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# The Current State of the Golgi Proteomes

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## 1. Introduction

The Golgi apparatus plays a central role in the eukaryotic secretory pathway shuttling products between a variety of destinations throughout the cell. It is a major site for the post translational modification and processing of proteins as well as having a significant role in the synthesis of complex carbohydrates. The Golgi apparatus exists as a contiguous component of the endomembrane system which encompasses the endoplasmic reticulum (ER), plasma membrane, vacuoles, endosomes and lysosomes (Morre & Mollenhauer, 2009). The Golgi apparatus is tightly linked to numerous signaling processes through membrane and vesicular trafficking throughout the endomembrane. This interconnection provides communication and recycling networks between the Golgi apparatus, the plasma membrane, vacuoles and lysosomes. Thus, the Golgi apparatus represents significant structure within the eukaryotic cell by regulating an array of complex biosynthetic processes.

The Golgi apparatus was first described at the end of the 19<sup>th</sup> century by Camillo Golgi using light microscopy on nerve tissue samples (Golgi, 1898; Dröschner, 1998). A half century passed and with the development of the electron microscope a more detailed picture emerged highlighting the extreme complexity and heterogeneity of the organelle in the eukaryotic cell (Dalton & Felix, 1953). The classic structure of the Golgi apparatus is that of a distinct membranous stack disassociated within the cytosol (Fig.1). This familiar image conceals the underlying complexity and interconnected nature of this organelle within the cell. With the development in the last decade of routine mass spectrometry-based proteomics, applying these methods to functionally characterize biological systems has been a major focus. The complexity and integrated structure of the Golgi apparatus and associated membrane systems makes analysis of this organelle one of the most complicated subcellular compartments to address with modern proteomics techniques. This chapter will highlight recent advances in our knowledge about the Golgi apparatus in eukaryotic systems that have been largely driven by the development of isolation procedures and subsequent proteomic analysis.

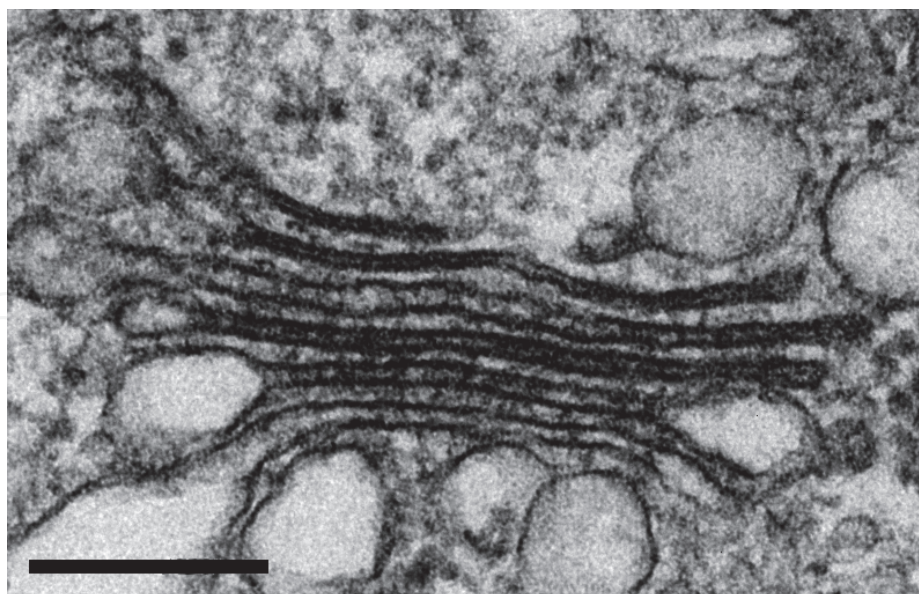


Fig. 1. Electron micrograph of a Golgi stack from the model plant *Arabidopsis thaliana* highlighting the integrative structure and membrane organization. Scale bar = 200nm.

## 2. Differential density enrichment of Golgi

The basic technique of differential density enrichment of Golgi membranes represents the most common isolation and enrichment process for downstream proteomic analyses. The technique was well-established in most eukaryotic systems prior to the development of mass spectrometry-based identification techniques. Consequently, analysis of the enriched Golgi apparatus fraction using this method was a logical approach, although one limited by contaminating organellar membranes and low gradient resolution. Yet, insights into membrane systems associated with trafficking, post-translational modifications and complex carbohydrate biosynthesis have been revealed.

Many of the initial approaches used to characterize Golgi associated proteins by differential centrifugation employed SDS-PAGE arraying techniques prior to identification of proteins by mass spectrometry. Nearly all of the early proteomic studies on enriched Golgi fractions are from easily accessible samples such as rat livers, likely reflecting the need for the development of purification techniques. The earliest 'proteomic' analyses of the Golgi employed two-dimensional gel electrophoresis (2-DE) to array enriched stacked Golgi fractions from rat livers (Taylor et al., 1997b). The study used cyclohexamide in an effort to clear transitory proteins from the secretory pathway and reduce non-specific Golgi proteins. While only a handful of proteins were identified by cross comparing reference maps and immunoblotting the study demonstrated the validity of a proteomic approach to analyze the Golgi and could discern resident proteins from cargo and cytosolic proteins (Taylor et al., 1997b). Significantly, the study employed a recently developed sequential sucrose gradient enrichment method (Taylor et al., 1997a) and enabled the reliable visualization of Golgi proteins by 2-DE with reduced contaminants. The sequential sucrose enrichment of stacked Golgi from liver samples involved initially loading a clarified homogenate (post-nuclear supernatant) between 0.86 M and 0.25 M sucrose steps followed by centrifugation. The resultant 0.5/0.86M interface (Int-2) was removed, adjusted to 1.15M sucrose and overlaid with 1.0M, 0.86M and 0.25M sucrose and centrifuged. The resultant 0.25/0.86 interface

represented the enriched stacked Golgi fraction (Fig. 2). This fraction was estimated to represent 200 to 400- fold enrichment over the post-nuclear fraction (Taylor et al., 1997a). With the development of reliable protein identification through tandem mass spectrometry this Golgi enrichment technique could be more readily exploited for functional proteomic studies. This was undertaken through 2-DE arraying of Golgi samples using the sequential sucrose technique on rat liver and mammary epithelial samples (Taylor et al., 2000; Wu et al., 2000). Both studies employed early liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods using iontrap MS and resulted in the identification of 71 proteins from 588 unique 2-DE spots of rat liver (Taylor et al., 2000) and over 30 distinct proteins from mammary epithelial cells (Wu et al., 2000). This later work outlined a comparative study of Golgi isolated from cells transiting from basal (steady-state) to maximal secretion. Proteins identified by this study were upregulated during this transition and comprised a series of Rab proteins, structural components such as microtubule motor proteins and membrane fusion proteins e.g. Annexins (Wu et al., 2000). Another early attempt at characterizing Golgi associated proteins by proteomic methods employed a previously developed strategy to enrich WNG fractions (nuclear associated Golgi fractions) from rat liver homogenates (Dominguez et al., 1999). The technique employs multiple rounds of low speed centrifugation steps in conjunction with a 0.25 to 1.10 M sucrose gradient. For analysis by mass spectrometry, proteins from the resultant rat liver WNG fraction were partitioned into a detergent fraction using Triton X-114 to enrich for membrane proteins and arrayed by SDS-PAGE or 1-DE (Bell et al., 2001). A total of 81 proteins were identified from 1-DE arrayed samples using MALDI-MS, nano-MS/MS or Edman sequencing and included a range of well-characterized Golgi proteins including lectins, KDEL receptor, glycosyltransferases and trafficking proteins (Rabs, SNAREs, SCAMPs). While the results demonstrated the applicability of the overall approach, a significant number of contaminants were also identified in the samples (Bell et al., 2001).

