Unifying Fluorescence Microscopy and Mass Spectrometry for Studying Protein Complexes in Cells.

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Abbreviations: GFP - green fluorescence protein, EYFP- enhanced yellow fluorescent protein,
MS - mass spectrometry, mTFP- monomeric teal fluorescent protein,
DNA - Deoxyribonucleic acid, PCR - polymerase chain reaction,
MAT - mating type, YEPD - Yeast Extract Peptone Dextrose,
SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis,
I-DIRT – Isotopic Differentiation of Interactions as Random or Targeted
Summary

We have developed and applied a method unifying fluorescence microscopy and mass spectrometry for studying spatial and temporal properties of proteins and protein complexes in yeast cells. To combine the techniques, first we produced a variety of DNA constructs that can be used for genomic tagging of proteins with a modular fluorescent and affinity tags. The modular tag consists of one of the multiple versions of monomeric fluorescent proteins fused to a variety of small affinity epitopes. After this step we tested the produced constructs by tagging two yeast proteins, Pil1 and Lsp1, the core components of eisosomes, the large protein complexes involved in endocytosis in *S. cerevisiae* with a variety of fluorescence and affinity probes. Among the produced modular tags we found several combinations that were optimal for determining sub-cellular localization and for purifying the tagged proteins and protein complexes for the detailed analysis by mass spectrometry. And finally, we applied the designed method for finding the new protein components of eisosomes and for gaining new insights into molecular mechanisms regulating eisosome assembly and disassembly by reversible phosphorylation and de-phosphorylation. Our results indicate that this approach combining fluorescence microscopy/mass spectrometry into a single method provides a unique perspective into molecular mechanisms regulating composition and dynamic properties of the protein complexes in living cells.
Introduction

Fluorescent proteins have become invaluable probes for studying molecular processes in living cells with the light microscopy techniques [1-3]. Proteins, organelles and the entire cells can be selectively visualized using a variety of fluorescent proteins fused to the proteins of interest [1-6]. Combined with genetics and molecular biology techniques fluorescence microscopy provides an efficient tool for observing molecular phenotypes useful for dissecting the pathways of cell cycle progression and cell response to the internal and external signals [7]. However, understanding the mechanism controlling the properties of proteins in the cells could be a challenging task, frequently requiring a comprehensive characterization of the proteins at the molecular level.

The proteins tagged with a green fluorescent protein (GFP) can be also purified using GFP antibodies. Cheeseman and Desai [8] and Cristea and co-workers [9] have enriched the GFP tagged proteins and protein complexes for further detailed analysis by mass spectrometry (MS). The MS based methods for protein analysis are fast, sensitive and able to identify both proteins in complex protein mixtures and residues bearing post-translational modifications [10, 11]. Thus, the addition of affinity purification and mass spectrometry steps enabled the researchers to study protein interactions and the post-translational modifications in the context of the protein sub-cellular localization. Juxtaposition of the protein localization, composition of the protein complexes and post-translational modifications frequently yields a unique perspective of the cellular processes and the molecular mechanisms of their regulation [12, 13].
Using fluorescent proteins also as affinity probes can be problematic in several instances. First of all, the good quality antibodies against the rapidly increasing number of fluorescent proteins [3, 6] are not yet readily available. Furthermore, rising antibodies specifically recognizing fluorescent proteins originating from the same organism but fluorescing at different color can be difficult or even impossible, because such proteins frequently differ by mutations of only few amino acids [1-6]. Thus, we seek an alternative approach to the design of the tags suitable for sub-cellular localization and purification of the proteins and protein complexes, which is (1) independent on the availability of antibody to a specific form of a fluorescent protein, (2) suitable for multiplexing, i.e. simultaneous observation of sub-cellular localization of several proteins and affinity purification of the proteins and stably associated protein complexes, (3) flexible and easy to modify to incorporate better versions of fluorescent proteins and affinity tags after they are discovered.

One possible solution which satisfies the stated requirements is to use a modular tag containing a version of a fluorescent protein fused to an affinity epitope. In this case we can decouple requirements to the both modules and optimize the performance of each one independently for fluorescence microscopy and affinity purification experiments. To our knowledge, this possibility was first realized by Thorn and co-workers [14] who have fused 3HA (3 repeats of YPYDVPDYA epitope from hemagglutinin protein) and 13Myc (13 repeats of EQKLISEEDL epitope, corresponding to a stretch of the C-terminal amino acids of the human e-Myc protein)
tags to several variants of fluorescent proteins. The authors have argued that the fusion of the fluorescent proteins to the affinity epitopes may enable fluorescence and immunochemical analysis but did not test this idea. Cheeseman and Desai fused the S-peptide and hexahistidine epitopes to the GFP protein to enable additional tandem purification steps [8]. Su and co-workers also fused a hexa-histidine tag (6xH) to GFP to purify the recombinantly produced proteins [15]. Although hexahistidine tag performs well for isolation of the over-expressed recombinant proteins, it works poorly for affinity purification of low abundant, endogenously expressed proteins [16]. A double affinity tag containing a single Myc epitope and hexahistidine was also used to purify the recombinantly produced fluorescent proteins [6].

Here we describe the design and implementation of the modular fluorescent and affinity tags. These tags contain a variety of fluorescent proteins, which can be used exclusively for obtaining sub-cellular visualization, and several small epitope tags utilized to perform two-step affinity purification. To test the performance of the produced constructs, we tagged two yeast proteins, Pil1 and Lsp1, the core components of eisosomes, with a variety of modular tags.

