Chapter 13

Extracting, Enriching, and Identifying Nuclear Body Sub-Complexes Using Label-Based Quantitative Mass Spectrometry

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Abstract

Determining the proteome of a nuclear body is a crucial step toward understanding its function; however, it is extremely challenging to obtain pure nuclear body preparations. Moreover, many nuclear proteins dynamically associate with multiple bodies and subnuclear compartments, confounding analysis. We have found that a more practical approach is to carry out affinity purification of nuclear body sub-complexes via the use of tagged nuclear-body-specific marker proteins. Here we describe in detail the method to identify new nuclear body protein sub-complexes through SILAC (stable isotope labeling by amino acids in culture)-based affinity purification followed by quantitative mass spectrometry.

Key words Nuclear bodies, SILAC, Mass spectrometry, Affinity purification, GFP

1 Introduction

Eukaryotic cells enclose their genome within a dedicated organelle, the nucleus, which is the site of major cellular events including DNA replication, mRNA transcription, and ribosome biogenesis. Like the cytoplasm, the nucleus is compartmentalized to facilitate efficient coordination of these pathways, albeit into membraneless subnuclear organelles called nuclear bodies. Examples include nucleoli, Cajal bodies (CBs), histone locus bodies (HLBs), PML nuclear bodies (PML-NBs), splicing speckles (also known as interchromatin granule clusters), and paraspeckles (*see* [1] for review). These bodies primarily occupy the interchromatin space, and several have been shown to be associated with specific gene loci and/or their RNA products.

It is generally agreed that most nuclear bodies serve to concentrate proteins (and in most cases, RNAs) involved in similar processes in a constrained space, presumably to enhance reaction

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efficiency and facilitate regulation. Although many of the mechanisms controlling their formation, organization, and movement remain unclear, cataloguing their respective components and characterizing specific multiprotein complexes targeted to these bodies have provided valuable clues to their distinct functions.

With the exception of nucleoli, however, nuclear bodies do not lend themselves readily to purification. To date, mass spectrometry-based analysis of protein composition has only been carried out on purified nucleoli [2–5] and enriched fractions of speckles/interchromatin granule clusters [6] and Cajal bodies [7]. In these latter cases, and with many other nuclear bodies, the steps required to obtain pure preparations are complex and numerous and require detailed individual optimization for each subnuclear body. Alternate approaches involve fractionating nuclei into multiple samples for analysis by mass spectrometry (MS), to look for clustering that indicates subnuclear complexes. Such techniques have been used to map the distribution of yeast nuclear proteins in sucrose gradient fractions [8] and cardiac nuclear proteins in nucleoplasm, chromatin, acid-extracted chromatin, and detergent-solubilized whole nuclei [9]. A promising approach is protein correlation profiling, which maps specific co-enrichment with organelle marker proteins and has been utilized to map sub-cytoplasmic organization [10]. However, given that most components of nuclear bodies are highly dynamic and thus detected in multiple locations, it is likely that mapping subnuclear organelle components in this way will not be as straightforward as it is for cytoplasmic organelles. Indeed, while fractionation achieved more comprehensive coverage of the yeast nuclear proteome [8], it was noted that known components of complexes had multiple peaks, which complicates analysis.

In the absence of proteome datasets, manual curation of the published literature has provided component lists for PML nuclear bodies [11], paraspeckles [12], and Cajal bodies. These lists have been further expanded by imaging-based screens that map subnuclear protein localizations on a genome-wide scale [13–15], identifying novel components of known bodies and, in some cases, highlighting previously undetected nuclear bodies.

A complementary approach for the functional characterization of nuclear bodies, which has been used successfully in numerous studies, is the mapping of sub-complexes that associate with a specific protein component of that structure. This is normally based on affinity purification (AP) of the marker protein (endogenous or tagged) and identification of associated interaction partners by MS analysis. Quantitative analysis is an increasingly popular choice for defining multiprotein complexes, as inclusion of a negative control provides a background of contaminant proteins (i.e., those that bind nonspecifically to the affinity matrix and/or the fusion tag) against which proteins that bind specifically to the protein of interest clearly stand out. In addition to mapping protein-protein interactions,

quantitative AP-MS has also been extended to the analysis of protein-binding partners for synthetic peptides [16], synthetic DNA oligonucleotides, and tagged RNA [17, 18].

Label-free quantitation methods are available; however, we will focus here on label-based methods, given their wide applicability and relative ease of use. A large number of differential labeling strategies have been developed over the years, and one in particular that has been successfully applied by our labs and others to the analysis of various subnuclear complexes is SILAC (stable isotope labeling by amino acids in culture; [19]), a metabolic approach that labels proteins in vivo through incorporation of isotopic amino acids. SILAC can be applied to the analysis of either endogenous or tagged proteins affinity purified from cell extracts [20].

Briefly, proteins are labeled directly in cells through growth in culture media containing either light (^{12}C, ^{14}N) or heavy (^{13}C, ^{15}N) isotopes of the essential amino acids arginine and lysine. Cells are fractionated and nuclear extracts prepared for the affinity purification step, with the heavy amino acid-encoded nuclear extract used for immunoprecipitation (IP) of the endogenous or tagged protein of interest and the light amino acid-encoded nuclear extract used to perform a control IP. For an endogenous protein SILAC IP, the control IP consists of incubating light amino acid-encoded cell extract with purified antibody backbone (e.g., IgG) bound to the affinity matrix at the same concentration as the antibody used to deplete the endogenous protein. For a tagged protein SILAC IP, the tag alone is expressed in cells encoded with light amino acids and then depleted using the same affinity matrix used to deplete the tagged fusion protein. The experimental and control affinity matrices are then combined and all of the proteins eluted together, separated by SDS-PAGE, trypsin-digested, and identified by MS.

Combining the experimental and control samples prior to MS analysis eliminates the variability that occurs between two independent MS runs. The shift in mass of peptides arising from the heavy amino acid-encoded cells can be resolved using certain high-end mass spectrometers, allowing direct comparison of the relative amounts of the two peptide peaks (heavy and light). These "SILAC ratios" clearly identify contaminant proteins that bind nonspecifically in both the control (light) and the experimental (heavy) IP because they are found in equal amounts (ratio heavy: light = ~1). In contrast, proteins that are enriched specifically in the experimental IP have higher SILAC ratios (ratio heavy: light > 1). Importantly, this quantitative approach, with its built-in negative control, allows for lower-stringency IPs that preserve a larger number of specific interaction events. We can thus identify not only core complex members and high-affinity binding partners but also proteins that bind with lower affinity and/or are present at a lower abundance (*see* [21] for Review).

We initially applied this strategy to identify and compare specificity of nuclear-binding partners for two different isoforms of protein phosphatase 1 [22], later optimizing and extending it to the analysis of both endogenous and tagged proteins in cytoplasmic and nuclear extracts [20] and the mapping of nucleolar protein interactomes [23, 24]. To demonstrate the power of this technique for profiling nuclear body sub-complexes, we identified 22 known and several novel interactors for the well-characterized SMN (survival of motor neurons) protein in a single tagged SMN pulldown experiment [20], highlighting which proteins SMN associates with in the nucleus (where it accumulates in Cajal bodies) vs. the cytoplasm (where it is predominantly diffuse under steady-state conditions). When properly designed and implemented, SILAC-based AP-MS experiments have a high success rate, and all of these studies highlight the two key points for reliably identifying interaction partners: (1) ensuring efficient isolation of the target protein under study (from initial extraction through affinity purification) and (2) achieving a high signal-to-noise ratio (i.e. filtering the noise that is the necessary consequence of combining lower-stringency IPs with high-sensitivity MS methods).