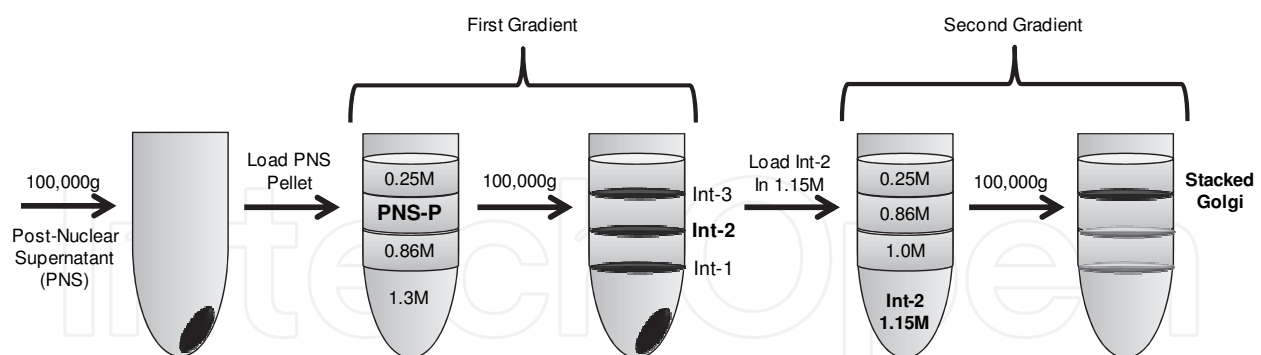


Fig. 2. Schematic outline of the sequential sucrose density gradient procedure developed for the isolation and purification of Golgi stacks from rat liver (Taylor et al., 1997a). The values within the tubes indicate concentration of sucrose (M: mol/L).

With the development of advanced gel-free approaches like multidimensional protein identification technology (MudPIT) for the analysis of complex lysates by mass spectrometry (Wu et al., 2003), a number of previously established enrichment strategies were again analyzed. The two independent and sequential sucrose step gradient technique (outlined above) was employed to isolate Golgi membranes from the livers of rats treated with cyclohexamide (Wu et al., 2004). These fractions were analyzed directly by MudPIT,



using a 12-step strong cation exchange (SCX) fractionation coupled to reverse phase LC-MS/MS by iontrap MS. From this approach, 421 proteins were identified from Golgi fractions isolated from rat liver homogenates (Wu et al., 2004). About 26% of the proteins (110) could be confidently allocated to Golgi-associated functions with about 10% allocated as unknowns. A large proportion of the identifications represented proteins from contaminating organelles including ER (23%), plasma membrane (9%) and cytosol (9%). Nonetheless, a large number of proteins were identified from major Golgi functional processes including transporters, SNARES, glycosyltransferases, G-proteins and clathrin proteins (Wu et al., 2004). Importantly, the gel-free approach enabled the detection of protein modifications with a total of 10 Golgi proteins identified with arginine dimethylation, a potential regulator of protein function (Wu et al., 2004). Stacked Golgi membranes isolated from rat liver homogenates were again analyzed by MudPIT (using a Q-TOF) in a separate study. After initially enriching the membranes by a discontinuous sucrose gradient, repeated centrifugations through 1.3M sucrose were used to clarify and purify the Golgi stacks (Takatalo et al., 2006). Replicate experiments were fractionated offline by SCX prior to analysis by LC-MS/MS. A total of 1125 proteins were identified in the two experiments by combining results from two separate MS/MS search algorithms (Takatalo et al., 2006). Analysis of these protein identifications found that 35% contained at least one predicted transmembrane domain and 201 proteins could be assigned as Golgi or transport related based on functional annotations. When compared to the similar approach of Wu et al., (2004) a total of 399 proteins were identified by both studies. The multiple isolation and analysis strategies enabled the number of unknown proteins from the rat liver Golgi fraction to be significantly extended by 89 proteins. Overall, proteins associated with glycosylation, Golgi matrix complexes, enzymes involved in isomerase and transferase activities and protein trafficking components were all identified (Takatalo et al., 2006). Nonetheless, limitations in the purification procedure are highlighted by positive allocations of this proteome to the cytosol (15%), contaminating membranes (20%) and the cryptically named 'undetermined location' (20%) (Takatalo et al., 2006).

In an effort to address contamination issues and to better tease apart and define the ER, quantitative proteomic strategies were subsequently employed on rat liver Golgi enriched by sucrose density centrifugation (Gilchrist et al., 2006). Previous methods used to isolate rat Golgi fractions (Bell et al., 2001) were used as well as rough and smooth microsomal preparations (Paiement et al., 2005). This was followed by 1-DE, band isolation and analysis by nanoLC-MS/MS (Q-TOF MS). Based on annotation information, Golgi fractions were estimated to comprise around 10% mitochondrial protein, although there was a distinction in proteins identified between the Golgi and microsomal fractions. Multiple biological replicates for the Golgi and microsomes were undertaken and proteins quantified by redundant spectral counting techniques (Blondeau et al., 2004). Hierarchical clustering with Pearson correlation was used to visualize protein abundance measures and this analysis identified four major groups which co-clustered with organelle markers sec61 (rough microsome), calnexin (ER), p97 (smooth microsomes) and mannosidase II (Golgi). This co-clustering with defined markers indicated that quantifying distinct fractions could be used to tease apart the secretory pathway (Gilchrist et al., 2006). Nonetheless only 43 additional proteins could be confidently assigned to the Golgi compared to a previous analysis that had identified 81 proteins (Bell et al., 2001). Consequently COP1 vesicle enrichment from the Golgi fraction was also undertaken to extend the proteome. Overall 1430 proteins were identified from the rat liver secretory system with quantitative proteomics enabling the

confident assignment of 193 proteins to Golgi/COP1 vesicles, with 405 proteins shared between the Golgi and ER fractions. The work identified a host of secretory proteins including Rabs, SNAREs, Sec proteins and unknowns (Gilchrist et al., 2006). A similar MudPIT approach in mouse was undertaken by employing spectral counting for quantification of proteins from multiple subcellular fractions (cytosol, microsomes, mitochondria and nuclei) to determine distinct proteomes (Kislinger et al., 2006). This study also isolated compartments from different organs and undertook organ specific transcript profiles to develop machine-learning techniques to classify subcellular localizations. The enrichment method for the microsomal fraction (Golgi) resulted from high speed centrifugation of a post-nuclear supernatant and thus represents a fairly crude membrane preparation. Nonetheless, by employing samples from multiple organs, transcript profiling and modeling techniques a significant number of secretory proteins were identified and expressions profiled (Kislinger et al., 2006).

The focus on cellular secretory system as an important component in agricultural milk production was explored with the analysis of microsomal fractions from bovine mammary glands (Peng et al., 2008). The study used a 100,000g microsomal pellet from homogenized mammary tissue clarified at 10,000g to isolate enriched microsomal fractions. Samples were arrayed by 1-DE and analyzed by nanoLC-MS/MS, by linear iontrap (LTQ). Of the 703 proteins identified from these fractions nearly half were designated as likely secretory components with a total of 48 (~ 7%) allocated as originating from the Golgi apparatus. These included ADP-ribosylation factors, coatamer protein complex components, transporters, Rab and Sec proteins (Peng et al., 2008).