Eisosomes are large heterodimeric protein complexes recently discovered in *S. cerevisiae* [17]. There are ~50-100 eisosomes in each grownup yeast cell, distributed uniformly in the characteristic, dotted, pattern at the cell surface periphery. Each eisosome contains ~2,000-5,000 copies of Pil1 and Lsp1. It was shown that eisosomes serve as portals of endocytosis in yeast. The function of eisosome is regulated by reversible phosphorylation [18, 19].
Among the tested constructs, we found several combinations of fluorescent protein and affinity tags which were optimal for determining sub-cellular localization and purification of the proteins and protein complexes. We applied these tags to further investigate eisosomes, and found several new protein components of the complexes and obtained the new insights into molecular mechanisms regulating eisosome integrity by reversible phosphorylation and de-phosphorylation. Our results indicate that an approach combining fluorescence microscopy/mass spectrometry into a single method provides a unique perspective into molecular mechanisms regulating composition and dynamic properties of the protein complexes in living cells.

**Experimental Procedures**

*Plasmid Vectors*—We have developed multiple plasmid vectors (see Supplemental Fig.1 and Table 1), which contain the DNA sequences of a variety of fluorescent proteins, i.e. GFP(S65T), mTFP1, mCitrine, mOrange, mCherry and YEFP [2-6], fused to several versions of small double-affinity tags, such as 3xFlag-6xH [20], 3xMyc-8xH [6], NxStrepTagII-8xH [16] (where N=1,3,4 is number of epitope repeats), terminators of transcription, bacterial and yeast selection markers, and some, LoxP recombination sites [21]. The vectors were produced by a standard cloning into a pEYFP-C1 vector (Clontech). The sequences of the vectors are shown in the Supplemental Data. The designed vectors can be used as templates for PCR-based genomic tagging [22, 23].

*Genomic Tagging, In vivo Disruption and In vivo Mutagenesis*—The PCR primer pair, 5’-gene-specific primer (51 bases) + GAT CCG CTA GCG CTA CCG GTC-3’ (sense) and 5’-gene-
specific primer (51 bases) TAA TAC GAC TCA CTA TAG GGA GAC -3’ (anti-sense), was selected to amplify the desired DNA construct coding for a specific modular tag. The amplified DNA was directly used for C-terminal tagging of the protein of interest by a homologous recombination technique [22, 23]. In vivo disruption and mutagenesis of the genomic DNA was performed essentially as described in [24].

A typical molecular weight of a modular fluorescent and affinity tag is ~30 kDa, which includes the molecular weight of a fluorescent protein, two affinity epitopes and spacers between the tags and the protein (see DNA sequences in the Supplemental Data).

Yeast Strains - All yeast strains were derived from MATa BY4741 bar1Δ::KanMX strain or MATα BY4742 strain. Transformed clones were selected based on the presence of a selectable marker and a fluorescence signal.

Cell Culturing - Cells were cultured in 0.5-1 liter YEPD media (MP Biomedicals) at 30 °C to the density (2-4) x 10⁷ cells/ml. For the cell-cycle arrest and release experiments, we cultured several liters of yeast cells. Each liter of a culture was incubated in YEPD media containing 50 μg/l α-factor (GenScript Corporation) for 3 hours, spun down and washed 3 times with double deionized water, then cultured in 1 liter of a regular YEPD media containing 50mg/l pronase enzyme (Sigma-Aldrich) added to the media to digest the remaining α-factor [25].
Combined Fluorescence Microscopy and Mass Spectrometry Experiment – The schematic diagram of the combined microscopy/mass spectrometry experiment is shown in Figure 1. The cultured cells were quickly collected by centrifugation of the media in two 500-ml-buckets of a Hermle Z383 centrifuge (Denville Scientific) at ~ 4000 x g for 5-10 minutes. A small portion of cells was immediately deposited on a glass slide for the fluorescence microscopy experiment performed on a Zeiss LSM510 confocal microscope. Images were later processed using Zeiss LSM Image Browser software.

The rest of the sampled cells were frozen by dripping cells in a 50 ml falcon tube filled with liquid nitrogen. The pellets of cells were stored at -80 °C until the proteins and protein complexes were affinity purified according to the procedures described below.

Tandem Affinity Purification of the Protein Complexes. Tandem affinity purification of proteins was performed essentially as described previously [20]. The detailed protocol can be also found in the Supplemental Data. Briefly, the fist purification step was performed by adding ~5-10 mg magnetic beads immobilized with antibody against a particular epitope to 5–10 ml of a crude cell extract. We used M-270 epoxy magnetic dynabeads immobilized with anti-Flag monoclonal antibody M2 (F3165, Sigma-Aldrich) when purifying proteins tagged with a fluorescent protein and the 3xFlag-6xH affinity tag (DYKDDDDKDYKDDDDKHHHHHH), and a mouse monoclonal anti-(c-Myc) antibody (clone 9E10, Roche) when purifying proteins tagged with a fluorescent protein and
3x(c-Myc)-8xH epitope (3 repeats of EQKLISEEDLG fused to octahistidine). Immobilization of the dynabeads with the antibody was carried on essentially as described in the manufacture's protocol, binding \(~10~\mu g~of~antibody~per~5~mg~of~beads\). After 60-90 min incubation, the beads were collected with a magnet and washed 3 times with 1 ml of IP buffer, 20 mM Hepes, 2 mM MgCl2, 250 mM NaCl, 0.05% Tween-20, and protease inhibitor cocktail (Sigma-Aldrich). The enriched proteins were eluted with \(~300~\mu l~of~IP~buffer~containing~a~3xFLAG~peptide~(Sigma-Aldrich),~at~a~concentration~of~\sim~400~\mu g/ml,~or~with~the~c-Myc~peptide~(Sigma-Aldrich)~at~a~concentration~of~\sim~800~\mu g/ml,~for~30~min~at~4°C~with~constant~rotation.~The~eluate~was~collected~and~diluted~in~1~ml~of~IP~buffer.~

The proteins tagged with a variety of fluorescent proteins and several repeats of StrepTagII epitope (WSHPQFEK) and octahistidine (8xH) were first purified using Strep-Tactin magnetic beads essentially as recommended by a manufacturer (Qiagen), then eluted with biotin (Sigma-Aldrich).

