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Identifying components of protein complexes in *C. elegans* using co-immunoprecipitation and mass spectrometry

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Abstract

Mass spectrometry-based proteomics is rapidly becoming an essential tool for biologists. One of the most common applications is identifying the components of protein complexes isolated by co-immunoprecipitation. In this review, we discuss the co-immunoprecipitation, mass spectrometry and data analysis techniques that have been used successfully to define protein complexes in *C. elegans* research. In this discussion, two strategies emerged. One approach is to use stringent biochemical purification methods and attempt to identify a small number of complex components with a high degree of certainty based on MS data. A second approach is to use less stringent purification and identification parameters, and ultimately test a longer list of potential binding partners in biological validation assays. This should provide a useful guide for biologists planning proteomic experiments.

1) Introduction

The power of proteomics is revolutionizing biological research. Many *C. elegans* scientists are wondering how to harness this emerging technology for their own studies. One of the most common uses of mass spectrometry based-proteomics is the identification of individual proteins from samples containing many proteins. This is especially useful for identifying members of purified protein complexes. Traditionally, a worm geneticist could identify genes acting in the same pathway by mutagenic or RNAi-based screens for animals with a mutant phenotype. However, such genetic screens are limited by an inability to determine if the gene products are interacting physically. Generally, a biochemical purification procedure that isolates a small selection of proteins is required to identify physically interacting components of a complex, although in practice the final sample often contains non-specific

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'background' proteins that are not members of the complex. With the development of efficient protein separation technologies, the extreme sensitivity of the modern mass spectrometer can aid in the identification of true complex members. Understanding the relationship between methods used for complex purification, protein identification and complex determination is of key importance in designing a successful experiment.

There are three critical decisions to be made when designing an experiment. 1) How will the protein complex be isolated? 2) How will proteins be identified? 3) How will members of the complex be distinguished from non-specific background proteins? After a discussion of these questions, we will examine published work to see how different approaches affect results.

2) Isolation of the protein complex by co-immunoprecipitation (co-IP)

This question focuses on the biochemical purification steps used to isolate the protein complex. The worm lysate contains thousands of proteins. The goal of purification is to generate a sample containing only the complex of interest. In this regard, the worm poses a challenge. Although the genome and proteome of *C. elegans* is smaller then those of a mammal, proteomic analysis may not be any easier. With larger animals you can reduce the variety of proteins in a sample by surgically removing a tissue of interest. However, when we lyse an adult worm all 959 somatic cells and the germ cells are present. (Imagine your response to a colleague grinding up an entire adult mouse to study kidney proteins.) This reduces the relative amount of desired complex and increases the number of background proteins that need to be removed.

Harvesting synchronized worm cultures can help reduce complexity. The groups studying the kinetochore during early development enriched for embryos by dissolving synchronized adults in bleach and collecting the bleach-resistant embryos[1–2]. Techniques such as gel filtration and differential centrifugation may be used to enrich for organelles[3]. Membrane-associated or DNA binding complexes present specific challenges to complex purification [4–5].

A popular way to isolate a protein complex from worm lysate is by co-immunoprecipitation. The co-IP can be performed in a single step or as part of a tandem purification. An antibody targeting a known member of the complex is bound to a bead and incubated with lysate in order to extract the target from the solution along with the other members of the complex. The antibody used for the co-IP can recognize the protein of interest or a protein tag that has been genetically fused to the protein of interest. The advantage of using an antibody against the protein of interest is that the protein expression is not altered; the disadvantages are mostly practical, e.g. more time required to synthesize and/or purify the peptide or protein antigen and then to immunize animals and harvest the antibodies. These polyclonal antibodies are limited in supply and unique to each immunized animal. Also, one cannot be certain that the antibodies will be specific enough for useful separation. Alternatively, a molecular "tag" can be added to a protein of interest expressed from a transgene and purified using an antibody against the tag. The tag can be used for a single-step purification or a multistep or tandem purification. Many different tags have been used in *C. elegans*[6–9]. As the functional impact of adding a tag usually is not known, different tags attached at either the N or C terminus can be constructed. The ability of a tagged protein to rescue the phenotype of a null mutant is a sign that the tagged protein is functional. Commonly, green fluorescent protein (GFP)-tagged proteins are generated to determine protein localization in *C. elegans*. GFP can also be used for single-step purification[10]. This is an exciting option as many strains generated solely to obtain localization data can now be used to identify binding partners. Combining a localization tag with a purification tag, as done with the

multipurpose Localization and Affinity Purification (LAP) tag [7], provides an improved purification ability without sacrificing the localization information *C. elegans* biologists have come to expect (see section 5.1, Identifying a kinetochore regulatory complex).