Although endogenous proteins in their native settings under physiological conditions would appear to be the ideal bait for mapping interactomes via quantitative AP-MS experiments, and indeed have been utilized successfully in numerous screens, interpretation of the data can be complicated by cross-reactivity of the antibodies. The interactors identified would in that case represent the mix of complexes that co-purified with both the intended bait and with any unrelated proteins that share the same epitope [21]. There are ways around this, including comparison of AP-MS datasets collected before and after knockdown of the target protein by siRNA [25].

An alternate approach, and the current method of choice for most AP-MS experiments, is the exogenous expression of epitope-tagged recombinant target proteins that can be efficiently recovered from cell extracts using affinity matrices or well-characterized antibodies. It should be noted that transiently overexpressing very high amounts of a bait protein is unlikely to increase interactome coverage unless there is a concomitant upregulation of all of its binding partners and also increases the risk that nonphysiological protein interactions will occur and be detected during MS analysis [21]. Ideally, a tagged protein should be expressed at near endogenous levels and rigorously validated to ensure that it behaves in a similar fashion to the endogenous protein.

The tag of choice for most cell biologists is green fluorescent protein (GFP) or one of its many variants, as they can be exploited to measure properties such as dynamic subcellular localization, colocalization of proteins in subcellular structures, rates of protein movement, and direct protein-protein interactions (*see* [26] for Review). Although it was originally a common practice to analyze

the properties of a GFP-tagged protein in vivo but then switch to a different tag for affinity purification and analysis of interaction partners, demonstration of its minimal nonspecific binding and the availability of highly efficient affinity reagents have led to the adoption of GFP as an affinity tag [20], permitting the direct comparison of imaging and proteomic data.

Using the quantitative SILAC-based proteomics strategy described here, endogenous or tagged protein complexes can be purified from nuclear extracts or specific subnuclear fractions and components identified by quantitative mass spectrometry. GFP-tagged nuclear body proteins will be used as examples, as a fluorophore tag provides the added benefit of being able to monitor protein localization throughout fractionation steps; however, this same approach can be applied to other epitope tags.

2 Materials

2.1 Cell Culture and Transfection

1. Enhanced GFP-C1 (EGFP-C1) (*see* **Note 1**).
2. HeLa and U2OS cell lines (available from ATCC, USA).
3. Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and 100 U/mL penicillin/streptomycin (1:100 dilution of a 10,000 U/mL solution). Add G418 to media used for generation and maintenance of cell lines stably expressing GFP fusion proteins.
4. Dulbecco's PBS (DPBS).
5. 1× Trypsin-EDTA solution (supplied as 0.05 % trypsin/0.48 mM EDTA; Invitrogen).
6. 90-mm-diameter tissue culture dishes.
7. 25- and 75-cm^2 flasks with filter caps.
8. Dimethyl sulfoxide.
9. Effectene transfection reagent (QIAGEN).
10. Geneticin (G418). Prepared as a 200 mg/mL stock in serum-free DMEM, filter-sterilized, and stored in 0.5 mL aliquots at −20 °C. Add 0.5-mL to a 500-mL bottle of DMEM for a 200 μg/mL stock or 1-mL to a 500-mL bottle of DMEM for a 400 μg/mL stock.

2.2 SILAC Encoding

1. Basal media: Dulbecco's Modified Eagle's Medium (DMEM) minus arginine and lysine (AthenaES, Maryland, USA) supplemented with 10 % dialyzed FBS (Invitrogen) and 100 U/mL penicillin/streptomycin.
2. "Light" media: basal media supplemented with L-arginine (84 μg/mL; Sigma) and L-lysine (146 μg/mL; Sigma). Filter through a 0.22-um filter (Millipore) using a suction pump and stored at 4 °C.

3. "Heavy" media: basal media supplemented with L-arginine ^{13}C (84 μg/mL; Cambridge Isotope Laboratories, USA) and D4-lysine (146 μg/mL; Cambridge Isotope Laboratories, USA). Filter through a 0.22-um filter (Millipore) using a suction pump and stored at 4 °C (*see* **Notes 2** and **3**).
4. PBS-based nonenzymatic cell dissociation buffer (Invitrogen) (*see* **Note 4**).

2.3 Preparation of Nuclear Extracts and Affinity Purification of Tagged Proteins

1. RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 % NP-40, 0.5 % deoxycholate and protease inhibitors). Prepare fresh and store on ice.
2. Cell-swelling buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and protease inhibitors) for Method 1 (Dounce). Prepare fresh and store on ice.
3. Mild detergent buffer (20 mM Tris-HCl, pH 7.4, 10 mM KCl, 3 mM $MgCl_2$, 0.1 % NP40, 10 % glycerol, and protease inhibitors) for Method 2 (permeabilize/centrifuge). Prepare fresh and store on ice.
4. S1 buffer (0.25 M sucrose/10 mM $MgCl_2$).
5. S2 buffer (0.88 M sucrose/0.5 mM $MgCl_2$). Prepare fresh and store on ice.
6. Wheaton Dounce homogenizer (Thermo Fisher Scientific).
7. Sepharose CL4B fast flow (GE Healthcare, Little Chalfont, UK).
8. Endogenous or tag antibody covalently conjugated to the affinity matrix of choice. For affinity purification of GFP-tagged proteins, GFP-TRAP® covalently conjugated to agarose (Chromotek, Martinsried, Germany) is the most efficient reagent (*see* **Note 5**).

2.4 Protein Elution and Separation by 1D Polyacrylamide Gel Electrophoresis

1. Vacuum centrifuge evaporator (e.g., Thermo Savant SpeedVac).
2. Sodium dodecyl sulfate (SDS; Sigma), prepared as a 1 % working solution.
3. Dithiothreitol (DTT; Sigma), prepared as a 1 M stock that can be stored at −20 °C.
4. Iodoacetamide (IAA; Sigma), prepared as a 1 M stock that can be stored at −20 °C.
5. NuPAGE 4× LDS sample buffer (Invitrogen).
6. Precast NuPAGE 10 % Bis-Tris gels, 1.5 mm, 10-well (Invitrogen).
7. NuPAGE MOPS SDS running buffer (Invitrogen), supplied as a 20× stock solution.
8. SeeBlue Plus 2 molecular weight standards (Invitrogen).
9. SimplyBlue SafeStain (Invitrogen).

2.5 Peptide Digestion

1. Sterile scalpels (Swann-Morton disposable scalpels, VWR).
2. Eppendorf LoBind microcentrifuge tubes (VWR).
3. Ammonium bicarbonate (VWR), prepared as a 1 M stock solution, aliquoted, and stored at −20 °C.
4. Acetonitrile (Sigma).
5. Trypsin Gold (Promega, Wisconsin, USA) is supplied as 100 μg of powder, which is resuspended in 200 μL of 20 mM ammonium bicarbonate as a 0.5 μg/μL stock solution and stored at −20 °C.
6. Formic acid (VWR), prepared fresh as both a 1 % and a 5 % stock solution in dH_2O.
7. Crimp vials, 11 mm PP, 0.3 mL (Kinesis, St. Neots, UK) and snap caps, 11 mm, PTFE (Kinesis).