The second purification step was performed using polyhistidine tag and 20 \(\mu l\) of Cobalt TALON Dynabeads (Invitrogen). The enriched proteins were efficiently eluted with an SDS running buffer containing 250 mM imidazole and separated by SDS-PAGE. Alternatively, the purified protein complexes were left on the beads for on-bead digestion.
Gel Electrophoresis - The eluted proteins were separated by SDS-PAGE using 4-20% gradient gels (Pantera S, B-Bridge) or 4-20% or microGels (Life-Gels, Life Therapeutics, Australia). The gels were stained with a colloidal coomassie stain (GelCode, Pierce). The bands were excised, digested with 5-7 \( \mu l \) of a 1 pmol/\( \mu l \) of trypsin solution in ammonium bicarbonate. The tryptic peptides were extracted according to a standard in gel digestion/peptide extract procedure [10]. The details of the protocol could be also found in the Protocol II in the Supplemental Data.

Digestion of the protein complexes. - After the final wash of Talon beads with two times 1 ml of IP buffer and two times 1 ml of 50 mM ammonium bicarbonate buffer, the proteins were digested directly on the beads with 10 \( \mu l \) of trypsin solution (1 pmol/\( \mu l \)) in 10–50 mM ammonium bicarbonate buffer for 6-12 hours at 37 C.

Sample preparation. 1–3 \( \mu l \) of a mixture of either synthetic peptides or tryptic peptides were deposited on the interchangeable MALDI target and allowed to dry. 2 \( \mu l \) of a saturated solution of 4HCCA matrix was then added to the spot and again allowed to dry. The sample spots were then washed two times with 10% MeOH in 0.1% TFA by applying a 5–7 \( \mu l \) droplet on the top of the sample for 15–30 seconds and then quickly aspirating it.

Identification of Pproteins by Mass Spectrometry. We used two MALDI mass spectrometers, the orthogonal time-of-flight instrument prOTOF 2000 (PerkinElmer) and the MALDI- ion trap mass spectrometer vMALDI-IT (Thermo Finnigan) to identify proteins. The two instruments can
be used separately or sequentially to interrogate the same samples deposited on the MALDI target plate, which can be loaded into either of the mass spectrometers [20].

Typical analysis started with acquisition of single-stage MALDI-MS spectra of the samples using the prOTOF mass spectrometer. This time-of-flight instrument provides accurate measurements of the m/z values of the monoisotopic peaks, with a typical accuracy 5–15 ppm in the m/z range 500 - 4000. The spectra then were analyzed with the “m/z” program (version 2002.10.01 by Ronald Beavis, Beavis Informatics Ltd., Canada), which can automatically find and label the first isotopes of ion peaks in the spectra. We set the major setting of the program, such as, peak centroid to 6, signal-to-noise ratio to 1.5 and resolution to 10,000. This procedure usually results in detecting 50-300 ion peaks in the single spectrum measured in the range 500-4000 m/z with intensities above signal-to-noise ratio 1.5 [20]. The detected values were saved as text files.

The monoisotopic values from the lists can be directly used to identify proteins in simple protein mixtures. We used the XProteo search engine [20] to identify proteins based on tryptic mapping. Searching for the *S. cerevisiae* proteins in NCBI non-redundant data base, version 06/10/16 was performed with the following typical settings - protein mass: 0-300 kD, protein pI: 1-14, mixture search: auto, enzyme: trypsin, maximum missed cleavage sites: auto, mass type: mono, charge state MH+, mass tolerance 0.025 Da, and allowed default modifications: meteoneine oxidation and phosphorylation. The XProteo search engine uses a signal detection theory to evaluate the search results and, for each protein candidate, outputs a quality index d’ (d prime) and ROC
(Receiver Operating Characteristics) curve. A “threshold of identification” is set at the d’-value equals to 4. In this case, a protein candidate is identified with the probability ~0.99 with false alarm rate 0.05. For complete information about the XProteo search scores refer to the “FAQ” section at the www.xproteo.com.

We confirmed all species detected in the single-stage MALDI by MS/MS analysis. A computer program “AutoMSMS”, written in house using AutoIt Basic-like scripting language (www.autoitscript.com) was used to create MS/MS data acquisition methods for the vMALDI-ion trap. The program automatically opens the text files containing lists of the detected m/z values and pastes them into the method files, together with the defined isolation width (3Da for ions with m/z < 2000 and, 4 Da for ions with 2000 < m/z < 4000), energy of activation (35%) and ion’s activation time (30ms). These method files can be later executed by native Xcalibur software controlling the ion trap. The v-MALDI-IT is capable of fast acquisition of high quality MS/MS spectra (~3 sec per spectrum) of peptides presented in the sample at a femtomole level [20].

The MSMS spectra were converted to DTA format using the in house written “DTA converter” program utilizing an original subroutine written by Yates [32]. The DTA data files containing accurate m/z values of parent peptides and their MS/MS spectra were supplied to the XProteo search engine set to operate in MS/MS mode. Searching was performed in the in NCBI non-redundant data base (version 06/10/16) of S. cerevisiae proteins, with the following typical settings, protein mass: 0-300 kD, protein pI: 1-14, mixture search: auto, enzyme: trypsin, maximum missed cleavage sites: auto, mass type: mono, charge state: MH+, precursor tolerance:
0.025 Da, fragment tolerance 0.35 Da, instrument: MALDI_1_TRAP. No possible modifications were considered in this mode. Xproteo search engine again produced a quality index d’ and ROC (Receiver Operating Characteristics) curve for each protein candidate based on the combined scores evaluated from accuracy of measurements of m/z values of parent ions and the accuracy and intensity of fragments detected in the MS/MS spectra using a proprietary, Bayesian-based, algorithm.

