (IP) experiments. This method addresses one of the most challenging problems in defining real signals within protein complexes analyzed by IP–MS.**

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