Implementation of Multidimensional Protein Identification Technology and its Application to the Characterization of Protein Complexes in Bakers Yeast

Thesis by

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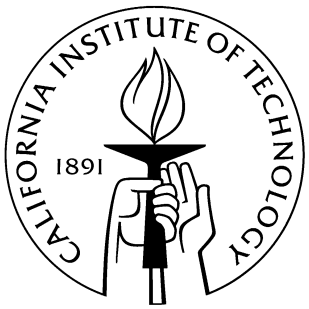

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"... when one is writing a letter, he should think that the recipient will make it into a hanging scroll."

Yamamoto Tsunetomo, "Hagakure"

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Many steps led to this work and every single one was helped along by people I would like to thank.

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Abstract

The analysis of complex polypeptide mixtures poses a central and ubiquitous problem to biochemistry, molecular and cellular biology. Historically the problem has been approached by means of gel electrophoretic separation, coupled to immune–chemistry or Edman degradation (Edman 1949) based identification of separated components. These approaches as well as those based on liquid chromatography are hampered by a central issue: the wide spectrum of polypeptide characteristics that renders their separation difficult. A recent strategy termed *multidimensional protein identification technology* (MudPIT) tackles this problem by capillary chromatographic separation of not the complete polypeptides, but rather peptides yielded by them through proteolytic digest and analyzing them in–line using ion trap mass spectrometry (Link et al. 1999; Washburn et al. 2001; and Wolters et al. 2001).

This work describes the implementation of MudPIT outside of the analytical chemistry environment of its inception. Robustness and generalizability of the technique are tested by analysis of polypeptide complexes copurifyed with 25 selected gene products from *Saccharomyces cerevisiae* (Graumann et al. 2004). The pilot study reveals MudPIT to be mature enough for use outside of specialized environments and, by yielding with Rtt102p a novel component of the Swi/Snf and RSC chromatin remodelling complexes, to have potential for delivering new insights even into extensively studied systems.

Subsequent application of MudPIT to the characterization of components of the ubiquitin–proteasome system (Verma et al. 2004; and Mayor et al. 2005) and mitochondrial fission (Griffin et al. 2005) in *S. cerevisiae* further emphasize its potential to contribute to biochemical research.

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1 Introduction

The work presented in this thesis describes the implementation of a set of techniques termed "Multidimensional Protein Identification Technology" or MudPIT (Link et al. 1999; Washburn et al. 2001; and Wolters et al. 2001), that enables the analysis of complex protein mixtures. This chapter provides an introduction to the significance of the analysis of complex protein mixtures in molecular biology and biochemistry, as well as describing MudPIT in detail.

1.1 The Problem of a Complex Protein Mixture

Polypeptides dominate the spectrum of biological functions as both mediators and catalysts. Although knowledge of biological processes mediated by nucleic acids has expanded dramatically as a result of whole genome sequencing projects (Storz 2002), polypeptides provide the greater variety of building blocks—20 amino acids vs. four nucleotides—and as a result the larger spectrum of possible conformations and chemistries. The array of possible posttranscriptional modifications of nucleic acids (Gott and Emeson 2000) is met by an equally extensive variety of posttranslational modifications in polypeptides (Creighton 1993) and does not shift the balance.

A significant part, if not the majority, of protein–mediated biological reactions is dependent not on a single functional polypeptide, but rather a group of polypeptides working together in a concerted manner, often forming subunits of one protein complex, one "molecular machine" (Alberts 1998). Gavin et al. (2006), for example, estimate that *S. cerevisiae* contains 800 core "protein complexes"—condition–

independent protein complexes, whose composition is modulated in a condition–dependent manner by "attachment proteins." Conservatively assuming two polypeptide chains per "core complex" and disregarding all transient interactions with "attachment proteins," this amounts to 27 % of all systematically named open reading frames in yeast being assembled into complexes. This number rises to 41 % for an average of three polypeptide chains per complex.[1]

These two points—the domination of biological processes by proteinaceous agents and the prevalence of these polypeptides in heterogeneous complexes—present a challenge: separation of a complex protein mixture and the identification of its components, even if one is interested in a single biological process rather than questions of global changes in a cellular or organellar protein complement.