The MS/MS data were also examined for the presence of the neutral loss of 98 Da in the fragmentation spectra, which is a characteristic signature of phosphorylation. Detection of neutral loss of 98 Da was facilitated by using the “Ion Map” function of the Qual Browser of Xcalibur program controlling the trap [20]. The interpretation and assignment of fragments in the fragmentation spectra of phosphopeptide was performed manually PROWL computer tools available from Brian Chait’s laboratory at the Rockefeller University.

Isotopic Differentiation of Interactions as Random or Targeted (I-DIRT) technique – was implemented exactly as described in [30] to distinguish between the specific and non-specific interactors in the affinity purified complexes (see also Supplemental Data).

Results

Development of a Modular Fluorescent and Affinity Tags – The produced DNA vectors were used as templates for PCR-based genomic tagging of proteins with a variety of modular fluorescent and affinity tags (see Supplementary Fig.1 and Table1). We tagged two yeast
proteins, Pil1 and Lsp1, i.e. the core components of eisosomes [17, 18] by incorporating different DNA cassettes at the C-termini of the protein genomic sequences. Then we experimented with cells expressing the tagged proteins as a model system to optimize the performance of the modular fluorescent and affinity tags. The schematic diagram of the combined fluorescence microscopy/mass spectrometry experiment is shown in Figure 1.

**Optimization of the Performance of Fluorescent and Affinity Tags** - The characteristic distribution of the eisosome particles at the cell periphery [17] was obtained in all cases of tested modular fluorescent and affinity tags (Supplemental Fig. 2). However, not all of the fluorescent probes performed well. We found that GFP(S65T), mCitrine, mTFP1 provide the brightest fluorescent signals. The mCherry protein produced a good signal when we used 568 nm wave length of an excitation laser. The fluorescence signals of EYFP and mOrange proteins were weak and many cells expressing the proteins did not produce fluorescence. An erratic performance of these proteins may be due to the several reasons, including long folding time compared to a typical cell cycle period in yeast (~90 min) and high sensitivity to the pH in micro-environment [3-5]. Interference of the affinity and the fluorescent probes could be also additional factor, which we discuss bellow.

We also investigated the performance of the affinity probes for purification of proteins and protein complexes. To carefully compare purification yields that can be achieved using different epitopes, we incorporated several affinity tags, i.e. 3xFlag, 4x-StrepTag II (4 repeats of
WSHPQFEK peptide) and octahistidine 8xH (HHHHHHHHH) in tandem at the C-terminus of Lsp1-GFP(S65T) fusion protein. The protein was purified from equal amount of yeast cells, but applying different tandem purification schemes. The results show that tandem purification using 4xStrepTagII-8xHis yields more protein after the two-step purification procedure (Supplementary Fig. 3A). However, the two-step tandem purification using a StrepTagII-8xHis tag usually resulted in noisier “pull outs” affected by endogenously biotinylated yeast proteins. Although the addition of increasing amounts of avidin to the cell lysates helped to compete out some of the biotinylated proteins from the Strep-Tactin resin [26], there was always a substantial amount of impurity proteins in the background. In general, the amount of impurity proteins was less when we used 3xFlag-6xHis and 3xMyc-8xHis tags resulting in higher signal/noise ratios of the enrichment procedure.

We also compared the purification yield of Lsp1 fused to the different modular tags, -mCherry-3xFlag-6xH, -mCherry-3xMyc-8xH, -mCherry-3xStrepTagII-8xH and mCherry-4xStrepTag II-8xH. The highest purification yield was again achieved using a tandem purification scheme 4xStrepTagII-8xH albeit with higher impurity content. The 3xMyc-8xH tandem purification produced less protein under the conditions identical to 3xFlag-6xH tandem purification, but still provided enough protein for staining and MS analysis. The efficiency of 3xStrepTagII dropped drastically compared with 4xStrepTagII (Supplementary Fig. 3B).

Compatibility of fluorescent and affinity probes is another important factor for designing the
modular tagging system. For example, 4xStrepTag II affinity tag strongly compromised and, in some cases, entirely quenched fluorescence when it was fused with a short or a long linker to the N- or C-terminus of GFP(S65T) or mCherry tag. The effect was reproducible in cases when other then Pil1 and Lsp1 proteins were tagged with the same tags. Interestingly, decreasing the number of StrepTag II repeats from 4 to 3 or 1 reduced this problem, but also drastically reduced the protein purification yield (Supplementary Fig. 3B). Perhaps, binding of one of the StrepTag II repeats to a fluorescent protein may unfavorably affect its structure and result in suppressing fluorescence [27].

Presently, we have selected a pair of modular tags, GFP-3xFlag-6xH and mCherry-3xMyc-8xH, among a variety of tested constructs (Table 1). The two modular tags enabled us to simultaneously track two proteins in a cell and to co-purify the proteins and associated complexes without noticeable cross-interference of the probes (Fig. 2). Using these tags we have performed the combined fluorescence microscopy mass spectrometry experiments to investigate the compositions and the dynamic properties of eisosomes.

Localization-driven exploration of the composition of protein complexes – Figure 3 shows the schematic diagram of several strategies that can be readily implemented using the combined fluorescence microscopy/mass spectrometry experiments. In one strategy (Fig. 3A), we use the detected pattern of protein sub-cellular localization as a clue of whether the identified proteins could be associated in the protein complexes. We start by tagging the protein of interest and first
explore the protein sub-cellular localization. In parallel, we purify the tagged protein and identify the co-purified proteins using mass spectrometry. The identified proteins are consequently tagged with a fluorescent and affinity tags and we, again, start with establishing sub-cellular localization of the identified proteins. Thus, in this walking strategy, the localization step is followed up by purification, separation and identification of the new interacting proteins. The new identified proteins either exhibit or do not exhibit the similar patterns of localization indicating the possibility that they could form either stable or transient complexes or even interact non-specifically. This information is treated as a valuable clue for further experiments.