2.6 Mass Spectrometry and Data Analysis

1. Liquid chromatography-mass spectrometry (LC-MS) on an LTQ Orbitrap XL Hybrid MS system with nanospray source (Thermo Scientific, Bremen, Germany) and an UltiMate 3000 RSLC nano-HPLC (Dionex, Camberley, UK), controlled by Xcalibur software version 2.0.7 (Thermo Scientific).
2. C18 CapTrap column (Bruker-Michrom, California, USA) in line with a 10-cm-long column with integrated emitter tip (Picofrit PF360-75-15-N-5; New Objective, Massachusetts, USA) packed with Zorbax SB-C18 5 μM (Agilent, California, USA).
3. HPLC interfaced to the mass spectrometer with a voltage of 1.1 kV applied to the liquid junction.
4. MaxQuant software v1.2.7.4 [27].

3 Methods

3.1 Selection of Marker Proteins for Nuclear Bodies

Marker proteins for nuclear bodies are used for a variety of reasons, including confirmation of the accumulation of a particular protein of interest within a specific body or the effect of a cellular perturbation on the body's integrity. Note that not all markers are considered defining components of the body and should therefore be chosen with care according to the experimental design [1]. Figure 1 provides a list of proteins that are commonly utilized as visual markers for the most widely studied nuclear bodies, either by immunofluorescence or by expression as tagged fusion proteins. For the analysis of sub-complexes within a specific nuclear body, the tagged marker protein will ideally accumulate primarily within this structure. If there is more than one subnuclear pool, validation of region-specific interactions will have to be carried out in follow-up analyses.

Nuclear Body	Marker Protein	Known/Predicted Function(s)
Nucleolus	UBF, B23	Ribosome biogenesis; stress response; regulation of cell cycle progression
Cajal body (CB)	Coilin, SMN	Maturation of snRNA and snoRNA; telomerase assembly
Speckle/IGC	ASF, Sm proteins	Storage and assembly of pre-mRNA splicing factors
Paraspeckle	PSPC1, NONO	Nuclear retention of specific mRNA, transcription factor sequestration
PML NB	PML, SP100	Stress response and viral defense
Histone locus body (HLB)	NPAT, LSM11	Transcription and processing of histone pre-mRNA

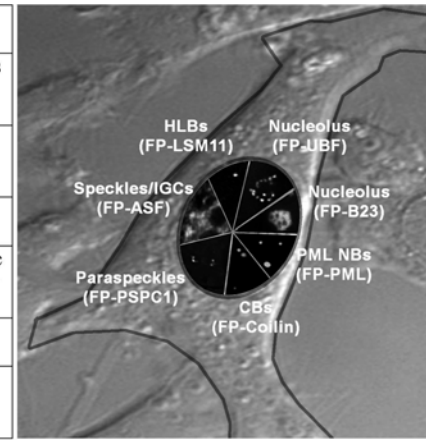

Fig. 1 Nuclear bodies and examples of marker proteins that accumulate within them. The table at *left* indicates a number of well-studied nuclear bodies, their functions, and the usual marker proteins for each structure. The composite fluorescence micrograph and light microscopy image on the *right* shows a HeLa cell with a number of fluorescent protein (FP)-tagged nuclear body marker proteins enriched in nuclear bodies

A caveat with all tagged protein approaches is that the presence of the tag can alter the properties and function of the molecule being studied. It is therefore important to control for the effect of the tag in the experimental design and, where possible, validate the results obtained using other methods. Figure 2 presents a flowchart that can help determine the suitability of a tagged protein for a quantitative proteomic approach. Although the ideal approach (and where a tagged protein is not a valid marker, the only approach) is direct analysis of endogenous protein complexes, in the absence of an efficient endogenous antibody, tagged AP-MS experiments can give rapid clues to cellular function. One advantage of using tagged proteins is that most tag antibodies are highly specific, whereas antibodies raised against endogenous proteins often cross-react with other proteins and can thus complicate interactome analyses with off-target hits. An additional advantage of using a GFP-tagged protein is that intracellular localization of the protein can easily be monitored by fluorescence imaging throughout the fractionation steps (Fig. 3) (*see* **Note 6**).

3.2 Encoding Cells by Growth in SILAC Media

The diagram in Fig. 4 shows the design of a typical SILAC AP-MS experiment.

Cell lines such as HeLa and U2OS are routinely passaged in SILAC media for at least 5–10 cell doublings prior to harvesting to ensure complete incorporation of isotopic amino acids (for Review *see* [19, 28]) (*see* **Note 7**).

1. Transfer 1 × 150 mm dish of cells into either light or heavy media to start incorporating the amino acids. For this size dish, 15 mL of media is sufficient to cover the cells, provided the incubator shelf is level.

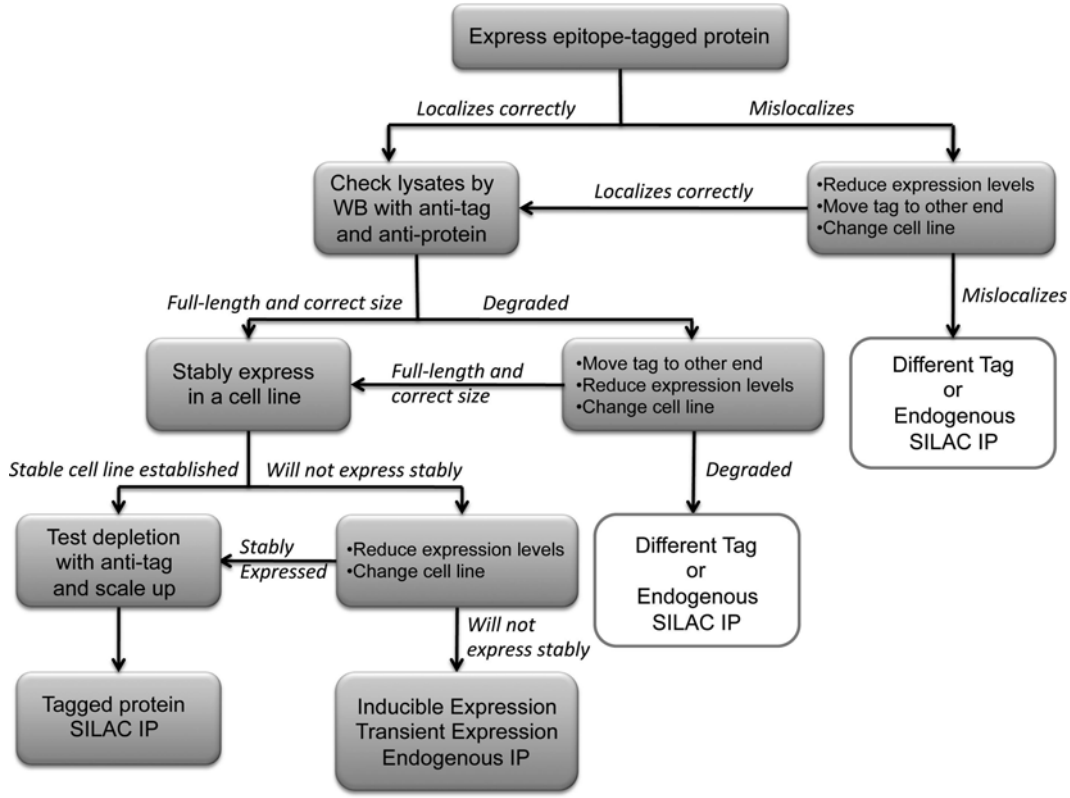

Fig. 2 Flowchart for the optimization of SILAC-based quantitative AP-MS experiments. This flowchart can be used to determine the suitability of a tagged protein for interactome mapping

2. When the cells are confluent, rinse with DPBS and add 3 mL of cell dissociation buffer. Incubate at 37 °C and monitor to determine when cells have released from the dish (*see* **Note 8**).
3. Expand the cells into 5 × 150-mm dishes, and plate at a confluency of ~20 %. When confluent, a final split of 1:2 dishes will result in the required 10 × 150-mm dishes for each condition (*see* **Note 9**).