1.1.1 Separation of complex protein mixtures

Separation of protein mixtures is commonly handled by one of two technically divergent approaches: gel electrophoresis or liquid chromatography. Gel electrophoresis separates proteins by a combination of their electrostatic and size properties, whether native or conferred by agents such as sodium dodecyl sulfate as introduced by Laemmli (1970). While immensely popular, the technique in both its one– as well as two–dimensional form (see, e. g., Klose 1975; and O'Farrell 1975), has inherent disadvantages:

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[1] Based on 5872 nondubious and nonpseudogene open reading frames present in the *Saccharomyces* Genome Database (Cherry et al. 1998) as of 03/31/2006.

1. Both one– and two–dimensional gel electrophoresis have severely reduced resolving power for polypeptides of extremely small or large sizes.
2. Similarly, the isoelectric–focusing–based first dimension of conventional two–dimensional gel electrophoresis biases against polypeptides with very high or low isoelectric points.
3. Gel electrophoresis is notoriously unsuited for the separation of polypeptides with extreme hydrophobicity, such as membrane proteins.
4. Although more mechanized approaches have been made (see, e. g., Gavin et al. 2002), selection of separated polypeptide chains is commonly done visually, opening the technique to bias against weakly staining or diffusely migrating polypeptides.
5. Gel electrophoresis delivers the separated polypeptide chains embedded in a gel matrix, which implies the potential for low extraction efficiency.
6. The conventional workflow (see, e. g., Shevchenko et al. 2002) of gel electrophoretic mixture separation, gel block excision and in–gel digest results in the case of complex mixtures in massive sample parallelization, requiring a significant degree of automation. This problem is partially remedied by slicing groups of polypeptide bands rather than individual bands and subsequent chromatographic separation of the electrophoretically prefractionated mixture (Gavin et al. 2002).

Traditional protein mixture separation by chromatographic methods—the mixture is carried through a column of chromatography matrix by a liquid phase and separated by differential interaction with the matrix—implies a similar set of problems, for example:

1. Depending on the polypeptide property by which the matrix separates in a given

experiment (e. g., hydrophobicity, hydrophilicity, charge, size), there are gel electrophoresis analogous problems in separating polypeptides in the extremes of the property spectrum. Extremely hydrophobic proteins are, for example, difficult to separate by reverse phase liquid chromatography, as are extremely hydrophilic ones.

2. Conventional chromatography workflows involving fraction collection potentially yield, just as in the case of gel electrophoretic separation, massive sample parallelization and the necessity of automation.
3. Varying with the liquid phase throughput through the column, chromatographic methods have the inherent problem of volume expansion, necessitating additional procedures as precipitation or lyophilization, implying the risk of sample loss by, e. g., surface coating.

The preceding lists concentrate on the drawbacks of the two most popular means to separate complex polypeptide mixtures. Evidently, the two approaches also have distinct advantages. Gel electrophoresis for example is uniquely suited for separation of posttranslationally modified polypeptide forms (for an example see Larsen et al. 2001), while liquid chromatographic methods are very well suited for subsequent biochemical manipulations as functional assays or crystallographic analysis of the separated polypeptides. Approach–specific problems aside, the methodologies essentially struggle with the same issue: the wide spectrum of biochemical/biophysical characteristics associated with polypeptides in the complex mixtures to be separated. Section 1.2 describes in detail how MudPIT tries to remedy this problem.

1.1.2 Identification of the components of a complex protein mixture

The second step in characterization of a complex polypeptide mixture is the identification of separated components. The methodologies to achieve this fall into two categories: before and after the application of mass spectrometry to the problem. Pre–mass–spectrometry methods for the analysis of an unknown proteinaceous agent include Edman degradation (Edman 1949) as well as raising antibodies against a purified polypeptide, which is then identified by screening through of a phage expression library and sequencing. The first mass spectrometric approach to join the canon of techniques applied to polypeptide analysis was peptide mass fingerprinting (Henzel et al. 1993; James et al. 1993; Mann et al. 1993; Pappin et al. 1993; and Yates et al. 1993). This technique is based on the proteolytic digest of the polypeptide to be analyzed with a site–specific protease and the subsequent mass spectrometric analysis of the resulting peptide mixture. The measured peptide masses are matched with the *in silico* digest of a protein database, yielding the protein with the closest hypothetical spectrum as the identification candidate. Peptide mass fingerprinting shares a major drawback with pre–mass–spectrometric methods: they require polypeptide mixture components to be highly purified, which poses a significant challenge when dealing with highly complex mixtures.