Analysis of the gel bands corresponding to affinity purified proteins of the eisosome complexes (see Fig.2B, Fig.4 and Supplementary Fig. 3) consistently indicates the presence of three major proteins, Pil1, Lsp1 and Mrp8. The first two proteins, Pil1 and Lsp1 are the core eisosome proteins [17]. The third protein, frequently detected in the purified complexes is Mrp8, a putative mitochondrial ribosomal protein (see, for example, in Saccharomyces Genome Database http://www.yeastgenome.org). This protein has been also identified as a possible interacting partner of Pil1 and Lsp1 proteins in high-throughput immunopurification [28] and two-hybrid experiments [29]. The analysis of the localization of Mrp8 (see Fig. 4A) indicates that it does not co-localize with the eisosomes, but rather resides in the cytoplasm of the cells. Nevertheless, a small portion of Pil1 and Lsp1 proteins can be co-purified with the Mrp8-GFP-3xFlag-6xH (Fig 3A).
To investigate whether Mrp8 protein interacts with Pil1 and Lsp1 protein in the specific or non-specific manner we implemented the I-DIRT technique [30]. We affinity purified the Pil1 protein from a ~1:1 mixture of ~2 g of yeast cells expressing the tagged protein and grown in the presence of normal, undeuterated, lysine in the yeast media, and the wild type of yeast cells (MATa BY4741) grown in the presence of deuterated DL-lysine-4,4,5,5-d4 in the yeast media (see Supplemental Fig 5.) The two identified proteins, Pil1 and Lsp1, did not produce the deuterated versions of the tryptic peptides containing lysine residues, indicating that the complexes of the two proteins were formed in the unlabeled cells. On the other hand, the other two identified proteins, Rsp17a and Mrp8, were represented by the doublets of peaks. These doublets contain the “light”, undeuterated, and “heavy”, deutarated, versions lysine residues, which indicates that these two proteins are fast exchanging components of the Pil1 and Lsp1 complexes, and thus, most probably, are the no-specific interactors.

On-bead digestion of the complexes co-purified with Pil1-GFP-3xFlag-6xH followed by mass spectrometry [20] resulted in identification of several additional proteins, Tef1, Pma1 and Ygr130c (Supplemental Fig.6). Tef2 and Pma1 are the impurities and are frequently found in control samples. However Ygr130c was not identified in the control. The pictures of the protein sub-cellular localization from the www.yeastgfp.ucsf.edu data base [31] suggested a dotted pattern of localization at the cell periphery. Intrigued by this, we implement the strategy for localization-driven exploration of the composition of protein complexes (Fig. 3A) and tagged
Ygr130c protein with a modular -GFP-3xFlag-6xH tag and investigated the protein sub-cellular localization and the protein interacting partners.

Figure 4B confirms eisosome-like, distribution of the protein tagged with GFP-3xFlag-6xH at the cell periphery. On-bead digestion of the co-purified proteins followed by mass spectrometric analysis of the tryptic peptide mixture produced several top candidates: Ygr130c, Ymr031c, Pil1, Lsp1 and Ymr086w (Supplemental Fig. 7). Figure 4B also shows that these proteins were also identified by the standard SDS-PAGE separation/in gel digestion/ MS procedure (Supplemental Protocol II).

Guided by detecting a characteristic pattern of localization, we, again tagged Ymr086w and Ymr031c with one of our modular tags and first, determined sub-cellular localization and then, interacting partners of those proteins. Figure 4 C and D show that both proteins localize to the cell periphery and that Pil1 and Lsp1 are reciprocally co-purified with the query proteins. Despite some other identified proteins may also exhibit the eisosome-like, pattern of sub-cellular localization as for example, one of the identified proteins Ycp4 (see the in the data base of localization of yeast proteins at www.yeastgfp.ucsf [31]), at this particular point of our research we decided to focus our efforts on the most prominent candidates Ygr130c, Ymr031c and Ymr086w, and investigate whether these proteins are indeed the new integral components of eisosomes.
Figure 5 A shows that Ygr130c, Ymr031c and Ymr086w tagged with GFP-3xFlag-6xHis co-localize with Lsp1 protein tagged with mCerry-3xMyc-8xHis tag. To further investigate whether the proteins interact physically with eisosomes, we introduced modifications to the DNA sequence of Pil1 protein. During implementation of the “delitto perfetto” in vivo mutagenesis technique [24], we accidentally found that removal of 114 amino acids from the C-terminus of Pil1 protein causes Lsp1 to re-localize into the cytoplasm and into several bright spots at the cell periphery (Fig. 5B). This effect is similar to the one observed after complete deletion of Pil1 protein from the cells [17]. It confirms the importance of Pil1 protein, especially the C-terminus, for proper localization of eisosome particles. We decided to use this phenomenon to deliberately change Lsp1 localization and to examine whether this, in turn, will change localization of the other proteins.

Ygr130c, Ymr031c and Ymr086w changed their localization together with Lsp1 in the cells in which the Pil1 protein was truncated at the C-terminus (Fig. 5B). This result suggests that the new discovered proteins physically interact at least with Lsp1, and thus, are the integral components of eisosomes. The biological function of Ygr130c, Ymr031c and Ymr086w and a possible involvement into endocytosis remains to be investigated.

*Study of the dynamic properties of protein complexes. Application to the studies of protein dephosphorylation dynamics* – Figure 3B the schematic diagram of the experiments for studying the dynamic properties of the protein complexes. In this approach, we sample cells at the different
stages of the cell cycle or cell response to a particular signal to obtain the dynamic profiles of changes in protein sub-cellular localization, composition of protein complexes and abundances of post-translational modifications. Analysis of such profiles can frequently yield unique clues about the cellular processes and the molecular mechanisms of their regulation. Here we demonstrate the usefulness of the combined fluorescent microscopy/mass spectrometry experiments for the studies of the dynamics of eisosome de-phosphorylation. We also show that from the measured changes in sub-cellular localization and abundances of phosphorylation sites we can deduce the existence of a phosphotase system involved in maintaining eisosome integrity during the cell cycle.