3.3 Preparing Nuclear Extracts

To prepare nuclear extracts, it is first essential to determine the most efficient method for purifying nuclei from your cell line of interest. For example, we have found that although HeLa cells are amenable to the use of Dounce homogenization to break them open and release nuclei, this method does not work for U2OS cells, and they remain intact. They are, however, amenable to an alternate method that is based on mild detergent permeabilization and high-speed centrifugation. These two methods can be used as starting points for optimizing the purification of nuclei from other cell lines.

3.3.1 Harvest of the Cells

1. For each batch (light and heavy), save 50 mL of the original media and clear it by centrifugation at 1,532 × *g* for 4 min. This will be used for the initial resuspension of harvested cells at **step 3**.

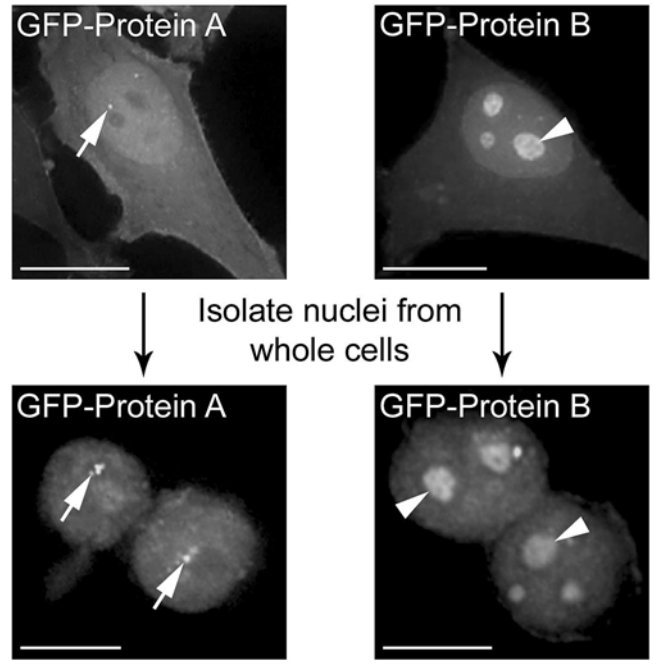

Fig. 3 Monitoring GFP-tagged proteins during subcellular fractionation. A major advantage of GFP fusion proteins is the ability to readily monitor their localization during fractionation protocols. In the example shown here, two GFP-tagged proteins retain their distinct subnuclear localization patterns when cells are broken open and nuclei purified. Protein A accumulates at Cajal bodies (*arrows*), while Protein B accumulates within nucleoli (*arrowheads*). These localizations can be further confirmed by directly staining purified nuclei with antibodies that recognize endogenous Cajal body and nucleolar marker proteins (*see* **Note 6**)

2. Remove the SILAC media.
3. Wash the cells once with DPBS, treat with either dissociation buffer or trypsin-EDTA as described in Subheading 3.2, **step 2,** and resuspend in 20 mL of the cleared original media, taking care to use the appropriate media for each batch. Pellet the cells by centrifugation at 1,532×*g* for 4 min.

3.3.2 Purification of Nuclei by Dounce Method

1. Wash the pelleted cells in DPBS and resuspend in 5 mL of ice-cold swelling buffer on ice for 5 min.
2. Break cells open to release nuclei using a prechilled Dounce homogenizer (20 strokes with a tight pestle). Monitor by light microscopy to ensure that the cells have broken open, and carry out additional Dounce steps if required.
3. Centrifuge dounced cells at 228×*g* for 5 min at 4 °C to pellet nuclei and other fragments. Retain the supernatant as the cytoplasmic fraction.

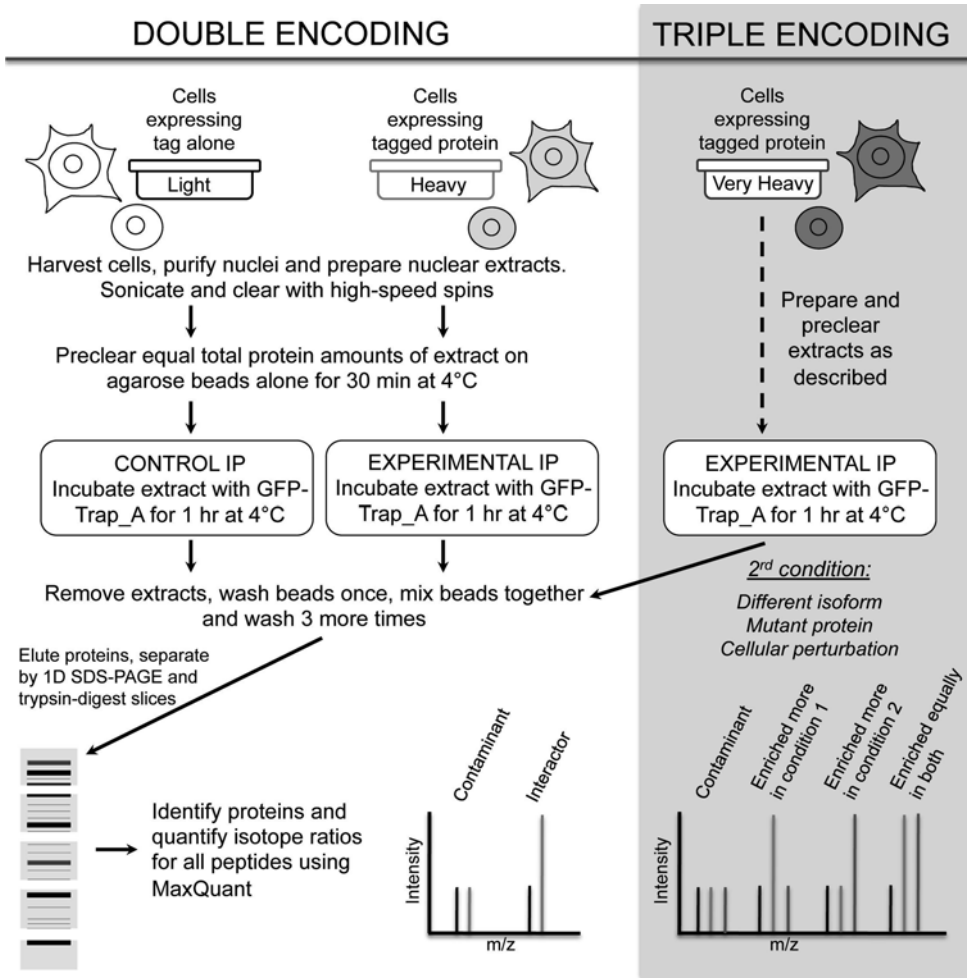

Fig. 4 Design of a typical SILAC-based AP-MS experiment to map the interactome of either a tagged or endogenous protein. The tagged protein experimental design can be modified for different epitope tags, using the appropriate control and affinity matrix. For endogenous protein pulldowns, experimental extracts are incubated with antibodies raised against the protein of interest covalently conjugated to beads, while control extracts are incubated with the same concentration of purified IgG, covalently conjugated to beads. If desired, a third condition can be added, by labeling cells with $^{13}C/^{15}N$ isotopes of Arg and Lys

3.3.3 Purification of Nuclei by Centrifugation Method

1. Wash the pelleted cells in DPBS and resuspend in 5 mL of ice-cold mild detergent buffer on ice for 10 min.
2. Centrifuge at 1,350 × *g* for 10 min at 4 °C to break open cells and release and pellet nuclei and other fragments. Retain the supernatant as the cytoplasmic fraction.