While peptide mass fingerprinting already took advantage of some of the following innovations (e. g., electrospray ionization), the application of the complete set was necessary in order for mass spectrometry to emerge as the dominating technique with respect to polypeptide mixture analysis:

1. The development of postsource decay (PSD, Spengler et al. 1992) and collision induced dissociation (CID, Hunt et al. 1986) changed the field dramatically: the

techniques allow the direct sequencing of the amino acid composition of peptides, which are not necessarily present in highly purified form but can be isolated from an injected peptide mixture by mass filtration in the mass spectrometer.

2. "Soft" ionization techniques such as matrix assisted laser desorption ionization (MALDI, Karas and Hillenkamp 1988) and electrospray ionization (ESI, Fenn et al. 1989) enable the analysis of chemically fragile biomolecules such as polypeptides without significant decomposition.
3. The introduction of a new class of mass analyzers to the characterization of biological samples proved to be crucial to the success of mass spectrometry: quadrupole–ion trap mass spectrometers (ITMS, Jonscher and Yates 1997) not only enable rapid rounds of selection of a single ion from an injected mixture of peptides, but also multiple stages of collision induced dissociation—and therefore sequencing of multiple fragmentation ions. Constant improvements of ITMS systems focus mainly on scan speed—crucial for example to the sampling depth in a chromatographic sample eluted via ESI directly into the mass spectrometer—and better ion statistics (Blackler et al. 2006) and mass accuracy, resulting in higher sequence confidence (Olsen et al. 2005). A small but important feature of these instruments designed for high–throughput analyses is the so called "dynamic exclusion," a mechanism preventing the refragmentation/sequencing of ions in an injected mixture by imposing a temporary exclusion of mass over charge values already attended to.
4. The last innovation to pave the way for mass spectrometry in the analysis of polypeptide mixtures was the creation of software which automatically matches experimental peptide fragmentation spectra to hypothetical spectra derived from organism specific protein sequence databases. Eng et al. (1994) pioneered this

approach with their program `Sequest`, but a number of competing programs as, e. g., `Mascot` (Perkins et al. 1999) and `X! Tandem` (Craig and Beavis 2004) have followed suit.

MudPIT incorporates a number of these innovations to tackle the problem of the analysis of a complex polypeptide mixture, which is described in detail in section 1.2.

1.2 Multidimensional Protein Identification Technology

Multidimensional protein identification technology (MudPIT) was introduced by Link et al. (1999) as "Direct Analysis of Large Protein Complexes" (DALPC). Generalization of the concept lead to the coining of the term MudPIT (Washburn et al. 2001; and Wolters et al. 2001). The workflow established by the authors combines multidimensional capillary chromatography of complex polypeptide mixtures digested in solution with in–line electrospray–ionization ion–trap tandem mass–spectrometry and automated matching of the acquired fragmentation spectra to translated genomic sequence via `Sequest` (Eng et al. 1994). The strategy addresses many of the challenges to the analysis of complex polypeptide mixtures laid out in section 1.1.

MudPIT strives to separate proteins that have been digested into peptides rather than the intact polypeptides. This approach—also termed *bottom up* (Wysocki et al. 2005) or *shotgun proteomics* (Wolters et al. 2001)—levels the biochemical/biophysical properties and therefore reduces the problems polypeptides pose to separation techniques with their wide property spectrums.

Reliance on capillary chromatography with low liquid phase flow rates[2] remedies the issue of volume expansion connected to conventional high pressure liquid chromatography (HPLC), while directly interfacing the separation setup to the mass spectrometer via electrospray ionization. The latter prevents the need for sample collection, thereby rendering further automation unnecessary, reducing manual intervention and preempting sample loss by surface coating. The use of a two–dimensional chromatography column significantly improves the resolution of the setup by utilizing two independent biophysical characteristics of the peptides to be separated: charge by the strong cation exchanger (SCX) phase and hydrophobicity by the reverse phase. It extends prior work (e. g., Lundell and Markides 1992; and Takahashi et al. 1985) and transfers the principles long utilized in twodimensional gel electrophoresis (O'Farrell 1975) to liquid chromatography. McDonald et al. (2002) further enhanced the approach by adding a third phase—a second reverse phase chromatography matrix—to the capillary column, allowing sample desalting in–line to the mass spectrometer, further reducing handling requirements and capturing a class of hydrophilic peptides missed when using the twophasic column layout.