There are several caveats to tagging proteins. Gene expression may not be regulated under the same promoter and the copy number of the gene could be much higher so the protein may be over-expressed, increasing non-specific interactions. Additionally, the tag itself may interfere with the protein's normal function, translation and turnover rates or prevent the formation of protein complexes. A major benefit to using tags is the commercial availability of well-characterized antibodies which should help to reduce the effort required to optimize the purification.

Isolation of protein complexes often fails due to inadequate sample preparation. Possible explanations for failure include acquiring undetectable amounts of the complex or destruction of the complex during purification. Although the expression level of the target protein and the characteristics of the antibody have a major influence, using a higher amount of worm lysate should increase the likelihood of success. For a co-IP of abundant proteins, 10–20 mg of lysate was adequate [11–12], while 40 g was used for a less abundant membrane protein [4]. Liquid culture of worms can be carried out to generate large quantities of worms [13]. However, as behavior and physiology may be altered by liquid culturing, it is possible that some complexes will be different compared to complexes isolated from worms grown on plates. Growing worms on multiple large (15 cm) plates allows for the plating of a precise number of synchronized L1 larvae that can be cultured for an exact amount of time to generate reproducibly homogenous samples [5]. Harvested worms can be flash frozen and stored at −80° C or lysed immediately.

Lysis conditions need to be harsh enough to break open the worms to free the complex but mild enough to prevent complex dissociation. Lysis methods often include a step to break the protective cuticle of the worm. A Dounce homogenizer[5], French press[14], mortar and pestle[15] or sonicator [16] have been used successfully to generate lysates for co-immunoprecipitation. To minimize degradative enzymatic activity and preserve the complex in the lysate, the lysis buffer often contains protease and phosphatase inhibitors and the lysate is kept ice-cold at all times[17]. Typically, lyates are cleared by centrifugation, protein concentration is determined, and the co-IP is then carried out. Freezing lysates before the co-IP may harm the complex and should be avoided.

3) Identifying members of the protein complex

Protein identification by mass spectrometry is the cornerstone of proteomics. The two choices for protein identification using a mass spectrometer are peptide fingerprinting or shotgun proteomics. For peptide fingerprinting, the eluted complex is separated using SDS-PAGE. The gel is either Coomasie-stained or silver-stained and bands unique to the test sample and hopefully containing a single protein are excised, enzymatically digested, and analyzed by mass spectrometry. The mass of these peptides is determined and matched to a peptide database to determine the source protein. The gel also provides a rough estimate of the molecular weight of the protein. Since only unique bands are cut out, background bands are not identified. Abundant background proteins may obscure target proteins while less abundant proteins may fall below the limits of detection by staining. This method works well with purified samples containing only a handful of proteins.

Alternatively, for shotgun proteomics the entire eluate, containing many proteins, is digested. Shotgun proteomics is currently the most powerful strategy for analyzing such complicated mixtures. Following enzymatic digestion of the eluted protein complex, the resulting peptides are chromatographically separated and analyzed by tandem mass

spectrometry[18]. Tandem mass spectrometry consists of three steps. In the first step, the *m/z* of the peptide is measured, in the second step the peptide is fragmented. The third step measures the *m/z* of the fragment ions. When a multi-step chromatographic purification is performed in line with a mass spectrometer this technique is commonly called Multi Dimensional Protein Identification Technology (MudPIT). Protein identification is usually achieved by comparing experimental tandem mass spectra with theoretically generated spectra and selecting the most likely sequence match via search engines such as SEQUEST [19], ProLuCID [20], or Mascot[21]. The identifications are then filtered according to quality scores and a false-discovery rate is estimated with a software such as DTASelect [22]. Since most *C. elegans* laboratories are not equipped with a mass spectrometer, analysis of the eluate or gel band is outsourced [23]. Institutional mass spectrometry core facilities can typically perform standard techniques for protein identification, although specialized proteomics laboratories with top of the line high-accuracy mass spectrometers may be able provide additional expertise to deal with challenging samples or identification of post-translational modifications.