Surprisingly, only one study has used basic density centrifugation techniques and proteomics to examine the Golgi apparatus of yeast, the most widely studied eukaryotic system (Forsmark et al., 2011). The *sec6-4* yeast mutant was identified several decades ago as a temperature sensitive mutant that accumulates post-Golgi secretory vesicles (Walworth & Novick, 1987) although until recently no proteomic analysis had been undertaken. Recently the *sec6-4* mutant was used to undertake comparative proteomics against the *sec23-1*, a yeast mutant that is depleted of vesicles (Kaiser & Schekman, 1990). Comparative proteomics was undertaken between these two yeast mutants using iTRAQ on fractionated vesicles purified on 9-step linear sorbitol gradients. After density centrifugation, fractions showing the highest signal for SNARE proteins from the two mutant lines were pooled and used for analysis by nanoLC-MS/MS by Orbitrap MS. A total of 242 proteins were identified from the pooled fractions with 91 having greater abundance in the *sec6-4* mutant lines. Many of the most highly differentially expressed proteins were cell wall associated and plasma membrane transporters all of which were likely cargo proteins. The analysis also identified many vesicle proteins which included SNAREs, GTPases, protein glycosylation components and V-ATPase complex components (Forsmark et al., 2011).

While there has been significant interest in plant cell wall biosynthesis for the past decade, only two studies have used proteomics to explore density enriched Golgi fractions from plants. The absence of many studies likely reflects the reported difficulties in isolating Golgi membranes from plants. With this problem in mind, one of the first attempts employed a rice suspension cell culture expressing a *cis*-Golgi marker (*35S::GFP-SYP31* construct) in an attempt to characterize enriched fractions from discontinuous sucrose density gradient (Asakura et al., 2006). The enrichment procedure utilized sequential discontinuous sucrose gradients on a microsomal fraction to greatly enrich membranes containing the *35S::GFP-SYP31* construct. The technique also employed the addition of  $MgCl_2$  which had previously

been shown to separate distinct Golgi compartments (Mikami et al., 2001). Twice purified Golgi membranes were arrayed by both 1-DE and 2-DE, but the 2-DE arrays yielded few identifications. Protein bands from 1-DE were excised and analyzed by MALDI-TOF MS. A total of 63 proteins were identified with 70% containing predicted transmembrane domains. A variety of Golgi proteins were identified including COP complex components, GTPases, Rab proteins, an EMP70 and phospholipase D (Asakura et al., 2006). Very little is known about the role of the Golgi apparatus in secondary cell wall formation in plants due to considerable technical issues in sample preparation. Recently, an analysis of membrane enriched fractions from developing compression wood of *Pinus radiata* has attempted to address this shortage in knowledge (Mast et al., 2010). Microsomal fractions were enriched from compression wood homogenates using discontinuous gradients with maximal Golgi marker activity found at the 8/27% interface. Fractions were further extracted using a TX-114 Triton phase separation technique in order to remove contaminating proteins (e.g., actin). Samples were analyzed using nanoLC-MS/MS by linear iontrap (LTQ). A total of 175 proteins were identified, 66 in the aqueous phase and 103 in the detergent phase. Only a handful of proteins were confidently allocated to the Golgi apparatus after functional annotations, further highlighting the inherent difficulties in working with this tissue. These included laccases, cellulose synthases and a xylosyltransferase (Mast et al., 2010).

### 3. Immunoaffinity purification of Golgi

The immunoaffinity purification (IP) of organelles has been a widely used technique for decades. Since the approach relies on the presence of differential epitopes rather than differences in physical parameters it is highly amenable for isolating functionally distinct subcellular organelles (Richardson & Luzio, 1986). This approach is often combined with sucrose gradient fractionation. The use of IP and proteomics to isolate and characterize subcellular compartments of the secretory system has been most effectively applied in animal systems. Early approaches investigating Golgi immunoisolation employed baby hamster kidney (BHK) cells infected with mutant vesicular stomatitis virus (VSV). After temperature induction the viral G protein accumulates at the *trans*-Golgi network (TGN) serving as bait for the immunoisolation of the corresponding compartment. Immunoisolated TGN was visualized with TEM confirming its structural integrity. Several polypeptides were enriched in the isolated fraction (de Curtis et al., 1988).

An early study which highlighted future approaches, Hobman et al., (1998) successfully used IP to identify ER exit sites in BHK cells. This study used the Rubella virus E1 glycoprotein, which normally localizes in a subset of Smooth ER (SER) and does not overlap with COPI coated ER. Tubular networks of SER were separated by cell fractionation, followed by immunoisolation using Dynabeads coated with an antibody against epitope tagged E1. Using electron microscopy, the authors demonstrated that the isolated membrane structures were distinguished from COPI coated ER. Western blot analysis against known markers identified the presence of proteins in this compartment and included ER-Golgi intermediate and transitional ER markers. These results indicated that this tubular distinct subdomain of SER provides the site for COPII vesicle biogenesis. A further early study demonstrating the viability of the approach used antibody-conjugated magnetic beads facilitating the isolation of peroxisomes from rat liver (Kikuchi et al., 2004). Peroxisomes separated by cell fractionation were further isolated with an antibody against PMP70 (70-kDa peroxisomal membrane protein). Proteomic analysis was carried out, using

in gel digestion and LC-MS/MS was accommodated in a hybrid type-Q-TOF mass spectrometer. The proteome contained 34 known peroxisomal proteins and a minor number of mitochondrial proteins. Two unknown proteins were added to the peroxisomal proteome by this study; a Lon protease and a bi-functional protein consisting of an aminoglycoside phosphotransferase-domain.

The application of IP followed by protein identification through mass spectrometry addressing the proteome of the secretory components was first undertaken to examine the ER-Golgi intermediate compartments or ERGIC of humans (Breuza et al., 2004). The approach used the drug Brefeldin A to cause an over accumulation of ERGIC clusters in human HepG2 cells. The post nuclear supernatant was loaded onto a linear 13-29% Nycodenz gradient and fractions enriched for the ERGIC-53 marker pooled. Dynabeads coupled to KDEL receptor monoclonal antibodies were used to IP the ERGIC membranes. Samples were arrayed by 1-DE and protein bands analyzed by MALDI-TOF MS. A total of 19 proteins were identified including ERGIC-53, the KDEL receptor, SEC22b, cargo receptors and membrane trafficking components.

The use of IP on recombinant lines expressing specific epitopes was first undertaken in yeast (Inadome et al., 2005). Strains expressing recombinant SNARE proteins Myc<sub>6</sub>-sed5 and Myc<sub>6</sub>-Tlg2 were employed to isolate vesicles associated with early (sed5) and late (Tlg2) Golgi compartments. Since recombinant Myc tagged proteins are employed the IP does not require protein specific antibodies. The approach utilized the supernatant from a 100,000g centrifugation step to purify both Myc<sub>6</sub>-sed5 and Myc<sub>6</sub>-Tlg2 vesicles using an anti-Myc monoclonal antibody. Vesicles were affinity purified from each strain using Protein A-Sepharose beads and resultant proteins arrayed by 1-DE. Protein bands were excised and analyzed by MALDI-TOF MS. A total of 29 proteins were identified from the Sed5 vesicles and 32 proteins from the Tlg2 vesicles. Both proteomes contained large proportions of known Golgi proteins including Rab GTPases, SNAREs, mannosyltransferases, V-ATPase proteins (Inadome et al., 2005). Interestingly a number of proteins were identified exclusively in either the early Sed5 vesicles, namely COPII components involved in transport from ER to Golgi and mannosyltransferases involved in protein glycosylation. This fraction included a protein of unknown function (svp26) that was shown to be involved in retention of membrane proteins in early Golgi compartments. Similarly, in the late vesicle Tlg2 proteome, a significant number of proteins with no known function were identified.