Eisosomes can exhibit the dynamic behavior. Eiossome assembly and organization is controlled through reversible phosphorylation of its core proteins [18]. It was shown that at least several kinases, Pkh1 and Pkh2 are involved in eisosome phosphorylation. However, there is no information indicating the existence of phosphatase system involved in eisosome’s de-phosphorylation.

We measured the dynamics of eisosome de-phosphorylation. First of all, we noticed that eisosomes of the MATa (BY4741) cells disassemble in response to the treatment with the alpha-mating pheromone (α-factor). At the same time, treatment of the control of MATα (BY4742) cells with α-factor did not affect the distribution of Eisosome particles (Supplementary Fig. 13A and 13B). Analysis of the mass spectra of Pil1 and Lsp1 purified from α-factor treated or non-treated haploid MATa BY4741 revealed a number of striking differences (Supplementary Fig. 13C and 13D). The abundance of a Pil1 phosphorylated peptide detected in the mass spectra at
m/z 2987.3 396 increased by more than a factor of 5 in the yeast cells incubated with alpha-factor. Phosphorylation of a homologous peptide from Lsp1 detected at m/z 2985.400 increased by more than a factor of 10 in the cells incubated with the alpha-factor. Fragmentation spectra of these peptides confirmed their identity and the position of the phosphorylated residues (Supplementary Figs. 14,15). Drastic increase of the phosphorylation level after the treatment of cells with alpha-factor was noticed only at the reported phosphorylation sites. However, it was recently shown that eisosomes can be phosphorylated at the larger number of sites [18].

What is the dynamics of de-phosphorylation of the detected sites? We have sampled yeast cells at 0, 20, 40, 60, and 80 minutes after release from the alpha-factor for fluorescence microscopy and mass spectrometry experiments. Surprisingly, a substantial portion of Pil1 and Lsp1 proteins remain in the cytoplasm of the cells for almost one hour after removing the pheromone. After one hour, the fluorescence of the cytoplasmic fraction of the protein quickly, within 10-15 minutes, dropped to the level observed in the cells with intact eisosomes (Fig. 6A). This process well correlates with de-phosphorylation of Pil1 and Lsp1. Analysis of the mass spectra of the affinity purified proteins indicates that the abundance of the peptides phosphorylated at S230 and T233, also drops abruptly at ~60-80 min after release from the α-factor block (Fig. 6C and 6D). Noticeably, the relative intensities of gel bands corresponding to the phosphorylated forms of the proteins also decrease at the same time.

The observed dynamics revealed a rather abrupt change in phosphorylation level of both proteins at around 60 min of cell cycle progression. We speculate that some unknown phosphatase is activated at that stage of the cell cycle, which dephosphorylates Pil1 and Lsp1. Intriguingly, this
process coincides with the biogenesis of new eisosomes particles [34], the process in which new eisosomes are created in the new budding cells. It is tempting to hypothesize that both processes, biogenesis and protein accumulation in the cytoplasm of the cells, are related and controlled by reversible protein phosphorylation and dephosphorylation. Identification of a phosphatase system and the mechanisms of its activation could be the goal for the future projects.

Using an in vivo mutagenesis technique [24], we produced several yeast cell strains containing a combination of the different mutant versions of Pil1 and Lsp1 proteins tagged with fluorescent and affinity probes (see the complete, 3-by-3, mutation matrix in Supplementary Fig. 16). Figure 6E shows images of Pil1 and Lsp1 co-localization the cells from the diagonal of the mutation matrix. Noticeably, simultaneous mutations of S230 and T233 in both proteins to either aspartic acid or alanine residues produced enhanced phenotypes. Mutations S230D and T233D caused partial disassembly of eisosome particles, or, perhaps, inability to assemble eisosomes particles during eisosome biogenesis causing accumulation of the mutant proteins in the cytoplasm of the cells. Mutations S230A and T233A caused formation of fewer and brighter eisosomes as was described previously by Walther et. al [18]. They showed that mutations of residues S45, S59, S230, and T233 to either aspartic acid or alanine residues in Pil1-GFP along have a profound effect on the integrity of eisosomes. Our experiments revealed the synergetic effect of simultaneous mutations Pil1-GFP-3xFlag-6xH and Lsp1-mCherry-3xMyc-8xH proteins, which had a stronger effect on eisosomes then the effects caused by mutation of the sites in a single protein (See Fig. 6E and Supplementary Fig.16).
Discussion

We have combined fluorescence microscopy and mass spectrometry techniques for studying the composition and dynamic properties of protein complexes in the cells. To combine both techniques we designed and tested a variety of modular tags containing a fluorescent protein for visualization and two small epitope tags for two-step affinity purification. The modular construction of the tag allowed us to decouple requirements to the fluorescence and affinity probes, and optimize the performance of each module independently for fluorescence microscopy and affinity purification experiments (see Table 1). However, several modular tags exhibited strong coupling causing compromised performance of either fluorescent protein or an affinity tag. It is one of our future goals to explore the cause and possible applications of this phenomenon.