4) Distinguishing true members of the complex from non-specific background proteins

Unfortunately, classifying an identified protein as a bona fide interactor or background contaminant is not always straightforward. Whereas only a handful of bands may be unique to a test sample when a co-IP eluate is separated by gel electrophoresis, in a shotgun proteomic analysis hundreds of proteins are often identified, even when the purification was successful. This might not be a consequence of suboptimal purification but rather of an extremely sensitive mass spectrometer. While a sample may benefit from additional or other means of purification, even using the most stringent purification methods finding hundreds of proteins in a sample is commonplace. Thus, ranking proteins by various selection criteria can help to focus a study on a few high quality candidates.

Percent coverage, the percentage of amino acids contained in the identified peptides for a given protein and spectral counting, the number of identified spectra related to each protein, provide rough estimates of protein abundance[24]. Often an arbitrary cutoff point is chosen below which proteins are not considered. Recently, powerful statistical methods have been developed to reduce the arbitrariness of the cutoff point[25]. These methods provide meaningful data from complex results and greatly increase the utility of the Co-IP to *C. elegans* scientists.

As the sensitivity of protein detection methods has improved, the number of proteins identified has increased. This is beneficial when detecting members of the complex but problematic when detecting background proteins, which include extremely abundant proteins such as actins, tubulins and vitellogenins[26] that although greatly reduced by the purification still remain detectable.. Other background proteins may become enriched during the purification by, for example, binding the resin or the antibody even though they are not members of the desired complex.

Negative controls can be crucial for ruling out non-specific background proteins as complex members. Worm researchers are fortunate that many knockout strains are available. These make great controls when using antibodies against the protein of interest. However, it cannot be assumed that a point mutation prohibits complex formation. For example, Gu et al. used the fact that an inactive complex was formed in a mutant strain to verify members of the complex[12]. It is tempting to use gene knockdown by RNA interference (RNAi) as a negative control but as gene knockdown may not be complete, data analysis could be

challenging. For Co-IPs using tags, wild-type worms, worms expressing only the tag or a tagged unrelated protein can be used as controls.

Once the shotgun proteomic data has been acquired, the next step is to pinpoint the differences between the complex peptide mixtures. A user friendly and freely available software for these tasks is the PatternLab for proteomics [25]. PatternLab is a graphical and integrated environment that aims to be a one-stop-shop for analyzing proteomic data. Here we focus on two of its modules that can be applied for analyzing protein complexes: the approximate area proportional Venn diagram (AAPVD) and the TFold module.

The AAPVD uses an “all or none approach” by comparing different conditions and generating a Venn diagram of circles with area proportional to the number of identified proteins. A highlight is that it offers data quality filters. One such filter is used when analyzing data from technical replicates. This filter can be used to remove proteins that are only identified by a single spectrum or in a single run. It may be important to require further evidence to remove a “one-hit-wonder” or a spurious identification. To eliminate such cases and allow the user to focus only on confident data, the AAPVD module can be set to consider proteins that were identified in at least two out of three replicates. The TFold module complements the AAPVD module as it can pinpoint proteins that are contained in both test sample and negative control yet are differentially expressed. This is a tricky task because it deals with a massive hypothesis-testing problem. To overcome such, the TFold module combines fold-change cutoffs with the t-test and the Benjamini-Hochberg (BH) theoretical false-positive rate estimator [27]. This type of analysis could be helpful when examining complexes containing very abundant proteins or common background proteins.

Other computational approaches such as determining interaction networks are valuable tools in the study of co-IPs. Sardiu et al [28]described a strategy to take advantage of label-free quantitative proteomics to determine protein interaction networks; this strategy is optimized for small-scale datasets. The quantitative approach employed in this study uses normalized spectral abundance factors (NSAF) [29](*1*) consisting of the total number of spectra identified from each protein, normalized for protein length and the total number of identified spectra for all proteins in the sample. The authors then contrast the protein abundance between affinity purifications of samples against the negative control purifications and separate proteins that are uniquely found and the ones that were quantitatively enriched over the negative controls. The later is mathematically performed using Singular Value Decomposition (SVD). Finally, the proteins from co-IPs are clustered using the Jaccard index as the similarity measure and a Bayesian approach is used to determine the “bait-to-prey” probability of intra and inter cluster interactions. The authors show that such quantitative deletion-interaction network are valuable tools to aid in the study of protein functions and in discerning sub-complexes within protein complexes.