Isolation of synaptic vesicles by immunopurification from mammalian systems is a well-developed procedure (Morciano et al., 2005; Burre et al., 2007). Due to the dynamic nature of synaptic vesicle trafficking, it has been challenging to isolate synaptic vesicles from their corresponding plasma membrane with conventional vesicle isolation techniques. Consequently an IP approach was employed on samples from rat brains and enabled the identification of several proteins that might be involved in the regulation of neurotransmitter release and the structural dynamics of the nerve terminal (Morciano et al., 2005). Synaptic vesicles were isolated from two synaptosomal enriched sucrose fractions, followed by IP with Dynabeads covered with antibodies against synaptic vesicle protein 2 (SV2). The isolated proteins were separated by BAC/SDS-PAGE and identified by MALDI-TOF MS. The free vesicle fraction contained 72 proteins while 81 were identified in the plasma membrane containing, denser fraction. Although many proteins, involved in vesicle trafficking and tethering were identified in both fractions, several proteins were specific to one fraction. For example, several isoforms of Rab2, Rab3, Rab11, Rab14 were only found in the free vesicles. The PM associated fractions contained several proteins that could modulate



presynaptic function, including GTPases and Na<sup>+</sup>/K<sup>+</sup>-ATPases, which are potentially associated with the plasma membrane. Several physicochemical conditions were evaluated to enrich low abundant proteins (Burre et al., 2007), among these the addition of SB/DTT or NP40 showed that it can improve the efficacy of protein elution from the magnetic beads. Moreover, the use of phase separation can divide proteins according to their hydrophobicity, resulting in the isolation of more integral membrane proteins.

The selection of bait proteins might be a crucial prerequisite for specific vesicle immunoisolation. Motor proteins could be excellent baits for dissecting a subset of post-Golgi membrane compartments. Recently, a kinesin motor, calsyntenin-1, has been successfully used to identify its cargo vesicles from neuronal axon in mouse brain (Steuble et al., 2010). This study involved IP from two distinct subfractionated organelle populations, using Dynabeads coupled with anti-calsyntenin antibodies. Solubilized proteins were analyzed by LTQ-ICR-FT MS. This approach allowed the identification of endosomes that contained calsyntenin-1 and identification of their specific resident proteins. A combination of biochemical analysis and immunocytochemistry lead to a model of two distinct, non-overlapping endosomal populations of calsyntenin-1. An early endosome population, containing  $\beta$ -amyloid precursor protein (APP) and second, a APP negative, recycling endosomal population.

In contrast with the studies in animal and microbial systems only one pioneering study has been undertaken in plants (Drakakaki et al., 2011). The SYP61 TGN and early endosome compartment was isolated, employing an IP approach from SYP61-CFP transgenic Arabidopsis plants (Fig. 3). This involved a two-step procedure, comprising sucrose gradient fractionation followed by IP using agarose beads coupled with antibodies against GFP, facilitating the SYP61 compartment isolation. In total, 145 proteins were identified by MudPIT nano-LC MS/MS. These include the SYP61 SNARE complex and its regulatory proteins (SYP41, VTI12, and VPS45), GTPases and proteins involved in vesicle trafficking. Other SYP4 members such as SYP43 were also found in the SYP61 vesicle proteome, establishing new protein association with SYP61. Several proteins of unknown function,

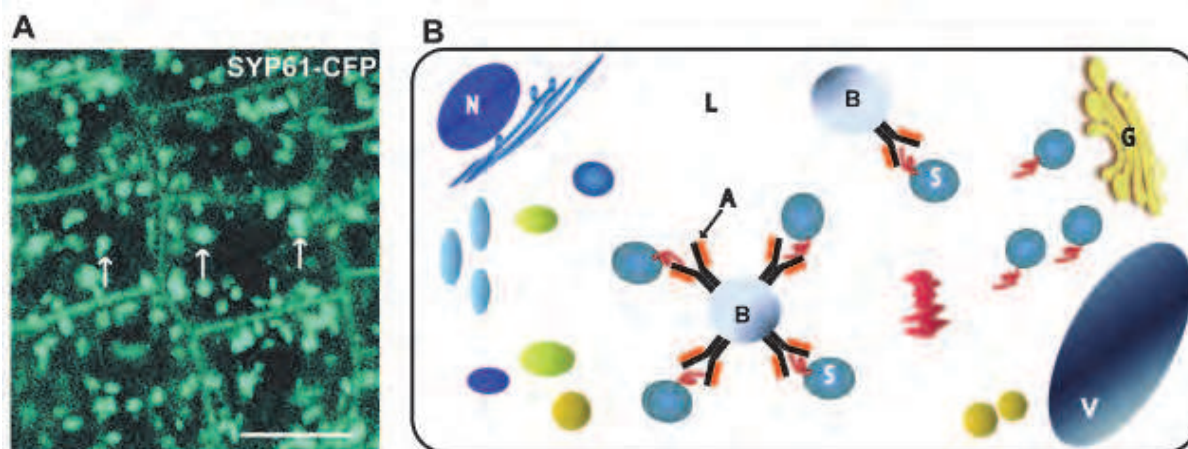


Fig. 3. Schematic representation of vesicle immunoisolation. A). Subcellular localization of SYP61-CFP in transgenic Arabidopsis root cells. Arrows indicate SYP61-CFP vesicles. Scale bar = 10 $\mu$ m. B). Diagram of vesicle IP. Vesicles or compartments are isolated from cell lysate with the aid of an antibody against a target protein. Compartments are not to scale. B: Protein A-Agarose bead; A: GFP antibody; G: Golgi apparatus; S: SYP61 containing vesicles; L: cell lysates, V: vacuole.

such as ECHIDNA were included providing the opportunity of new cargo identification. Plasma membrane associated proteins such as the SYP121-complex and members of the cellulose synthase family were also present. These findings suggested a role of SYP61 in exocytic trafficking and possibly in transporting of cell wall components.

Immunoisolation of TGN vesicles can not only facilitate the identification of its proteome but also other components. A recent study in yeast has analyzed the lipid profile of TGN vesicles transporting a transmembrane raft protein. It demonstrated that the isolated compartment was selectively enriched with ergosterol and sphingolipid, thus providing evidence that TGN can sort membrane lipids (Klemm et al., 2009). As more different subcompartments of Golgi are purified, we will gain greater insights into the nature of the sorted components and their transported cargo.

#### **4. Subcellular correlation analysis of Golgi from complex lysates**

For proteomic analysis of an isolated organelle, it is critical that the sample is of high purity to minimize the inclusion of contaminant proteins. If the organelle sample is only partially enriched, far greater scrutiny is required to confidently differentiate between proteins that genuinely reside in the organelle and contaminants. Highly purified samples are relatively straightforward to obtain for proteomic analyses of mitochondria (Sickmann et al., 2003; Taylor et al., 2003; Heazlewood et al., 2004), chloroplasts (Friso et al., 2004; Kleffmann et al., 2004) and nuclei (Bae et al., 2003; Turck et al., 2004; Mosley et al., 2009), mainly through the use of differential centrifugation techniques. However, obtaining pure organelles of the endomembrane system such as the Golgi, endoplasmic reticulum (ER) and plasma membrane is difficult as they are highly interconnected and are of similar sizes and densities (Wu et al., 2004; Hanton et al., 2005). To avoid the task of having to isolate pure endomembrane system organelles, several groups have instead developed techniques to analyze multiple subcellular compartments simultaneously with crude organelle samples. One such technique is the localization of organelle proteins by isotope tagging (LOPIT), where organelles are partially separated by equilibration centrifugation in a self-forming iodixanol density gradient (Dunkley et al., 2004; Sadowski et al., 2006; Lilley & Dunkley, 2008). Protein distribution patterns are quantified by differential isotope tagging of proteins across organelle fractions with LC-MS/MS on a Q-TOF instrument. Finally, multivariate analysis of isotopically-tagged peptide fragment ion data is used to correlate proteins with similar density gradient ion distributions with that of known organelle marker proteins to determine their subcellular location.