3.3.4 Preparation of Nuclear Extract

1. Resuspend the nuclear pellet (from Subheading 3.3.2 or 3.3.3) in 3 mL of S1 buffer and layer over a 3-mL cushion of S2 buffer.
2. Centrifuge at 2,800 × *g* for 10 min at 4 °C. This results in a cleaner nuclear pellet.

3. Resuspend purified nuclei in 5 mL of 1× RIPA buffer and sonicate on ice, using a microtip sonicator (6×10 s pulses, with 10 s rest in between). Monitor by light microscopy to confirm efficient disruption of nuclei, adding further sonication steps if required. As a final clearing step, centrifuge the nuclear extract at 2,800×*g* for 10 min at 4 °C.
4. Move the soluble extract to a new tube. Solubilize any remaining insoluble material (visible or not) by adding 8 M urea to the tube.
5. Monitor the efficiency of extraction at the level of total protein or at the level of your individual protein of interest, by separating equivalent volumes of soluble extract and insoluble pellet by 1D SDS-PAGE. For example, if a 5 mL soluble nuclear extract is prepared, solubilize the remaining pellet in 0.5 μL of 8 M urea and load 10 μL and 1 μL of each, respectively. Total protein can be visualized by Coomassie staining the gel. Figure 5b, d demonstrates the efficiency of extraction of total protein with sonication in RIPA buffer for nuclei purified using the two methods outlined above. Efficiency of extraction of the protein of interest can be confirmed by Western blot analysis. If necessary, extraction efficiency can be improved by changing the stringency of the extraction buffer (e.g., higher salt and/or detergent concentration), with the caveat that the increased stringency may disrupt certain protein-protein interactions.
6. Verify efficiency of fractionation by Western blot analysis of cytoplasmic and nuclear extracts with antibodies raised against proteins specific to each compartment. Western blot analysis of HeLa and U2OS fractions confirmed selective enrichment of alpha-tubulin in cytoplasmic extracts and lamins A/C in nuclear extracts (Fig. 5b, d). Figure 5 also shows a whole proteome analysis of cytoplasmic and nuclear extracts prepared from HeLa (using Method 1; Fig. 5a) and U2OS (using Method 2; Fig. 5c) cells, with commonly utilized compartment-specific markers highlighted. As expected, tubulins and GAPDH distribute to the cytoplasmic extract, while histones and lamins distribute to the nuclear extract. Note that caution should be used with actins as cell compartment markers, given that they can show a more equal cytoplasmic-nuclear distribution.

3.4 Affinity Purification

1. Measure total protein concentrations for all extracts.
2. Aliquot equal amounts of nuclear extract from the two differentially labeled cell lines, based on total protein concentrations.
3. Preclear extracts separately by incubating with affinity matrix alone for 30 min at 4 °C. For sepharose beads, pellet by centrifugation at 3,716×*g* for 2 min. Subsequent affinity purifications are also carried separately, and the beads combined at the final wash steps (*see* Fig. 4).

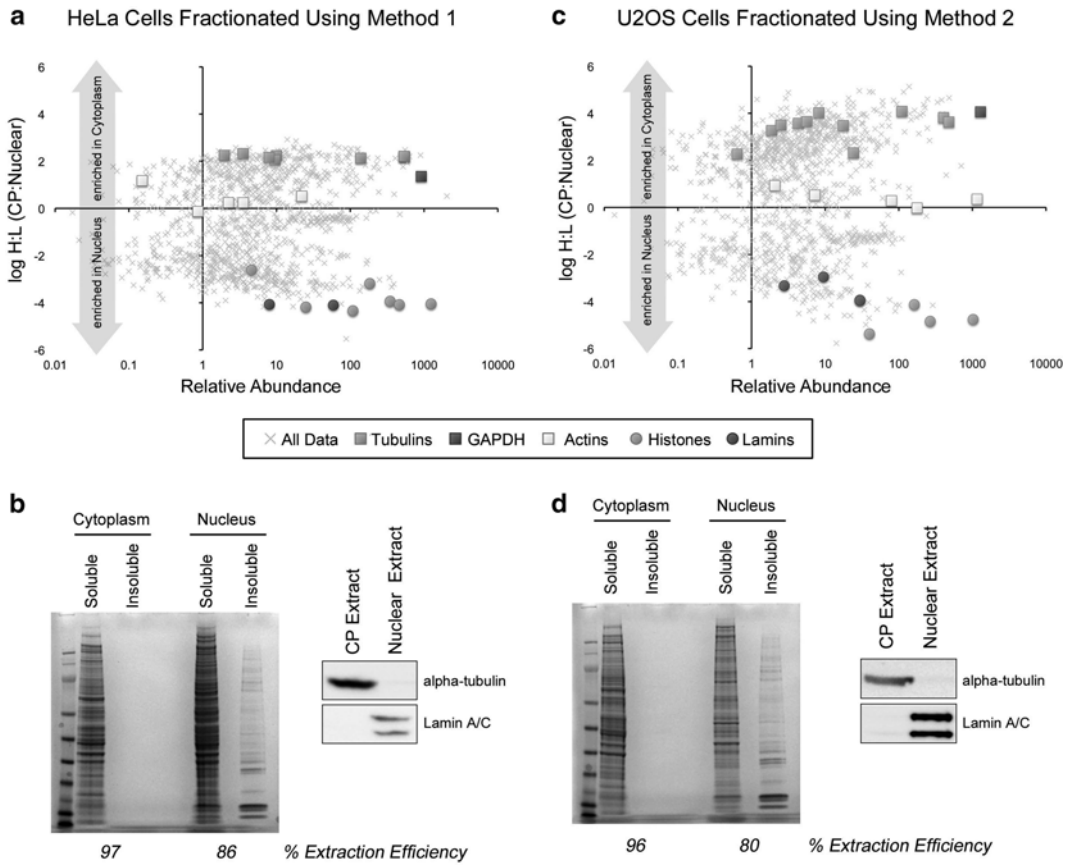

Fig. 5 Verifying efficient fractionation and total protein extraction. (**a**) The efficiency of fractionation and protein extraction using Method 1 for HeLa cells was demonstrated both by Coomassie staining (*left panel*) and Western blot analysis (*right panel*). Alpha-tubulin and lamin A/C are cytoplasm- and nucleus-specific markers, respectively. Note that total nuclear protein extraction is not 100 % efficient with sonication in RIPA buffer. This can be improved with increased extraction stringency; however, this carries with it the risk of disrupting protein interactions. (**b**) Quantitative proteomic mapping to assess the distribution of all identified proteins between the cytoplasmic and nuclear extracts. Cell-equivalent volumes of heavy isotope-labeled cytoplasmic extract (H) and light isotope-labeled nuclear extract (L) were combined and subjected to mass spectrometric analysis. The graphs plot log H-L (i.e., cytoplasmic-nuclear) ratio vs. relative abundance for all proteins identified. Ratios >1 (log ratios >0) indicate selective enrichment in the cytoplasm, while ratios <1 (log ratios <0) indicate selective enrichment in the nucleus. Common compartment-specific markers are *highlighted*. (**c**, **d**). Same analysis for the fractionation/extraction of U2OS cells using Method 2

4. For endogenous protein pulldowns, incubate experimental (heavy amino acid encoded) extracts with purified endogenous protein antibodies covalently conjugated to the affinity matrix (*see* **Note 10**). For the built-in negative control, incubate the same amount of control (light amino acid encoded) extract with an equivalent amount of control affinity matrix (purified IgG from the same species covalently conjugated to the affinity matrix at the same concentration). Retain a 10 μL aliquot of extract before and after IP to confirm efficient depletion.