The utilization of iontrap mass spectrometers—for reasons of patent protection of key scan features predominantly ThermoElectron's line of Deca, DecaXP, and LTQ mass spectrometers (historically successive in this order)—enables the analysis of ions eluting into the mass spectrometer with increasing speed and sensitivity (for the LTQ see Blackler et al. 2006). Together with the mechanism of dynamic exclusion discussed above, this renders possible increasingly comprehensive analysis

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[2] 50 μl/min in the original publication, in further works reduced to the order of 100 nl/min.

of peptides of ever lower abundance eluting into the spectrometer.

All the advantages of MudPIT aside, the technique also meets with valid criticisms. Through the projects described in this work, the hand–crafted, single–use capillary chromatography columns used emerged incontestably as the weekest link in the chain of procedures constituting MudPIT. Packing the 100 μm inner diameter columns on customized pressure vessels is tedious work, often requiring multiple attempts. After successful packing, some columns clog during sample loading[3] or produce suboptimal electrospray due to inadequate tip shape. Custom capillary columns are commercially available (e. g., New Objective, Woburn, MA), but the high price together with the triphasic nature of the columns, which interferes with effective cleaning and leave the column a single–use item, did prohibit their use for this work. Although there are promising microfluidic approaches emerging (e. g., Xie et al. 2005), mass production of multiphase capillary columns seems far in the future. The single–use characteristic of the capillary chromatography columns implies (together with the stochastic nature of peak sampling by the ion trap mass spectrometer) relatively low reproducibility when analyzing the same sample on different columns. Multidimensional chromatography is also possible with traditional HPLC columns, but their reliability, reproducibility, reusability and ready commercial availability comes with a significant hit to analysis sensitivity[4]—a fact very much undesirable when analyzing highly complex mixtures with low polypeptide

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[3] Promoted by the high urea concentrations regularly present.

[4] According to Abian et al. (1999), the maximum peak concentration of the sample eluate $C_{\max}$ increases by a factor of 100 when reducing the column diameter from the commonly used 1 mm to 100 μm.

abundance (as, e. g., polyubiquitin conjugates; see appendix C).

Another critical point is the use of in–line electrospray ionization itself. While reducing manual intervention and analysis time by directly linking the chromatography setup with the mass analyzer, this methodology also requires extreme spectrum acquisition speeds to be able to sample deeply into the injected ion mixtures. It also possesses the inherent drawback of producing multiply charged ions (which complicates subsequent spectrum matching) and—in conjunction with in–line chromatography—restricts the time window for analyzing a chromatographically separated peptide peak to its actual elution from the column.

The speed of spectrum acquisition in an ion trap mass spectrometer traditionally comes at the expense of mass accuracy, but this criticism is slowly disappearing due to the combination of ion traps with high mass accuracy mass analyzers as Fourier–transform mass spectrometers (e. g., ThermoElectron's LTQ–FTMS, see Olsen et al. 2004) and orbi–traps (e. g., ThermoElectron's LTQ–Orbitrap, see Olsen et al. 2005).

Shotgun shotgun proteomic data pose significant analysis challenges (Steen and Mann 2004). Improved precursor scan mass accuracy, as delivered by instruments similar to the ones described in the previous paragraph, remedies a part of that problem, but what remains—especially when dealing with higher eukaryotes—is the problem of polypeptide isoform multiplicity due to differential splicing, alternative promoter usage and other mechanisms, as well as often extensive groups of homologous polypeptides, which make pinpointing a polypeptide from a collection of sequenced peptides very difficult (Nesvizhskii and Aebersold 2005; and Godovac-Zimmermann et al. 2005). While this problem is triggering the field to revisit *top down* proteomics with its significant separation challenges (see above), the work pre-

sented here concentrates on the model organism *Saccharomyces cerevisiae*, which carries introns in only $\approx 4\,\%$ of its open reading frames (Spingola et al. 1999) leaving a *bottom up* approach as MudPIT in a favorable light.