The first LOPIT study was performed on twelve crude membrane fractions from *Arabidopsis* callus culture that were selectively tagged with light or heavy isotope-coded affinity tags (ICATs) (Dunkley et al., 2004). ICATs are chemical probes containing 1) a reactive group targeting free cysteine residues, 2) an isotopically coded linker to distinguish between heavy (deuterium or  $^{13}\text{C}$ ) and light tags (protons or  $^{12}\text{C}$ ) and 3) an affinity tag such as biotin or avidin to capture the labeled peptide or protein (Gygi et al., 1999). The twelve membrane fractions were organized into six pair-wise comparisons of ICAT light- and heavy-tagged fractions. The six ICAT protein sample pairs were pooled, digested with trypsin and the ICAT-labeled peptides were avidin-affinity purified and analyzed by LC-MS/MS (Q-TOF). Multivariate analysis of LC-MS/MS data determined the relative abundances of 170 identified *Arabidopsis* proteins. Of these, a subset of 28 known or predicted *Arabidopsis* organelle marker proteins were used by multivariate analysis to

highlight the clear separation between the known and predicted Golgi- and ER-localized protein clusters. Significantly a number of cell wall biosynthetic enzymes were identified including a number of glycosyltransferases. This confirmed LOPIT as a valid method for discriminating between Golgi- and ER-localized proteins from Arabidopsis crude membrane fractions (Dunkley et al., 2004). Further development of the LOPIT technique replaced ICAT with isotope tagging of Arabidopsis membrane peptide fractions for both relative and absolute protein quantitation (iTRAQ) (Dunkley et al., 2006) (Fig. 4). The iTRAQ method is a progression of ICAT by labeling the free primary amines of peptides with four different iTRAQ reporter tags (114, 115, 116 and 117 m/z). They are detectable by MS/MS, which allows for simultaneous quantification analysis of up to four peptide samples (Wiese et al., 2007). Arabidopsis membrane peptide fractions were differentially tagged with the four iTRAQ reporters, fractionated and analyzed by MudPIT and Q-TOF MS. The addition of SCX to RP LC-MS/MS provided superior peptide separation and identification, resulting in 689 Arabidopsis protein identifications. Multivariate analysis of iTRAQ-labeled MS/MS data revealed 89 proteins in the Golgi density gradient cluster. This more extensive analysis further validated the approach as further cell wall biosynthetic enzymes such as glycosyltransferases and sugar interconverting enzymes were identified as well as transporters, V-ATPase components and a variety of proteins with likely Golgi functions (Dunkley et al., 2006). This was a significant improvement on the initial LOPIT set of ten Arabidopsis Golgi-localized proteins by ICAT and LC-MS/MS (Dunkley et al., 2004).

To test its robustness in other biological system, LOPIT was used to investigate the subcellular distribution of proteins from *Drosophila* embryos. A total of 329 *Drosophila* proteins were identified and localized to three subcellular locations; the plasma membrane (94), mitochondria (67) and the ER/Golgi (168) (Tan et al., 2009). The lack of distinction between ER- and Golgi-residing *Drosophila* proteins by LOPIT underscored the significant challenges faced when dissecting complex and heterogeneous biological samples, as opposed to a simplified system of crude membranes from a relatively homogenous Arabidopsis cell culture.

A similar strategy to LOPIT but employing label-free quantitation techniques is protein correlation profiling (PCP). PCP uses quantitation of unmodified peptide ions by MS to bypass the chemical modification step in ICAT and iTRAQ, which results in less complicated MS/MS spectra and higher confidence in peptide identifications (Andersen et al., 2003; Foster et al., 2006). However, it is heavily reliant on invariable conditions in 2D LC-MS/MS for reproducible quantitation between samples. Proof of concept for PCP was first demonstrated with purified human centrosomes (Andersen et al., 2003) and in the cellular context with sucrose density gradient separations of mouse liver homogenate (Foster et al., 2006). A total of 1,404 mouse liver proteins were identified by 2D LC-MS/MS (LTQ-FT) and their MS ion distribution profiles were mapped by PCP to ten different subcellular locations. These results were corroborated with MS ion distribution profiles and enzymatic assays of known organelle marker proteins and immunofluorescence staining of mouse liver cells for visual confirmation of select proteins with overlapping or non-overlapping PCPs. While this study reported rates of 61 to 93% overlap from comparing its mitochondrial-localized protein set with previous human and mouse mitochondrial proteomes, the rates of overlap were considerably lower for proteins localized to the plasma membrane (49%) and Golgi (36%). Nonetheless, they made significant inroads in characterizing the mouse Golgi proteome and identified a series of Rab proteins, mannosyltransferases, COP components, transporters and a diverse range of transferases (Foster et al., 2006).

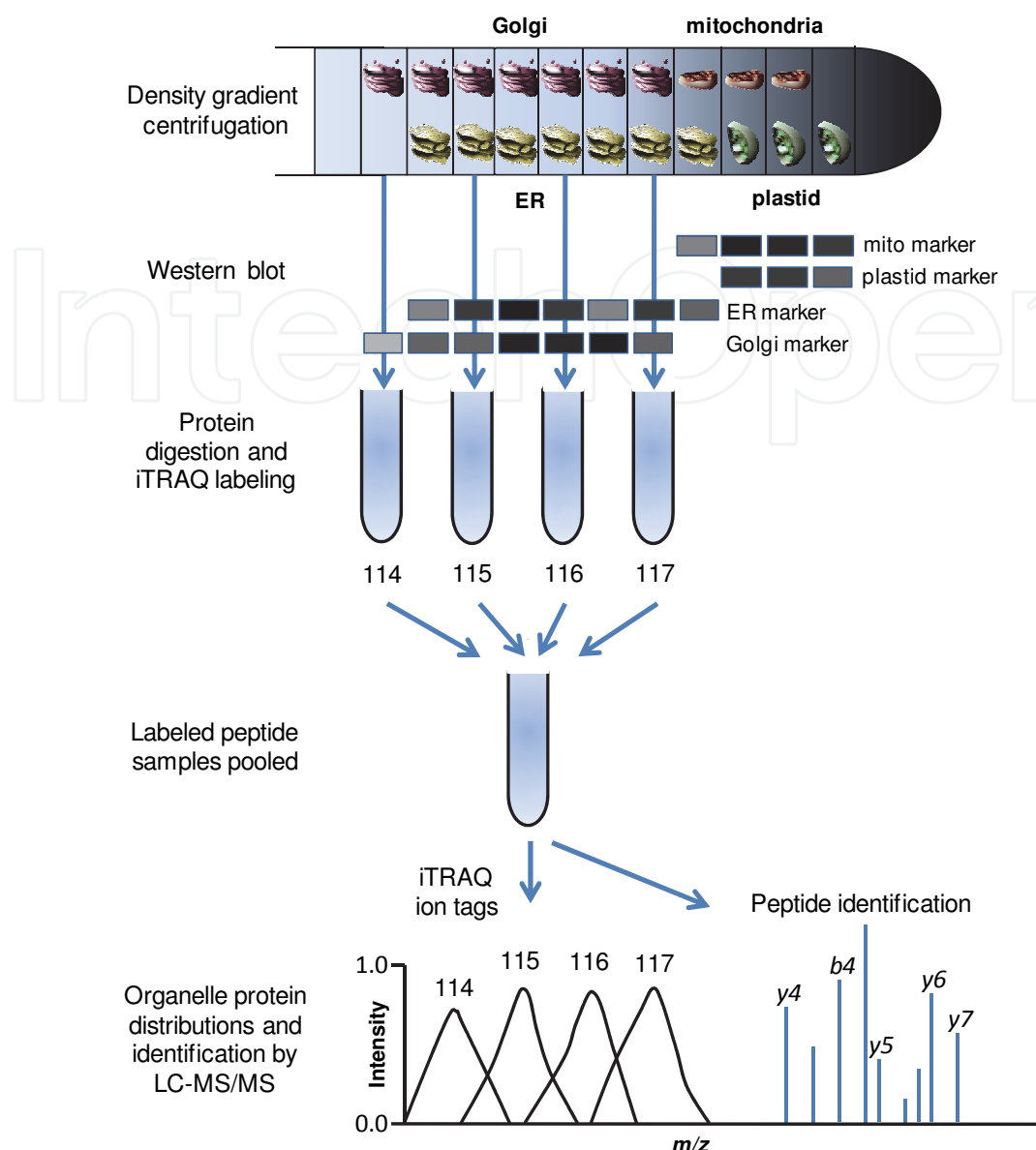


Fig. 4. Outline of the LOPIT technique using crude cellular extracts. LOPIT employs centrifugation of a self-forming iodixanol density gradient to partially resolve organelle fractions. Western blotting of the fractions for known Golgi and ER marker proteins show that in most cases, there is overlap between them. A series of four protein fractions are digested with trypsin and treated with iTRAQ reagents containing the labels 114, 115, 116 or 117 m/z and pooled for LC-MS/MS analysis. Ion intensity measurements of the iTRAQ reporter ion fragments 114 to 117 m/z providing the basis of protein quantitation with simultaneous analysis of the major b, y and other fragment ions for protein identification.