Our results confirm the usefulness of the produced reagents and the combined fluorescence microscopy/mass spectrometry approach for studying composition and dynamic properties of eisosomes [17, 18, 34]. The designed method enabled us to find the new protein components of eisosomes and gain the new insights into the molecular mechanisms regulating eisosome assembly and disassembly by reversible phosphorylation and de-phosphorylation. In our future work, we will investigate the applicability of the method for studying proteins from different organisms, localized to different compartments and present at low amounts in the cells. Our recent results with yeast anaphase promoting complexes (APC) [20] (~5000-1000 copies per cell [31]) and the securin-separase complexes [33] (~ 100-10 copies per cell, unpublished data) indicate a significant potential of the developed modular tags for studying the proteins exhibiting a dynamic pattern of localization and which are present at exceedingly low abundance in the cell.
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Figure captions

Figure 1. The schematic diagram of the combined fluorescence microscopy and mass spectrometry experiment. After the cells are grown and quickly collected by centrifugation, a small portion of cells (~10-100 μl) is sampled for fluorescence microscopy experiment. At this point, the experiment is performed by at least two investigators. One investigator obtains the images of sampled cells to establish sub-cellular localization of the studied protein, while the other one quickly collects the rest of the cells (~1-4 g of cell pellet) and freezes them in liquid nitrogen. Frozen cells later processes essentially as described in the experimental section to determine the composition of the purified protein complexes and possible posttranslational modifications on the purified proteins.

Figure 2. (A) Sub-cellular localization/co-localization and (B) purification/co-purification of the yeast proteins Pil1-GFP-3xFlag-6xH and Lsp1-mCherry-3x(cMyc)-8xH genomically tagged with different modular tags. The order of the tags used for affinity purification is indicated. The control and the principal experiments are indicated with – and +. The identities of proteins corresponding to the major bands are shown. The details on the identified proteins can be found in the Supplemental Material.

Figure 3. Schematic diagram of the strategy for (A) localization-driven exploration of the composition of the protein complexes and , (B) investigation of the dynamic profiles of changes in protein sub-cellular localization, composition of protein complexes and abundances of post-translational modifications.
**Figure 4.** Sub-cellular localization of the tagged proteins and the composition of the protein complexes associated with them in yeast cells. The co-purified proteins were separated in the SDS-PAGE gel and stained with colloidal coomassie. Proteins were identified by a standard in gel digestion/ mass spectrometry procedure. **(A)** Sub-cellular localizations of Mrp8-GFP-3xFlag-6H and Lsp1-mCherry-3xMyc-8xH and the major components of the protein complexes purified with the tagged Mrp8. **(B)** Sub-cellular localization Ygr130c-GFP-3xFlag-6H and the major components of the complexes co-purified with the tagged protein. **(C)** Sub-cellular localization of Ymr031c-GFP-3xFlag-6H and the composition of the major components of complexes co-purified with the tagged protein. **(D)** Sub-cellular localization of Ymr086-GFP-3xFlag-6H and the major components of the complexes purified with the tagged protein. The full list of the identified proteins, unique peptides found and protein coverage can be found in the Supplemental Material.

**Figure 5.** **(A)** Co-localization of Pil1, Ygr130, Ymro31c and Ymr086w proteins tagged with a -GFP-3xFlag-6xH modular tag and Lsp1-mCherry-3xMyc-8xH protein. All proteins co-localize in eisosomes [17]. **(B)** Proteins Ygr130, Ymr031 and Ymr086 physically interact with Lsp1 protein, whose sub-cellular localization was changed by removing the last 114 amino acids at C-terminus of Pil1 protein. Truncation of Pil1 causes re-localization of Lsp1 and other physically interacting protein partners into several bright spots at cell periphery.

**Figure 6.** **(A)** Subcellular localization of Pil1-GFP-3xFlag-6xH after release from alpha-factor (αF) **(B)** SDS-PAGE of Pil1 and Lsp1 proteins purified at the corresponding time points after release from alpha-factor. Note doubling of bands as the result of post-translational
modifications of both proteins. (C) Dynamics of the decrease of Pil1 phosphorylation. Note the decrease in abundance of phosphopeptide ALLELLDDp(SPVT)PGEAR m/z 2987.395 relative to unphosphorylated form of the peptide at m/z 2907.423 (D) Dynamics of the decrease of Lsp1 phosphorylation. Note the decrease in abundance of a phosphopeptide ALLELLDDp(SPVT)PGEAR m/z 1875.885 relative to unphosphorylated form of the peptide at m/z 1795.946. (E) Subcellular localization of the wild type (WT) and mutant forms of Pil1 and Lsp1. Simultaneous mutations of the phosphorylated residues S230 and T233 in both proteins (Pil1WT and Lsp1WT) to the aspartic acid (Pil1D and Lsp1D) and alanine residues (Pil1A and Lsp1A) indicate the importance of phosphorylation/dephosphorylation events for the controlled assembly and disassembly of eisosomes.
Cells are grown and quickly collected

Cells are sampled for fluorescence microscopy and images obtained immediately

The rest of cells are quickly frozen and stored at -70°C. Proteins purified and analyzed by mass spectrometry afterward

Protein localization

Composition of protein complexes

Post-translational modifications

Figure 1
Figure 2

A  

Purification  
3xFlag  
6xHis  

Co-localization  

B  

Purification  
3x(c-Myc)  
8xHis  

Co-Purification  
3xFlag  
3x(c-Myc)  
8xHis  

3xFlag  
6xHis  

Pil1, Lsp1 Fragments of Pil1 and Lsp1  
Mrp8  

Uba4  

kDa  
250  
200  
150  
100  
50  
37  
25  
20  

by guest on May 8, 2020
https://www.mcponline.org
Downloaded from

Downloaded from
https://www.mcponline.org by guest on May 8, 2020
Figure 3
Figure 4
Figure 5

A

- Pil1-GFP-3xFlag-6xH
- Ygr130-GFP-3xFlag-6xH
- Ymr031-GFP-3xFlag-6xH
- Ymr086-GFP-3xFlag-6xH