After evaluation of these arguments, MudPIT emerges as a viable candidate for complex polypeptide analysis—especially for *Saccharomyces cerevisiae* as a model organism—for the foreseeable future.

A more trivial data analysis problem also arises in conjunction with *shotgun* approaches: the sheer scale of spectra to be searched and their computational handling. The original `Sequest` Eng et al. (1994) read in input files containing information for a single spectrum and produced an output file for every single one of those. Given the tens of thousands of spectra a single MudPIT experiment produces, this strategy taxed even industry grade `UNIX` file systems to their limits. For `Sequest` the problem was fixed with unified input and output formats (Eng et al. 1994; Sadygov et al. 2002; and McDonald et al. 2004), providing all spectral information from one MudPIT step in one single file and the results inferred from it in another. J. G. was involved in the setup of this infrastructure, which is covered in detail in appendix B (p. 103). All spectrum matching programs in use today apply similar approaches.

1.3 Mass Spectrometric Quantification of Polypeptides

Knowing the constituents of a complex mixture of polypeptides represents valuable information in itself, but a large group of biological problems require the identification of differences in polypeptide representation between different biological states, such as wildtype versus mutant or untreated versus pharmacologically manipulated. MudPIT alone delivers excellent data on the composition of a polypeptide mixture,

but the issues of reproducibility discussed above render comparison of independent MudPIT analyses for different biological states difficult (see appendix C), which leaves MudPIT largely incompatible with so–called label–free approaches (Old et al. 2005) to the problem of polypeptide quantification that rely either on measurements and comparison of ion intensities (Bondarenko et al. 2002; Chelius and Bondarenko 2002; and Wang et al. 2003) or spectral counting (Liu et al. 2004).

The major class of solutions to the quantification problem that remains available is isotope or mass tag labeling (Old et al. 2005). The different approaches that can be combined in this category follow one theme: isotopically marking the states to be compared differentially and comparing the abundance of different forms of the same peptide mass spectrometrically—in the same analysis, using the same column, which implies compatibility with MudPIT despite its low reproducibility.

The first subclass in this collection includes the approaches termed ICAT (isotope coded affinity tags; Gygi et al. 1999), its successor cICAT (cleavable ICAT; Hansen et al. 2003; and Yu et al. 2004) and iTRAQ (isobaric tags for relative and absolute quantification; Ross et al. 2004).

(c)ICAT works by mass–differential chemical derivatization of peptides on cysteine residues. The restriction to cysteine–containing peptides along with differential reverse–phase elution behavior of heavy and light forms (Goshe and Smith 2003; Leitner and Lindner 2004; and Wu et al. 2006) are major criticisms facing the techniques. iTRAQ overcomes the residue specificity problem by targeting amines, so that all peptide N–termini, along with lysine side chains, are potential tag receptors. It also enables the direct comparison of up to four samples in the same experiment—a feat no other technique described here accomplishes. Since the tagged peptides have the same mass independent from which of the up to four tested conditions

they arise, the peptide mixture complexity is not increased (all other isotope labeling strategies described here raise it by a factor of two), which relieves the scan burden of the mass spectrometer (Wolff et al. 2006). However, iTRAQ requires high mass resolution fragmentation spectra, since quantification is achieved from small, low m/z–difference daughter ions of the fragmented linkers the peptides are derivatized with (114, 115, 116, 117 kDa), which in turn takes a toll in the achievable sequencing speed. (c)ICAT and iTRAQ share a central disadvantage: samples to be compared using these techniques have to be prepared in parallel, independently derivatized and then mixed, which obviously opens the door to asymmetric processing errors.

This caveat is not present with the second subclass of isotope tag labeling techniques: metabolic labeling. These approaches are based on the utilization of isotopically different polypeptide precursors in one of the biological samples to be compared. Polypeptides from the tagged and untagged samples are as a result available *in vivo* in mass spectrometric distinguishable populations and analytes are prepared from mixed samples rather than in parallel.