The introductions of LOPIT and related organelle purification-free methods were intended to address the issue of separating Golgi from other endomembrane system components, but this still remains rather difficult to achieve with complex biological systems. Refining these methods by optimizing density gradient conditions to enhance the resolution of Golgi, along with continuing development of multivariate techniques are seen as pivotal to expand the set of genuine Golgi-residing proteins in semi-purified samples (Foster et al., 2006; Trotter et al., 2010).



## 5. Free flow electrophoresis (FFE) purification of Golgi

Free Flow Electrophoresis, though 50 years old has adapted well to contemporary research fields, recently filling a particular niche in subcellular proteomics, in combination with mass spectrometry. This section explores the role of FFE in isolation of the Golgi apparatus from plant and mammalian tissues. Essentially, an electric field is applied perpendicular to a sample as it moves up a separation chamber in a liquid medium. Subcellular components are therefore separated according to surface charge and organelle streams collected as 96 fractions (Fig. 5). Hydrodynamic stability of the liquid is crucial; convection currents arising from localized joule heating can disrupt organelle streams. Apparatus design has consistently advanced along with the fields to which FFE has been applied. MicroFFE apparatus designs (Turgeon & Bowser, 2009) have overcome some of the imperfections inherent in the technique. Entirely liquid phase and continuous, FFE is appropriate for large scale, preparative fractionation of cells, organelles, proteins and peptides. The apparatus can be operated in two modes: zonal electrophoresis (ZE), or isoelectric focusing (IEF) mode. ZE-FFE is becoming recognized for its impressive separation and purification capacity of plant, mammalian and yeast organelles (reviewed by Islinger et al., 2010).

The first use of FFE for Golgi was applied to mammalian Golgi membranes and lead to separation of sub-Golgi compartments, demonstrated by a series of enzyme assays (Hartelschenk et al., 1991). However, this was prior to the proteomic era and was never

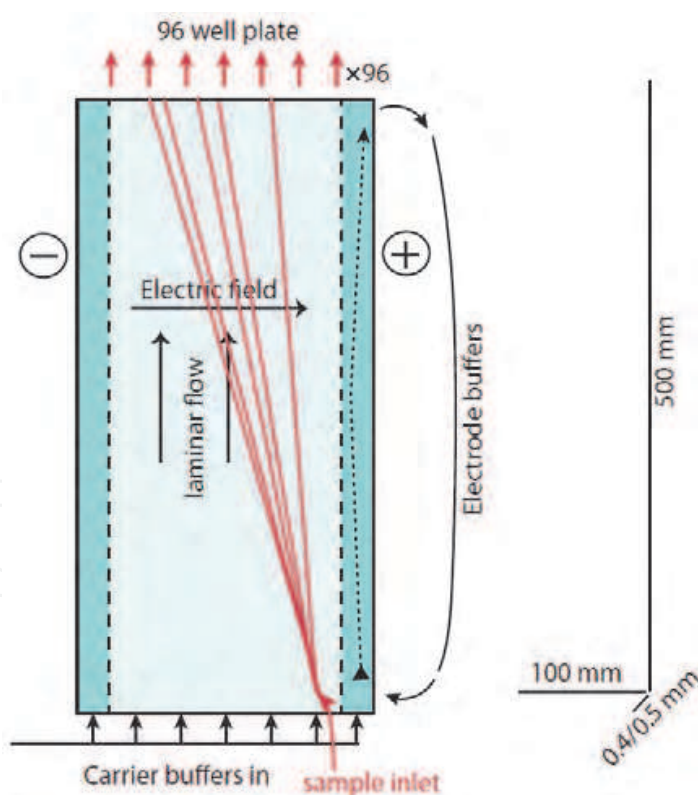


Fig. 5. A schematic diagram of a large scale FFE setup with dimensions shown on the right. The diagram outlines a late commercially model available through BD Diagnostics, with counter flow at sample outlets and stabilization buffers at the extreme anodic and cathodic carrier buffer inlets (Islinger et al., 2010). MicroFFE apparatus are similar with 56.5 mm × 35 mm × 30 mm dimensions (Turgeon & Bowser, 2009).

revisited with modern mass spectrometry tools. Plant homogenates were first subjected to FFE some decades ago (Kappler et al., 1986; Sandelius et al., 1986; Bardy et al., 1998) but these first forays demonstrated little potential for Golgi isolation. With plant Golgi antibodies then, as now, commercially unavailable, enzyme assays were the primary means of determining fraction composition. Profiling by enzyme assays was not sufficiently precise or efficient for tracking lower-abundance Golgi proteins amidst a relatively complex background of contaminants, although the distribution of enzyme activities reported by (Sandelius et al., 1986) are broadly consistent with later proteomic analyses.

The first isolation of plant Golgi membranes has depended on both FFE and proteomic advances (Parsons and Heazlewood, unpublished data). Semi-high throughput mass spectrometry was used to track the electrophoretic migration of Golgi membranes. The proteins identified in individual fractions were matched against markers protein lists for each subcellular location, including the cytosol, compiled from SUBA, the SUBcellular Arabidopsis database (Heazlewood et al., 2007). This allowed simultaneous monitoring of over 50 proteins in most fractions without recourse to antibodies or enzyme assays. Overlaid on the total protein output for all 96 fractions, marker lists revealed a detailed picture of organelle migration (Fig. 6). Once the shoulder peak corresponding to the purest Golgi fractions had been identified, parameters could be fine tuned, exploiting the electronegativity of Golgi vesicles and enhancing the cathodic migration of this area relative to the main protein peak. Total protein output from this targeted Golgi purification study showed a broader main protein peak and a prominent shoulder on the cathodic edge when compared to earlier studies on plant homogenates (Kappler et al., 1986; Bardy et al., 1998). Careful balancing of the carrier buffer flow rate to voltage ratio maximized the separation range of organelles whilst organelle streams remained focussed. Cathodic migration increased with voltage but was limited by increasing the flow rate as exposure time to the electric field was shorter. Lateral diffusion of organelle streams dictated the lower flow rate