5. For tagged protein pulldowns, incubate the same amount of experimental nuclear extract (heavy amino acid encoded, from cells expressing the tagged protein) and control nuclear extracts (light amino acid encoded, from cells expressing the tag alone) with tag antibodies covalently conjugated to the affinity matrix (*see* **Note 11**). Retain a 10 µL aliquot of extract before and after IP to verify efficiency of depletion. We recommend a maximum incubation time of 1–2 h, to minimize loss of low affinity and low abundance binding partners.
6. Pellet the beads, carefully remove the extract, and wash the beads once with 1 mL of RIPA buffer.
7. Add 0.5 mL of RIPA buffer to each tube and carefully transfer both sets of beads to a new tube. Wash the combined beads two more times with 1 mL RIPA buffer.

3.5 Eluting Proteins from Beads and Separating by 1D SDS-PAGE

1. Add a bead-equivalent volume of 1 % SDS, boil the beads for 10 min, and then add a 4× volume of dH_2O. For example, if eluting from a total of 100 µL beads, first add 100 µL of 1 % SDS, boil, and then add 400 µL of dH_2O. This helps to avoid retention of high concentration eluant in the inter-bead volume.
2. Vortex the beads, remove the supernatant, and reduce it to its original bead-equivalent volume (and 1 % SDS concentration) by vacuum centrifugation (Thermo Savant SpeedVac, 65 °C, monitor volume over time).
3. Add DTT to a final concentration of 10 mM DTT (add 1 µL of 1 M stock to 100 µL) and boil for 10 min. to reduce proteins.
4. Add IAA to a final concentration of 50 mM (add 5 µL of 1 M stock to 100 µL) and incubate at room temperature in the dark for 30 min to alkylate proteins.
5. Add 5 µL of 4× Laemmli sample buffer and load into two adjacent wells of a 10-well NuPAGE 10 % Bis-Tris gel. Run halfway down the gel (200 V for ~20 min).
6. Wash the gel 3 × 5 min in dH_2O in a dedicated glass box (cleaned with dH_2O and ethanol) and then stain by incubating for 1 h in SimplyBlue SafeStain solution. Wash 3 × 5 min in dH_2O and continue to destain overnight by incubating in dH_2O.
7. Scan the gel before cutting out the bands, by transferring onto the glass plate of a scanner (cleaned with dH_2O and ethanol). Do not handle the gel any more than necessary (and never without gloves), to minimize keratin contamination. The scan can be printed out to mark where the gel lanes will be cut in Subheading 3.6, **step 1**.

3.6 In-Gel Digestion and Peptide Recovery

From this point onward, avoid the use of autoclaved glassware and plasticware (which can be highly contaminated with proteins and detergents). For MS solutions, use dedicated glassware that is washed by hand using dH_2O. Perform all steps of the digestion in a laminar flow hood, and store all dedicated MS materials and solutions in this same hood. Wear gloves at all times.

1. To excise the bands from the gel, cut away all unnecessary parts (top, bottom, molecular weight marker lanes) with a sterile scalpel, leaving only the two sample lanes. Treat these two adjacent lanes as one lane and cut into five horizontal slices.
2. Once the slices are cut (and marked on a printout of the scanned gel), mince each slice further into 1×1-mm pieces using a scalpel and transfer to a 1.5-mL Eppendorf LoBind microcentrifuge tube (*see* **Note 12**).
3. Wash the band pieces with 300 µL of dH_2O for 15 min on a shaking platform. Add 300 µL of acetonitrile and wash for a further 15 min on a shaking platform.
4. Remove the supernatant using a P1000 tip with a P10 tip on the end. This ensures that gel pieces are not sucked into the pipette tip.
5. Wash the gel pieces with 300 µL of 20 mM ammonium bicarbonate for 15 min on a shaking platform. Discard the supernatant.
6. Wash the gel pieces with 300 µL of 20 mM ammonium bicarbonate/acetonitrile (50:50 v/v) for 15 min on a shaking platform. The gel pieces should shrink and look opaque. Discard the supernatant.
7. If the band pieces are still blue, repeat **steps 5** and **6**.
8. To dehydrate the gel slices, incubate with 100 µL of acetonitrile for 5 min on a shaking platform. The gel pieces should shrink and look completely white. Discard the supernatant.
9. Dry the gel pieces in a SpeedVac for 5 min.
10. For trypsin digestion, add 50–100 µL /band of a 6.3 ng/µL working stock of Trypsin Gold prepared fresh in 20 mM ammonium bicarbonate (e.g., to prepare 1 mL of trypsin digestion buffer, add 12.6 µL of Trypsin Gold stock to 987.4 µL of 20 mM ammonium bicarbonate) (*see* **Note 13**).
11. Allow bands to rehydrate in this trypsin digestion buffer for 30 min. The gel pieces should be restored to the original sizes, and there should be just enough solution to cover all the gel pieces. If required, add more 20 mM ammonium bicarbonate (minus the trypsin) to cover the band pieces. Keep a note of how much liquid is added to each slice, which will be required at **step 13**.

12. Incubate at 30 °C overnight (>16 h).
13. To start extraction of the peptides, add an equal volume of acetonitrile to the digest and incubate at 30 °C for 30 min on a shaking platform.
14. Transfer supernatant to a new Eppendorf LoBind microcentrifuge tube. This supernatant contains the peptides that you are going to analyze.
15. Add 100 µL (or sufficient volume to cover the gel pieces) of 1 % formic acid to the gel pieces. Prepare the 1 % formic acid solution fresh in the fume hood. Incubate for 20 min at room temperature on a shaking platform.
16. Transfer supernatant to the tube at **step 14**.
17. Repeat **steps 15** and **16**.
18. Add 200 µL of acetonitrile to the gel pieces and incubate for 10 min at room temperature on a shaking platform. The gel pieces should shrink and turn white.
19. Transfer supernatant to the tube at **step 14**.
20. SpeedVac to dry the peptides in the tube at **step 14** completely.
21. Resuspend the (invisible) pellet in 40 µL 1 % formic acid. To optimize solubilization of peptides, first add 8 µL of 5 % formic acid, vortex, and then add 32 µL of dH_2O. Peptides should now be stored in the freezer.
22. For injection on the MS, thaw the peptides, centrifuge at 13,000 × *g* for 10 min, and transfer 15–20 µL to a crimp vial with a lid. Store the remainder in the freezer.

3.7 Mass Spectrometry

Analyze an aliquot of the tryptic digest (10–15 µL) by LC-MS/MS on a Thermo Scientific LTQ Orbitrap XL Hybrid MS system with nanospray source coupled to a Dionex UltiMate 3000 RSLC nano-HPLC (*see* **Note 14**).

1. Load the peptide mixture onto a trap column (C18 CapTrap) for 5 min at 15 µL/min.
2. Separate the peptides by eluting over a 60 min discontinuous gradient of 3–45 % acetonitrile with 0.1 % formic acid at 0.3 mL/min onto a 10-cm-long column with integrated emitter tip packed with Zorbax SB-C18 5 mM.
3. Acquire MS survey scans in the Orbitrap module at 60,000 resolution and MS^2 scans in the ion trap module using data-dependent acquisition of the top five ions from each duty cycle.
4. Search the raw files against the UniProt human database using MaxQuant software v1.2.7.4 ([27]; http:/www.maxquant.org) and the following criteria: peptide tolerance = 10 ppm, trypsin as the enzyme (two missed cleavages allowed), and

carboxyamidomethylation of cysteine as a fixed modification. Variable modifications are oxidation of methionine and N-terminal acetylation. Heavy SILAC labels are Arg6 (R6), Arg10 (R10), Lys4 (K4), and Lys8 (K8). Minimum ratio count is 2 and quantitation based on razor and unique peptides. Peptide and protein FDR is 0.01. Further statistical, graphical, and bioinformatic analyses can be carried out following initial identification/quantitation (*see* **Note 15**).