Metabolic labeling is generally available in two flavors: SILAC–like (stable isotope labeling by amino acids in cell culture; Ong et al. 2002) approaches based on the incorporation of selected, isotopically labeled amino acids and approaches providing solely heavy nitrogen (^{15}N) in the form of ammonium acetate to the organism under study (Oda et al. 1999; and MacCoss et al. 2003). SILAC approaches elegantly combine applicability to difficult model systems such as culture cells (see acronym) with easy predictability of sister ion mass: when using arginine and lysine as the isotopically tagged amino acids, each peptide produced by trypsin—which hydrolyzes polypeptides specifically c–terminally of those two amino acids— will

carry the additional mass conferred by its c–terminal residue. Isotopically modified amino acids are, however, very expensive and imply the problem of being rerouted by the organisms metabolism, potentially resulting in the isotopic labeling of unintended amino acids, posing problems to accurate peptide sequencing. Approaches based on minimal diets solely providing heavy nitrogen (^{15}N, mostly in the form of ammonium acetate) are in comparison significantly more affordable and, since all nitrogen atoms indiscriminately represent the heavy or light form, do not suffer from metabolic rerouting. This makes them applicable to all systems able to grow on minimal media (a minimal diet). Wu et al. (2004) even managed to raise a rat (*R. norwegicus*) on a diet including ^{15}N–grown algae as the only source of nitrogen.

Given the modell organism this study centers on—*S. cerevisiae*—and the considerations above, metabolic labeling by ^{15}N on minimal media was implemented in the course of the work, additionally profiting from seamless integration of the appropriate quantification software `RelEx` (MacCoss et al. 2003) in the data analysis infrastructure consisting from `Sequest` (Eng et al. 1994; Sadygov et al. 2002; and McDonald et al. 2004) and `DTASelect/Contrast` (Tabb et al. 2002).

1.4 MudPIT at Caltech

Motivated by the advantages of MudPIT laid out in the preceding sections, we set out to implement the technique in a biochemistry laboratory at Caltech. MudPIT was—and may still be—considered experimental technology and had not spread far beyond the labs of John R. Yates III at The Scripps Research Institute (TSRI) and Torrey Mesa Research Institute (TMRI) in La Jolla, California, where the Yates groups were honing the technique (Washburn et al. 2001; and Wolters et al. 2001)

after having moved there from its birthplace at the University of Washington in Seattle (Link et al. 1999). The main challenge in doing so was to implement the setup without the analytical chemistry environment that had bred it: our laboratory had, as is to be expected for the majority of biochemistry/molecular biology laboratories, no expertise in mass spectrometry and very little in information technology and HPLC separation, yet all three fields are required for running a MudPIT facility. To overcome this obstacle J. G. spent 9 months in the Yates lab at TSRI, intensely immersed in all aspects of the labs operation: sample preparation, mass spectrometric and data analysis, as well as hardware maintenance.

Back at Caltech, we proceeded to emulate the Yates lab setup in small scale. The setup initially consisted of

- A P–2000 LASER needle puller by Sutter Instrument Co. (Novato, CA). This instrument is used to outfit the fused silica capillaries from which capillary chromatography columns are constructed (inner diameter: 100 μm) with a ≈ 5 μm diameter tip required for electrospray ionization and chromatography matrix retention.
- Two capillary chromatography column packing stations. These stainless steel pressure vessels, which were produced in–house according to drawings provided by the Yates lab, utilize helium pressures of up to 7×10^6 N/m^2 to pack chromatography matrices into tipped capillary chromatography columns and after equilibration load sample onto the finished columns.
- ThermoElectron's DecaXP$^+$ ion trap mass spectrometer for spectrometric analysis of sample peptides eluted from MudPIT columns.
- An HP–1100 HPLC pump and solvent degasser combination with four solvent

channels by Agilent (Palo Alto, CA). The extremely low flow rates used by MudPIT (100 nl/min and less) in combination with the required solvent gradients between low and high organic solvents with preceding salt bumps cannot be delivered by standard HPLC pumps. The HP–1100 system is therefore used at 100 μl/min and interfaces to the mass spectrometer via