B

- Pil1 [1-225]aa
- Ygr130-GFP-3xFlag-6xH
- Ymr031-GFP-3xFlag-6xH
- Ymr086-GFP-3xFlag-6xH

merge

merge

7 μm
Figure 6
Table I. Performance of the tested modular fluorescent and affinity tag constructs

| #   | Name of cassette used for genomic tagging | Subcellular Localization | Affinity Purification | Comments |
|-----|------------------------------------------|--------------------------|-----------------------|----------|
|     |                                          |                          |                       |          |
|     |                                           |                          | Fluorescent Protein   | Performance | Affinity Tags | Performance |
| 1   | pmTFP1-3F6H/TADH1/URA3                   | mTFP1                    | 3xFlag-6xHis          | ★★★★★    | 3xFlag-6xHis  | ★★★★★     |
| 2   | pmTFP1-3M6H/TADH1/His3MX6               | mTFP1                    | 3xFlag-6xHis          | ★★★★★    | 3xFlag-6xHis  | ★★★★★     |
| 3   | pmTFP1-3S8H/TADH1/His3MX6               | mTFP1                    | 3xStrep-Tag II-8xHis  | ★★★★★    | 3xStrep-Tag II-8xHis | ★★★★★ |
| 4   | pGFP(S65T)-3F6H/TADH1/URA3              | GFP(S65T)                | 3xFlag-6xHis          | ★★★★★    | 3xFlag-6xHis  | ★★★★★     |
| 5   | pGFP(S65T)-3M6H/TADH1/His3MX6           | GFP(S65T)                | 3xFlag-6xHis          | ★★★★★    | 3xFlag-6xHis  | ★★★★★     |
| 6   | pGFP(S65T)-3S8H/TADH1/URA3              | GFP(S65T)                | 3xStrep-Tag II-8xHis  | ★★★★★    | 3xStrep-Tag II-8xHis | ★★★★★ |
| 7   | pGFP(S65T)-3F6H/TADH1/His3MX6           | GFP(S65T)                | 3xFlag-6xHis          | ★★★★★    | 3xFlag-6xHis  | ★★★★★     |
| 8   | pGFP(S65T)-3M6H/TADH1/His3MX6           | GFP(S65T)                | 3xFlag-6xHis          | ★★★★★    | 3xFlag-6xHis  | ★★★★★     |
| 9   | pGFP(S65T)-3S8H/TADH1/His3MX6           | GFP(S65T)                | 3xFlag-6xHis          | ★★★★★    | 3xFlag-6xHis  | ★★★★★     |
| 10  | pEYFP-3F6H/TADH1/URA3                   | EYFP                     | 3xFlag-6xHis          | ★★★★★    | 3xFlag-6xHis  | ★★★★★     |
| 11  | pEYFP-3S8H/TADH1/URA3                   | EYFP                     | 3xStrep-Tag II-8xHis  | ★★★★★    | 3xStrep-Tag II-8xHis | ★★★★★ |
| 12  | pEYFP-3F6H/TADH1/His3MX6                | EYFP                     | 3xFlag-6xHis          | ★★★★★    | 3xFlag-6xHis  | ★★★★★     |
| 13  | pEYFP-3S8H/TADH1/His3MX6                | EYFP                     | 3xStrep-Tag II-8xHis  | ★★★★★    | 3xStrep-Tag II-8xHis | ★★★★★ |
| 14  | pmCitrine-3F6H/TADH1/URA3               | mCitrine                 | 3xFlag-6xHis          | ★★★★★    | 3xFlag-6xHis  | ★★★★★     |
| 15  | pmCitrine-3M6H/TADH1/His3MX6            | mCitrine                 | 3xFlag-6xHis          | ★★★★★    | 3xFlag-6xHis  | ★★★★★     |
| 16  | pmCitrine-3S8H/TADH1/His3MX6            | mCitrine                 | 3xStrep-Tag II-8xHis  | ★★★★★    | 3xStrep-Tag II-8xHis | ★★★★★ |
| 17  | pmOrange-3F6H/TADH1/URA3                | mOrange                  | 3xFlag-6xHis          | ★★★★★    | 3xFlag-6xHis  | ★★★★★     |
| 18  | pmOrange-3M6H/TADH1/His3MX6             | mOrange                  | 3xStrep-Tag II-8xHis  | ★★★★★    | 3xStrep-Tag II-8xHis | ★★★★★ |
| 19  | pmOrange-3S8H/TADH1/His3MX6             | mOrange                  | 3xFlag-6xHis          | ★★★★★    | 3xFlag-6xHis  | ★★★★★     |
| 20  | pmOrange-3S8H/TADH1/His3MX6             | mOrange                  | 3xStrep-Tag II-8xHis  | ★★★★★    | 3xStrep-Tag II-8xHis | ★★★★★ |
| 21  | pmCherry-3F6H/TADH1/URA3                | mCherry                  | 3xFlag-6xHis          | ★★★★★    | 3xFlag-6xHis  | ★★★★★     |
| 22  | pmCherry-3M6H/TADH1/His3MX6             | mCherry                  | 3xFlag-6xHis          | ★★★★★    | 3xFlag-6xHis  | ★★★★★     |
| 23  | pmCherry-3S8H/TADH1/URA3                | mCherry                  | 3xStrep-Tag II-8xHis  | ★★★★★    | 3xStrep-Tag II-8xHis | ★★★★★ |
| 24  | pmCherry-3S8H/TADH1/His3MX6             | mCherry                  | 3xFlag-6xHis          | ★★★★★    | 3xFlag-6xHis  | ★★★★★     |
| 25  | pmCherry-3S8H/TADH1/His3MX6             | mCherry                  | 3xStrep-Tag II-8xHis  | ★★★★★    | 3xStrep-Tag II-8xHis | ★★★★★ |
| 26  | pmCherry-3S8H/TADH1/His3MX6             | mCherry                  | 3xStrep-Tag II-8xHis  | ★★★★★    | 3xStrep-Tag II-8xHis | ★★★★★ |

a average intensity and variation from cell-to-cell for Lsp1 protein tagged with modular tags. The data are based on at least three biological replicates.

b relative protein purification yield. The best results correspond to five stars and the worst to one star.