3.8 Data Analysis

1. Graphical analysis of the data provides a great deal of information, as detailed in Fig. 6. Plotting the relative abundance of all proteins identified vs. the distribution of the log SILAC (H-L) ratios (Fig. 6a) confirms expected trends, such as the very low ratios of keratins and other environmental contaminants (found only in the non-isotopic form) and the large group of proteins that bind nonspecifically to the affinity matrix and cluster around a log value of approximately 0 (i.e., SILAC ratio 1:1). This cluster can deviate in either direction but should be close to 0. A large deviation can result from incomplete incorporation of the isotopic label, errors in the original calculation of total protein concentration in the cellular extracts, or unequal mixing of the beads after IP. Based on the median log SILAC ratio (X in Fig. 6a), an initial threshold value can be manually chosen (i.e., the SILAC ratio above which a protein is considered a putative real hit). We generally accept log ratios > 1 log above this median value as representing likely interactors. The Perseus module of MaxQuant can be used to calculate "significance B" *p*-values, which are outlier significance scores that are normalized for the more focused spread of highly abundant proteins. These are demonstrated in Fig. 6a.
2. While these initial thresholding steps increase confidence in putative binding partners, we can also mine data close to the threshold value by annotating known contaminants.

   Having noted that certain proteins reliably bind nonspecifically to the sepharose affinity matrix, we identified these proteins and created a sepharose "bead proteome" [20]. We also compared the nonspecific binding of proteins to sepharose, agarose, and magnetic beads in a series of quantitative proteomics experiments and revealed that these different matrices enrich specific classes of proteins (*see* **Note 16**). This initial list has since been extended to protein frequency libraries that help to distinguish bona fide interactors from common contaminants [29].
3. Triple-encoding SILAC IP experiments offer the option to compare two experimental conditions to a built-in negative control, such as the interactome of the same protein under two different conditions, the interactomes of a mutant vs. wild-type protein, or two isoforms of the same protein (Fig. 4).

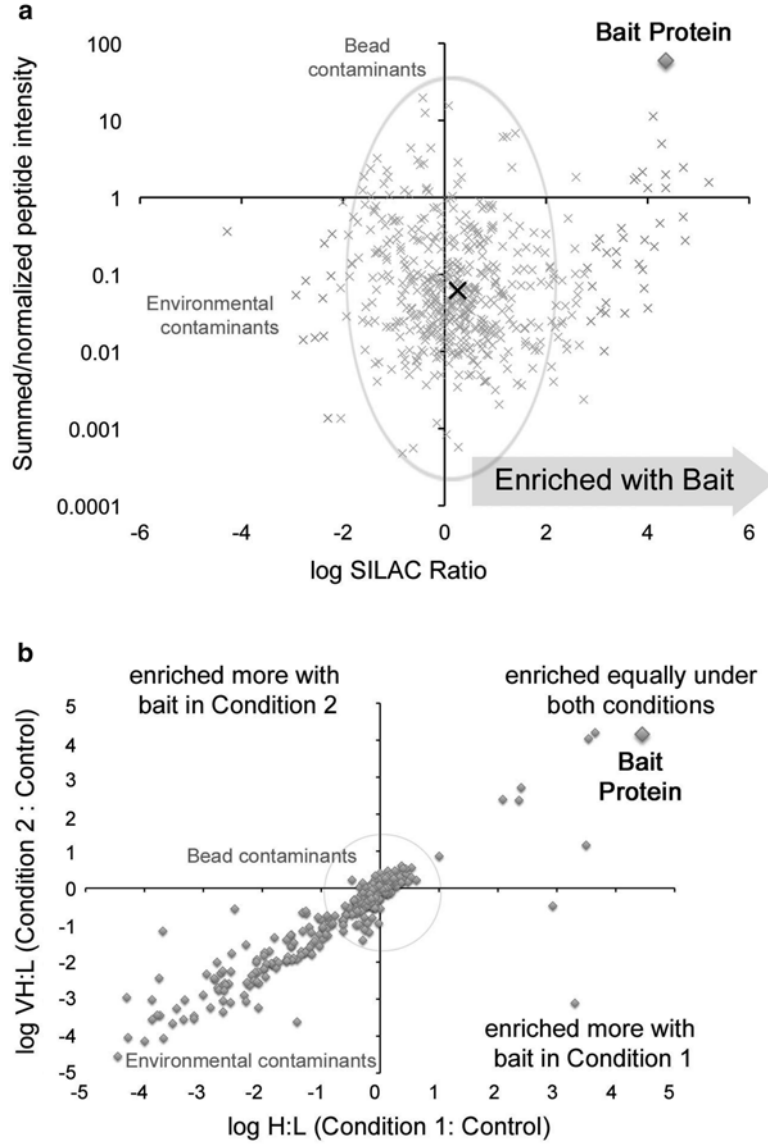

Fig. 6 Examples of SILAC-based AP-MS datasets. (**a**) For a simple pulldown experiment (bait IP vs. negative control), the enrichment of bona fide interactors above the background can be visualized by plotting the relative abundance (summed and normalized peptide intensity) of all identified proteins vs. their log H-L ratios. The majority of identified proteins bind nonspecifically to the affinity matrix, clustering around the median ratio (X) of ~1:1 (log ratio 0). Environmental contaminants such as keratins and trypsin have very low log H-L ratios, confirming that they were only detected in the light (non-isotopic) form. In the example shown here, the bait protein is the most abundant protein detected in the IP and highly enriched above background, as expected. Putative interactors are those proteins that are significantly enriched above the median. Significance can be determined manually by choosing a threshold value, or the Perseus plug-in for MaxQuant can be used to calculate significance *p* values (indicated here). These initial analyses, along with annotation of common contaminants, help to prioritize putative interactors for follow-up validation experiments. (**b**) For this experiment, triple SILAC encoding was utilized to compare enrichment of interactors with the bait protein under two different conditions: Condition 2 (log VH:L) vs. condition 1 (log H:L). The bait protein is *highlighted*, and the quadrants are marked to indicate putative interactors that were enriched more in a specific condition or equally under both conditions

The data can be analyzed in a variety of ways, such as plotting the log H-M ratios vs. relative abundance, to demonstrate the distribution of proteins between the two experimental conditions (with the caveat that any protein that is equally enriched with both will be buried in the contaminants, near the median log H-M ratio). Another option, which clarifies which proteins are bead contaminants while highlighting any selective enrichment, involves plotting log VH-L vs. log H-L (Fig. 6b). Enriched proteins fall into specific quadrants, based on whether they are enriched more in condition 1 or condition 2, or equally under both conditions.