Another technique for identifying background proteins employs metabolic labeling using stable isotopes[30]. In this approach, bacteria are grown with non-radioactive heavy nitrogen (^{15}N) as their only nitrogen source. Worms fed these bacteria will incorporate the ^{15}N into their proteins[31]. These heavy-labeled proteins behave identically to their standard light counterparts, but can be distinguished by the mass spectrometer. Equal amounts of lysate derived from a light-labeled test sample and a heavy-labeled negative control are combined before co-IP. After elution, digestion, and analyses by MS, proteins with relatively higher amounts in the light sample are likely interacting proteins, whereas proteins found with equal abundance in both samples may be background proteins.

Often a major advantage of using *C. elegans* is the ease of biological validation. The cost associated with testing candidates in worms is often so low as to allow for the application of

less stringent data filtering criteria. A mouse researcher with a long list of candidates may need very stringent filtering criteria to select a few strong candidates for biological validation. The worm researcher can use low stringency filtering to generate a long list that can quickly be tested in an RNAi screen.

5) Case Studies

Table 1 summarizes these studies in terms of the targets chosen for immunoprecipitation, types of negative controls, methods used to identify complex members, and numbers of true complex members versus background proteins. In reviewing these reports, two strategies emerged. One approach is to use stringent biochemical purification methods and attempt to identify a small number of complex components with a high degree of certainty based on MS data. A second approach is to use less stringent purification and identification parameters, and ultimately test a longer list of potential binding partners in biological validation assays. The stringent approach may miss weakly interacting proteins that could be detected with less stringent purification. It remains up to individual researchers to decide if time and resources are better spent improving protein complex purification or performing an increased number of assays for biological validation.

5.1 Identifying a kinetochore regulatory complex

The kinetochore is an organelle required for chromosome segregation. In a study that used mass spectrometry to identify kinetochore regulatory complex proteins, Desai et al. used a rabbit antibody against KNL-1, a protein required for kinetochore formation, for co-immunoprecipitation followed by mass spectrometry. Non-specific rabbit IgG was used as the negative control. The eluted proteins were separated by gel electrophoresis, which revealed three unique bands and six bands present in both the anti-KNL-1 and the rabbit IgG co-IPs. The three unique bands were excised, enzymatically digested, and identified by peptide fingerprinting. In addition to KNL-1 and the previously known HIM-10, Desai et al. identified a novel protein that they designated NDC-80[1].

Cheeseman et al. used the same KNL-1 antibody as used in the Desai study for their co-IPs. However, protein identification was performed by MudPIT and 54 proteins were identified using a 5% sequence coverage cutoff; 24 proteins were found bound to the GST negative control antibody. Two methods were employed to distinguish non-specific from bona fide binding partners. First, co-IP of another complex member, KNL-3, was performed, and of 83 proteins identified, only the 11 proteins found in both KNL-1 and KNL-3 IPs were considered to be true interactors[2]. Second, in order to reduce the association of background proteins, more stringent purification of the immunoprecipitated complex was performed using LAP-tagged complex members MIS-12 and KBP-1. The LAP tag consists of three parts: GFP, a TEV cleavage site, and an S peptide domain[7]. A GFP antibody was used to immunoprecipitate the complex, which was then released by the addition of TEV protease such that GFP remained bound to the beads. This eluate was used as the input for the second step of purification in which the S peptide domain was used for affinity purification. With this method, 10 and 11 proteins were identified using MIS-12 and KBP-1 as IP targets, respectively. Only one protein in the LAP eluates was shown to be a background protein. Conversely, one complex member identified using direct IP of endogenous protein was missing from the LAP IPs (CENP-C). These data clearly demonstrate the effectiveness of the tandem affinity purification strategy for removing background proteins, but at the cost of losing weak binding partners.

A similar outcome was observed in a comparison of tandem-tagged versus untagged protein Co-IPs in a study of the mechanism controlling kinetochore-microtubule interactions[32]. In that study, a sequence coverage cut-off of 20% was used to focus on five likely interactors

identified among over 150 proteins from an anti-ZWL-1 co-IP. On the other hand, purification using LAP-tagged ZWL-1 identified only three interactors with a less stringent 5% sequence coverage cut-off.