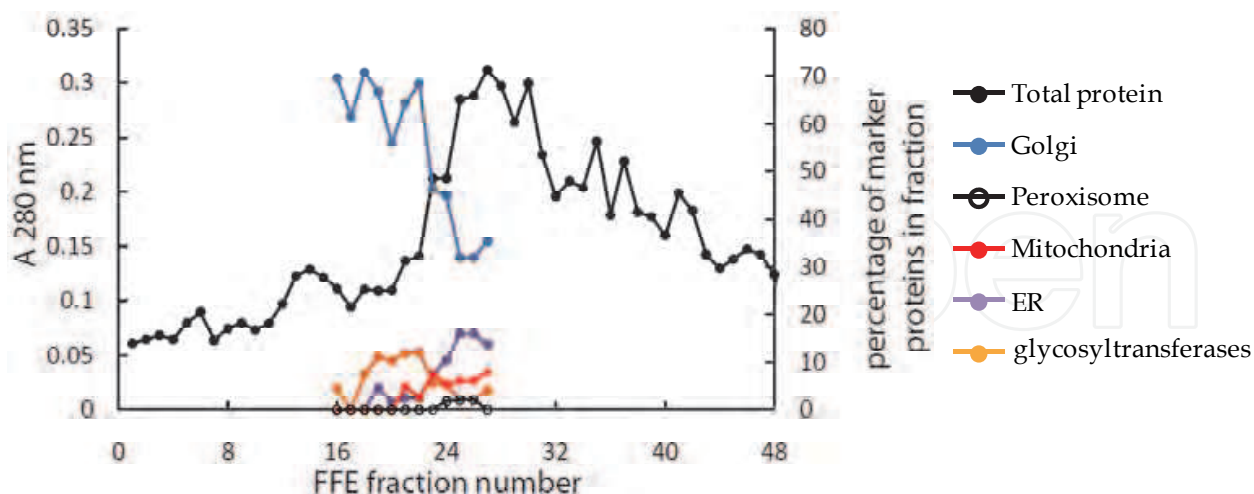


Fig. 6. Golgi membrane migration profile after FFE separation. A portion of the total protein output, measured at 280 nm (fractions 1 to 48) is shown. Around 50 proteins were identified in each fraction scanned using semi-high throughput LC-MS/MS. Overlaid are matches from marker protein lists compiled from the SUBA subcellular database and the ~50 identified proteins from each fraction. Many glycosyltransferases are located in the Golgi and were used as a further guide for Golgi membrane migration.

limit. Golgi fractions with minimal contamination were identified through continued monitoring and selected for detailed proteomic characterization (Parsons and Heazlewood, unpublished data).

The application of FFE, mass spectrometry and proteomic data as tools for Golgi isolation and characterization marked a precedent for plant Golgi proteomics. Previously, relatively few plant Golgi proteins had been identified by proteomic techniques (Dunkley et al., 2006). The application of FFE to isolate high purity Golgi fractions resulted in a Golgi proteome of 425 proteins identified in at least two of three biological replicates. This included over 50 glycosyltransferases, 25 transporters, the entire V-ATPase complex, a variety of trafficking components, methyltransferases and acetyltransferases (Parsons and Heazlewood, unpublished data). While proteins identified in a single preparation were excluded from the final proteome, they nevertheless present a useful resource for functional analysis of the plant Golgi apparatus. With so little Golgi proteomic data resources, common contaminants originating from the Golgi in other proteomes were difficult to identify. This therefore represents both significant progress in our potential to understand Golgi processes and consolidation of the current state of subcellular protein localization in plants. As an example the ectoapyrase protein APY1 is currently classified as a plasma membrane protein involved in extracellular signaling through the hydrolysis of phosphate from ATP (Wu et al., 2007). The APY1 protein was identified in all three replicates and YFP tagging confirmed its Golgi localization. Heterologous expression of this protein in the yeast nucleoside diphosphatase (NDPase) mutant *gda1*, rescued the glycosylation phenotype in this mutant, thus functionally characterizing the APY1 protein as a Golgi-resident NDPase (Parsons and Heazlewood, unpublished data). Since most glycosylation occurs in the Golgi, the APY1 protein represents a resident and functional Golgi protein, rather than a transitory plasma membrane localized protein. Furthermore, plasma membrane and Golgi compartments are easily separated using FFE (Bardy et al., 1998) with Golgi and ER compartments partially separated (Fig. 6). Thus, selectively pre-enriching organelles and tailoring FFE parameters for maximal separation has considerable potential in distinguishing between resident and transitory proteins in the secretory system. Some proteins observed after FFE purification of the plasma membrane were present in all three Golgi preparations and can be readily classified as 'transient proteins' rather than contaminants (Parsons and Heazlewood, unpublished data).

Given the success achieving high purity fractions (Taylor et al., 1997a) and sub-compartmental resolution of Golgi structures (Hartelschenk et al., 1991), it is surprising that a corresponding proteomic study has not been undertaken in rats. FFE was foremost amongst techniques compared for purification of mouse mitochondria (Hartwig et al., 2009) whilst impressive results were achieved after separating populations of PM vesicles (Cutillas et al., 2005), suggests that FFE still has much to contribute to both Golgi and other subcellular proteomes. In Arabidopsis, the Golgi proteome was characterized from only two to three fractions out of approximately 15 fractions over which Golgi proteins were detected. Further studies suggested this reflects medial to *trans*-Golgi separation (Parsons and Heazlewood, unpublished data). Could FFE separate the remainder of the Golgi from contaminating membranes or even Golgi sub-compartment? Chemical modification of Golgi compartments holds some promise; addition of ATP was found to enhance migration of membrane compartments towards the cathode (Barkla et al., 2007). Unfortunately no mass spectrometry was undertaken in this study. A low ionic strength two-component buffer system permits separation at lower currents, reducing convection from joule heating,

as could the use of microFFE setups, enhancing sub-compartment separation. FFE has already enhanced our knowledge of Golgi proteomics but its role is clearly far from over and there is much potential for further advances using FFE.

## 6. Comparative analysis of the Golgi proteomes

The characterization of the Golgi apparatus and associated secretory components by mass spectrometry has been undertaken on a range of species. While most of these organisms represent model systems with extensive genetic resources and well annotated genomes, analyses have been undertaken in less tractable systems, namely pine trees (Mast et al., 2010). Nonetheless, with the exception of work undertaken in rat, only a handful of analyses have focused on the proteomic characterization of the Golgi and its associated membranes from model systems. This is in contrast to the extensive series of proteomic studies undertaken on organelles from many of these systems. For example, in the model plant *Arabidopsis* over ten separate proteomic analyses have been undertaken on plasma membrane fractions, six studies on mitochondria and eight analyses of the plastid (Heazlewood et al., 2007). These facts further highlight the technical challenges when attempting to isolate high purity Golgi fractions and associated structures, even from well studied model systems. Overall, searches of the literature were able to identify over twenty separate studies that have employed proteomics techniques to address the characterization of the Golgi apparatus and associated secretory components. These studies have been undertaken using a diverse collection of isolation and enrichment techniques over the past decade and have employed a range of proteomics approaches including 2-DE (Taylor et al., 1997b; Morciano et al., 2005), 1-DE (Peng et al., 2008), iTRAQ (Dunkley et al., 2006), spectral counting (Foster et al., 2006) and MudPIT (Wu et al., 2004). These studies also covered the range of protein identification methods namely Peptide Mass Fingerprinting (Morciano et al., 2005), Edman degradation (Bell et al., 2001) and MS/MS (Gilchrist et al., 2006).

The protein identifications outlined in these works were extracted from the published manuscripts and online supplementary material to produce a collection of proteins identified in each study. Protein sequences were obtained from GenBank or UniProt for each accession and consolidated at the species level using BLAST analysis tool against minimally redundant protein sets where available. These comprised the International Protein Index (Kersey et al., 2004) for human, mouse, rat and bovine, The *Arabidopsis* Information Resource (Swarbreck et al., 2008) for *Arabidopsis*, the *Saccharomyces* Genome Database (Cherry et al., 1997) for yeast, FlyBase (Tweedie et al., 2009) for *Drosophila* and the Rice Genome Annotation Project (Ouyang et al., 2007) for rice. This enabled the classification of the total number of proteins identified from the Golgi apparatus and associated membranes based on each isolation method and by each species (Table 1). Finally, the total number of non-redundant proteins currently assigned to the Golgi apparatus and associated membrane components for each species could also be ascertained (Table 1). Where possible, we relied on annotation information and classifications outlined in each manuscript to determine whether a protein should be included in the final lists. This included early endosome, secretory and unknowns (when efforts to classify contaminants had been undertaken). The largest number of proteins assigned to the Golgi of any one species is that of rat. This reflects the number of individual studies and the fact that this represented the major system used to study the Golgi proteome for a number of years.