4 Notes

1. The EGFP mammalian expression vector was originally developed by Clontech. Current versions are available from Invitrogen (California, USA) and other companies. All are based on the original GFP protein from the jellyfish *Aequorea victoria*. The EGFP vectors contain an SV40 origin for replication and a neomycin resistance gene for selection (using G418) in eukaryotic cells. A bacterial promoter upstream of the neomycin gene expresses kanamycin resistance in *E. coli*.
2. These amino acid concentrations are based on the formula for normal DMEM (Invitrogen). All cell lines should first be tested in light (non-isotopic) SILAC media. Some do not adapt well to dialyzed FBS, which lacks certain low molecular mass growth factors. In this case, exogenous growth factors can be added or serum dialyzed using a lower molecular mass cutoff. Other types of media such as RPMI can also be custom ordered for use in SILAC experiments by providing the companies with the standard formulation for the media and requesting that arginine and lysine be left out. Although SILAC kits are available, the most affordable option is to purchase the media, serum, and amino acids directly from suppliers, with additional savings if larger quantities are purchased (e.g., to share between several laboratories).
3. Although double-encoding SILAC is standard for comparing an affinity-purified protein complex to a built-in negative control, triple-encoding SILAC offers the option to compare two experimental conditions to a negative control (*see* Fig. 4). This can include a protein purified under two different conditions (e.g., from untreated cells vs. cells treated with UV light to induce DNA damage), comparison of a wild-type and mutant protein, comparison of two mutant proteins (e.g., a phosphomimic vs. phosphomutant version), or comparison of two different isoforms of the same protein. For triple encoding,

L-arginine and L-lysine are added to the "light" (R0K0), L-arginine ^{13}C and L-lysine 4,4,5,5-D4 to the "heavy" (R6K4), and L-arginine $^{13}C/^{15}N$ and L-lysine $^{13}C/^{15}N$ to the "very heavy" (R10K8) media. All isotopes are available from Cambridge Isotope Laboratories.

4. Use this buffer to passage cells grown in SILAC media, as trypsin-EDTA solutions may contain amino acids.
5. The GFP-Trap recognizes both GFP and YFP, but not CFP (or any derivatives of dsRed, such as mCherry and mRFP). The epitope is lost when GFP is mutated to CFP, but a single amino acid substitution in CFP (I147N) restores binding to GFP binder, demonstrating the high selectivity of this reagent [30].
6. Partially purified fractions can be directly stained using antibodies raised against endogenous proteins (directly conjugated to fluorophores or detected using fluorophore-conjugated secondary antibodies), to demonstrate selective enrichment of a particular nuclear body. No permeabilization is necessary, and stained fractions can be embedded in a hard-drying mounting medium such as DABCO containing Mowiol for imaging.
7. Complete incorporation of isotopic amino acids is achieved in HeLa and U2OS cells after 5–6 cell doublings, even for proteins with no significant turnover. Incorporation should be tested when new cell lines are used, by growing cells in heavy media and sampling at different time points for MS analysis [19].
8. The dissociation buffer is not as efficient as trypsin-EDTA, and thus, the time varies for different cell lines. If necessary, cells can be released by treatment with trypsin-EDTA but then pelleted and rinsed with DPBS prior to passaging back into the SILAC media.
9. Passaging cells for a SILAC experiment should be done over 1.5–2 weeks, with cells passaged at confluencies that they normally tolerate. For example, HeLa cells grow rapidly and will grow to confluency even if diluted to low concentrations during splitting (e.g., can passage 1 × 150-mm dish in SILAC media for a week and then do a 1:10 split to generate the 10 × 150-mm dishes to harvest for the experiment). U2OS cells, however, cannot be passaged as aggressively, and the final 10 × 150-mm dishes must be accumulated more gradually (e.g., passage 1 × 150-mm dish for a week, then split 1:5. When confluent, split these 1:2 to generate the final 10 × 150-mm dishes).
10. It is important to covalently conjugate antibodies to the affinity matrix for these experiments because all non-covalently bound proteins will be eluted from the matrix and subjected to MS analysis. If the very abundant antibodies are included in this mix, they will dominate the MS analysis and mask underlying proteins that are less abundant. To covalently conjugate

the antibodies, first bind to beads at desired concentration (we normally bind at 1 mg/mL agarose) and then wash well with PBS to remove unbound antibody. Wash beads twice with 10 volumes of 0.1 M sodium borate pH 9. Prepare 10 volumes of borate buffer containing 20 mM dimethyl pimelimidate (DMP; Sigma), pellet the beads, and resuspend in this solution. Shake or mix end-over-end for 30 min at room temperature. Pellet beads, remove buffer, and resuspend beads in 1 mL fresh DMP/borate solution. Shake or mix end-over-end for 30 min at room temperature. Pellet beads, remove buffer, and wash beads twice with a 10× volume of 50 mM glycine pH 2.5. Wash several times in PBS and store at 4 °C as a 50 % bead/PBS slurry. Important note: DMP should be stored at –20 °C prior to use and not reused once it has been opened. To prepare 1 mL of 20 mM DMP in borate buffer, weigh out 5.2 mg and add borate buffer immediately before adding to the beads.

11. Our original GFP SILAC IP method was based on mixing the extracts before the IP. Although we minimized the incubation time, we still saw evidence of exchange of light and heavy proteins in protein complexes, with several real hits found to be close to the threshold value. These same proteins had much higher ratios when the lysates were not mixed prior to IP. This potential for exchange during IP was confirmed and directly measured by other groups. It only occurs for the more dynamically associated members of the complex and has been exploited to distinguish stable interactors from dynamic interactors in SILAC IP experiments [31–33]. For an initial experiment, it is recommended that separate IPs be carried out and combined at the bead stage, as described in Subheading 3.4, **step 2** (Fig. 4).
12. Try to work as quickly as possible because the gel becomes stickier as it dries out. If it starts to dry out, add a drop of water on top.
13. Trypsin is not stable, especially when pure and in nonacidic condition, so it must be diluted down from the original 0.5 μg/μL stock solution just before use. The amount of trypsin buffer added per gel slice can vary depending on the average amount of protein per gel slice (e.g., 50 μL for lighter bands and up to100 μL for darker bands), although we recommend that the slices are cut to try to normalize for this (larger slices for less stained regions of the gel and smaller slices for more highly stained regions). To ensure that larger slices are fully covered by the digestion buffer, 20 mM ammonium bicarbonate (minus trypsin) can be added as described in Subheading 3.6, **step 11**.
14. The success of a SILAC IP experiment relies on the quality of the dataset. New mass spectrometers with high mass accuracy, such as the linear ion trap-orbitrap combination, allow for very

high performance in a compact and robust format. Many proteomic facilities now have these systems and will process external samples on them for a fee. Because a standard SILAC IP experiment only comprises five samples, and they generally only need to be run once (provided that follow-up analyses such as co-IP/WBs are used to validate hits), this is an affordable option. In addition, open-source programs such as MSQuant and MaxQuant are available for quantitation of data and interpretation of results.

15. Analysis of MaxQuant generated datasets (text files) can be carried out using spreadsheet software such as Microsoft Excel. At this step, common environmental contaminants as per http://maxquant.org and proteins identified via the decoy database can be manually removed. Further analysis can also be carried out using the Perseus module of MaxQuant.
16. Many laboratories employ magnetic beads for IP experiments, with the understanding that they show less nonspecific protein binding. We confirmed that this is the case for certain magnetic beads (e.g., Dynabeads; Invitrogen), although interestingly our comparison of contaminant proteins enriched with different affinity matrices revealed that specific classes of proteins, namely, cytoskeletal, structural, and motility proteins, are more enriched with Dynabeads. In comparison, nucleic acid-binding proteins are enriched nonspecifically on sepharose and agarose. Thus, there is no affinity matrix that is clearly superior for all IP experiments. Having also noted a higher capacity of many agarose matrices to their magnetic counterparts, we have chosen to use the agarose-bound form of the GFP affinity reagent (GFP-TRAP®_A) for all of our SILAC GFP IP experiments, utilizing the bead proteome as a specificity filter to highlight likely contaminants.

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