5.2 Defining proteins associated with the nicotinic acetylcholine receptor

Another type of tandem purification was used to identify 33 proteins that interact with the nicotinic acetylcholine receptor[4]. The tandem affinity purification (TAP) tag used in this example contains Protein A followed by a TEV protease site and calmodulin-binding peptide (CBP)[33]. To prevent the purification of tagged proteins not assembled into complexes, a split tag was used in which Protein A and the TEV site were fused to one complex member, UNC-29, while the CBP domain was fused to a different complex member, LEV-1. A total of 225 proteins, all the proteins identified in this purification as well as proteins identified by other methods, were selected for further analysis. First, to determine if the identified proteins were enriched during purification, the list was compared to a published data set of 1616 abundant worm proteins[26]. Sixty-nine proteins were considered normally abundant proteins and thus given low priority. Second, the effect of RNAi-mediated protein depletion on nicotinic receptor sensitivity was tested for 157 genes. Thirty-three proteins were enriched by purification and affected receptor function, and were thus declared receptor-associated proteins.

This experiment highlights the advantage of combining the sensitivity of mass spectrometry for protein identification with *C. elegans* for biological validation. Since measuring receptor sensitivity of RNAi-treated animals can be done relatively easily, loose data filtering criteria could be used. Of the 33 receptor-associated proteins, 30 were identified by only one peptide. Approximately 80 other proteins were identified by one peptide but RNAi depletion had no effect on receptor sensitivity. In this case even though the MS data supporting receptor association was weak, the ease of biological validation permitted the identification of 30 true protein associations.

5.3 The Dicer complex

The mammalian RNA endoribonuclease Dicer is required for generating multiple types of small RNA. To gain insight into Dicer function, the *C. elegans* homologue DCR-1 was subject to immunoprecipitation and co-purifying proteins were identified by mass spectrometry[11]. This study isolated complexes containing DCR-1 from both gravid adults and embryos. Purification from wild-type adult worm lysate was performed using a DCR-1 antibody, while the purification from embryos used anti-HA antibodies and lysate from a *dcr-1* deletion mutant containing an HA tagged rescuing transgene. Each purification was repeated two to three times and 108 proteins identified in DCR-1 purifications from adults and embryos, but not control co-IPs, were considered potential interactors. Of these, 20 were found in at least one of the purifications and biological validation demonstrated that eleven were either required or enhanced RNAi. A known DCR-1 interactor, RDE-1, was present at very low levels of abundance, suggesting that other true interactors may be among the remaining 88 untested putative members of the complex. Interestingly, the co-IP of tagged protein using HA antibody identified 70 potential interactors whereas the co-IP using DCR-1 antibody found only 38[11]. Different effectiveness of the two antibodies and/or reduced complexity of the embryo lysate may account for this difference.

7) Conclusions

Combining purification by co-IP with protein identification by MS is a powerful method for determining members of protein complexes. Modern mass spectrometers are able to detect low abundance proteins, which is useful for identifying weak interactors but with the

drawback of increased detection of background proteins. Biochemical methods to improve the purity of the purification and bioinformatics filtering techniques are available to extract meaningful interactions. These improvements should increase the use of co-IPs by *C. elegans* biologists.

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Table 1

Comparing methods and results

IP target	Control used for comparison	Detection method	Positive Interactors	Background Proteins	Ref
Endogenous protein					
KNL-1	Random IgG	Gel	3	6	[1]
KNL-1	Other members of complex	LC/MS/MS	11	>40	[2]
DCR-1	Deletion mutant	LC/MS/MS	108	N/A	[11]
DRH-1	Deletion mutant	Gel	3	5	[12]
DRH-1	Point mutation	Gel	3	5	[12]
ZWL-1	LAP-ZWL-1	LC/MS/MS	5	>150	[32]
Tagged fusion protein					
AIN-1::GFP	Strain without tag	Gel	~2	N/A	[15]
AIN-2::GFP	Wild-type/untagged GFP	LC/MS/MS	36	N/A	[10]
TAP::UNC-29	Published list	LC/MS/MS	33	>200	[4]
IP followed by second affinity purification					
LAP::KBP-1	Random IgG	LC/MS/MS	10	1	[2]
LAP-ZWL-1	Anti-ZWL-1	LC/MS/MS	2	1	[32]