– A custom capillary column electrospray ionization source, which splits the column flow (100 nl/min) from the pump–delivered solvent flow (100 μl/min). The source also provides a liquid phase/voltage junction, applying the 2.4 kV electrospray ionization voltage to the waste arm of the split flow, which prevents gas bubbles resulting from electrochemistry on the electrode from entering the capillary chromatography column. The original design for this ionization source was also provided by the Yates lab and the source manufactured in–house.
– A `Linux` cluster for data analysis. The cluster consists from twenty 1.8 GHz RS–1200 computation nodes, provided by Verari Systems (formerly RackSaver, San Diego, CA). Mass spectrometric data is transferred to a central file server and undergoes charge state analysis as well as data quality filtration by `2to3` (Sadygov et al. 2002). `Sequest` search jobs using unified input and output formats (Eng et al. 1994; Sadygov et al. 2002; and McDonald et al. 2004) can than be queued on the cluster, using `GridEngine` (http://gridengine.sunsource.net/). The queuing mechanism as well as user account dependence are significant enhancements in comparison with the original Yates lab setup, where all members logged on as one user to execute `Sequest`, verbal agreements were necessary concerning how many jobs to run at one time and manual checking of running processes with low–level system commands provided the only handle on available slots. In order to coerce the experimental `Sequest` binary provided by

the Yates lab through a collaboration (Graumann et al. 2004) into conforming to the requirements of this system, `GNU screen` (http://www.gnu.org/software/screen/) has to be used to mimic a terminal interactively open to the binary—on the original setup remote users had to leave a terminal open on the computer they were acessing the cluster from. After `Sequest` analysis on the cluster, data filtration and annotation is performed by `DTASelect` (Tabb et al. 2002) on the fileserver and the results are immediately available for browsing through a `html` interface from the outside.

The MudPIT setup was later enhanced by the acquisition of ThermoElectron's next generation ion trap mass spectrometer: the LTQ linear ion trap instrument (Blackler et al. 2006). This instruments provides much higher scan rates as well as higher sensitivity and better signal–to–noise ratios due to a bigger ion capacity of the trap. It interfaces to a Surveyor four solvent channel HPLC pump (ThermoElectron, Waltham, MA) and a MicroAS autosampler (ThermoElectron, Waltham, MA). In this setup the HPLC pump only provides the low to high organic solvent gradients specific to the reverse phase parts of a MudPIT column, while the salt bumps necessary to elute subsets of peptides from the SCX phase are provided by injection of defined volumes and concentrations through the autosampler—potentially delivering much sharper salt peaks. Beyond that, quantitative mass spectrometric polypeptide analysis via metabolic incorporation of ^{15}N and the program `RelEx` (MacCoss et al. 2003) have been included as well.

To test the MudPIT setup established, we proceeded to analyze a diverse collection of affinity purified polypeptide complexes using baits mainly involved in cell cycle progression and transcription in the yeast *Saccharomyces cerevisiae* (Grau-

mann et al. 2004). This pilot study, which represents the core of this thesis, is documented in detail in chapter 2 (p. 28). The ORFs (open reading frames) to be studied were chromosomally tagged with a tandem affinity purification tag (TAP tag) analogous the the pioneering construct by Rigaut et al. (1999) and purified under native conditions from whole cell extract. Twentytwo out of 26 attempted chromosomal taggings succeeded. The study revealed 102 previously known and 279 potential new physical interactions to the set of tagged gene products. It includes among other things the characterization of a new subunit of the intensely studied Swi/Snf (Fry and Peterson 2001) and RSC (Sanders et al. 2002; Damelin et al. 2002; and Cairns et al. 1998) chromatin remodelling complexes. MudPIT proved mature enough for migration into less specialized environments and presented immediately new insights into systems extensively studied with more traditional techniques.

The technique has consequently been applied to a variety of problems linked with moderately complex polypeptide mixtures as delivered by affinity purified protein complexes. Appendices A (p. 67) and D (p. 153) present two such examples in detail.

We have also extended the use of MudPIT—in the spirit of Washburn et al. (2001), who analyzed whole cell lysates from *S. cerevisiae* by MudPIT—to much more complex mixtures with low abundant components. Appendix C (p. 121) presents an example for this with the analysis of affinity purified multiubiquitin conjugates from whole cell lysate.

1.5 References

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