Species	Density centrifugation	Immuno-affinity	Free Flow Electrophoresis	Correlation Analysis	Total
Pine	10				10
Human		24			18
Rice	49				43
Drosophila				168	168
Bovine	252				238
Yeast	241	52			276
Mouse	2711 <sup>a</sup>	56		490	428
Arabidopsis		145	425	92	534
Rat	1117	57			996

Table 1. The total number of proteins, by species and technique, currently identified by proteomic approaches from the Golgi apparatus and associated membrane systems. <sup>a</sup>The analysis of mouse microsomes by density centrifugation (Kislinger et al., 2006) has not been included in the final total for this species as it represents a crude microsomal fraction.

The set of non-redundant protein sequences compiled from the proteomic analyses of the Golgi were assembled for cross species orthology analysis. In order to remove identical genes and splice variants, these sequences were first clustered at 95% sequence identity and only one representative from each cluster carried over for subsequent analysis. Following this, the sequences were clustered at 30% identity. All clustering was performed with the program uCLUST (Edgar, 2010). A protein was mapped to an ortholog of another species if at least one representative of that species was present in the same cluster. Proteins were considered paralogs when two or more sequences from the same species were present in a cluster in which sequences from no other species were present (Fig. 7)

After homology matching, a number of gene families were found across the Golgi proteomes of most species. These included Rab GTPases, heat-shock proteins, alpha-mannosidases, thioredoxins, and cyclophilins. Apart from the Rab GTPases, which mediate vesicle trafficking, the other families are involved in protein folding and protein glycosylation. There were a number of large clusters containing only Arabidopsis genes and these clusters were contained glycosyltransferases associated with synthesis of the plant cell wall (Scheller & Ulvskov, 2010). In addition, there was a cluster of pine sequences containing laccases, which may be associated with the synthesis of lignin in woody tissue (Ranocha et al., 2002). In general, when only a few proteins had been reported in a species, those proteins were more likely to have orthologs in the other species in the set. This suggests that the most easily detected proteins in proteomics studies are abundant proteins involved in core Golgi-related functions that have not diverged as greatly over evolutionary history as the less abundant and harder to find proteins.

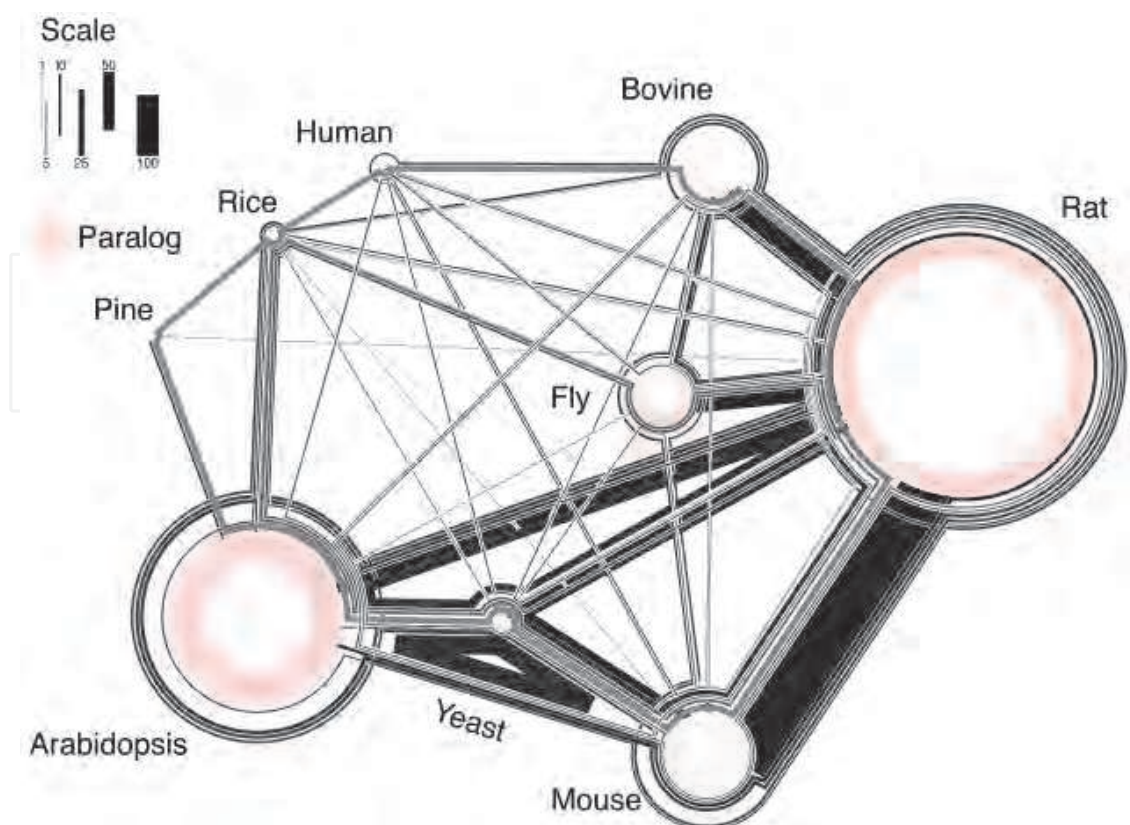


Fig. 7. Orthology interaction map of the non-redundant Golgi proteome sets. The size of the species circle indicates the number of proteins identified in the Golgi proteome of that species. The pink shading indicates the number of paralogs for a given species. The lines indicate orthology connections between the species with the thickness indicating the number of proteins. The Scale refers to the number of proteins represented by the thickness of the line.

## 7. Conclusion

The characterization of the Golgi proteome from various systems represents an important technical and biological achievement. Its central role within the cell in functions ranging from cell wall biosynthesis to protein glycosylation to secretion is of significant importance. Knowledge about these functions contributes to both our fundamental understanding of complex eukaryotic systems to their exploitation in areas of biofuels (cell wall manipulation) and agriculture (milk production). While there is clearly more basic knowledge required to understand the functionally complex roles of the Golgi apparatus, advances made by work outlined in this chapter demonstrate that the first decade of proteomics has been fruitful and improvements to isolation and analysis methods are promising for the field going forward.

## 8. Acknowledgment

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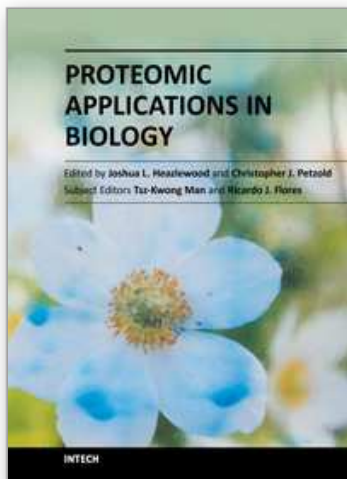
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The past decade has seen the field of proteomics expand from a highly technical endeavor to a widely utilized technique. The objective of this book is to highlight the ways in which proteomics is currently being employed to address issues in the biological sciences. Although there have been significant advances in techniques involving the utilization of proteomics in biology, fundamental approaches involving basic sample visualization and protein identification still represent the principle techniques used by the vast majority of researchers to solve problems in biology. The work presented in this book extends from overviews of proteomics in specific biological subject areas to novel studies that have employed a proteomics-based approach. Collectively they demonstrate the power of established and developing proteomic techniques to characterize complex biological systems.

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