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Strategies for organelle targeting of fluorescent probes

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Fluorescent tools have emerged as an important tool for studying the distinct chemical microenvironments of organelles, due to their high specificity and ability to be used in non-destructive, live cellular studies. These tools fall largely in two categories: exogenous fluorescent dyes, or endogenous labels such as genetically encoded fluorescent proteins. In both cases, the probe must be targeted to the organelle of interest. To date, many organelle-targeted fluorescent tools have been reported and used to uncover new information about processes that underpin health and disease. However, the majority of these tools only apply a handful of targeting groups, and less-studied organelles have few robust targeting strategies. While the development of new, robust strategies is difficult, it is essential to develop such strategies to allow for the development of new tools and broadening the effective study of organelles. This review aims to provide a comprehensive overview of the major targeting strategies for both endogenous and exogenous fluorescent cargo, outlining the specific challenges for targeting each organelle type and as well as new developments in the field.

Targeting subcellular structures

The first life forms were primitive, single-celled organisms with a phospholipid membrane that enclosed vital molecules. Over time, cells evolved to carry out more complex functions and even work together. They began to secrete chemicals, communicate with nearby cells, gain energy from their surroundings, and sense environmental stimuli. To carry out these functions with greater efficiency, the cell interior became compartmentalised into different types of membrane bound organelles and subcellular structures.[28] Each organelle deals with specific tasks and contains different chemical contents (Figure 1). Most importantly, biologically significant chemical reactions occur in isolation within organelles, ensuring that higher concentrations of reactants and greater reaction efficiencies can be achieved.[31] Furthermore, organelle membranes enable electrochemical gradients to be established, and the storage of potential energy in this manner drives many active cellular processes.[34]

The numerous organelles within human cells add greatly to their complexity. Best studied are the nucleus - the site of DNA storage, and mitochondria - the centres of energy production. Other key organelles are responsible for specific roles and functions; these include cellular digestion performed by lysosomes and related vesicles, energy storage in lipid droplets (LDs), oxidation reactions performed in in the peroxisomes, the

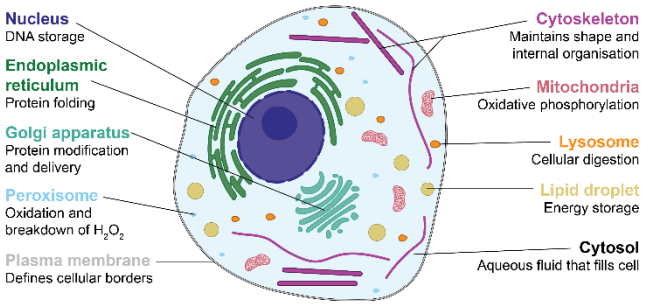

Figure 1. Schematic representation of a cell showing function of key cellular organelles and substructures.

synthesis and glycosylation of proteins within the endoplasmic reticulum (ER) and the further post translational modification and sorting of proteins in the Golgi apparatus. In addition to membrane-bound organelles, the plasma membrane and cytoskeleton also govern key cellular functions, such as nutrient uptake and motility.

Since organelles are specialised in their function, the chemical environments within each organelle (*e.g.* oxidation state, pH, and cation concentrations) must be kept at optimal levels, and protein catalysts and metabolites must be transported to the correct organelle. When a cell becomes abnormal or diseased, the chemical composition inside an organelle can change and disrupt its function, affecting overall cell health.[35] Therefore, it is vital to consider these diverse microenvironments within organelles when studying physiology and pathology.

Fluorescent tools to study organelles

The techniques used to study organelles can be either classified as destructive methods applied to study fixed cells or isolated

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organelles, or non-destructive techniques applied to monitor live cells. Destructive methods such as cell fractionation and organelle isolation often involve cell lysis to separate components, whilst fixation protocols can compromise membrane integrity. These destructive methods inherently alter the chemical contents within organelles and can potentially produce artefacts in resulting analysis. Non-destructive methods are less invasive and enable study of live cells with intact organelles. In recent times, fluorescence imaging has emerged as a powerful tool for studying the environment of live cells, due to excellent spatial and temporal resolution.[36] It has emerged as one of the most popular non-destructive methods to study organelles, enabling the visualisation of biomolecules within subcellular structures. Fluorescence imaging describes the array of techniques that involve measurement of a fluorescence signal emanating from a biological system. The most commonly-used instruments are fluorescence or confocal microscopes, but imaging plate-readers and flow cytometry set-ups are also popular tools for studying live cells.

Fluorescence imaging generally requires the application of fluorophores to the cell: in the context of organelle studies, these fluorophores must be targeted to the organelle of interest. In general, fluorescent dyes can be divided into two classes: stains and sensors. Fluorescent stains are markers for specific subcellular locations or species, with a fluorescence output that is generally constant. In contrast, fluorescent sensors are able to report on an analyte or chemical environment of interest, with a fluorescence output (whether emission intensity or wavelength) that is varied in the presence of this analyte.[37] Fluorescent stains and sensors may be based on one of a number of fluorescent scaffolds. Endogenous labels take the form of genetically-encoded fluorescent proteins such as the green fluorescent protein (GFP) and its analogues and derivatives.[38] The cell can also be stained with exogenously-added systems based on small molecule organic fluorophores, fluorescent metal complexes or fluorescent nanoparticles.

An advantage of fluorescence imaging is its high spatial resolution, sufficient for studying chemical species and processes within individual organelles. However, in order to gain specific information about organelle environments, it is crucial that fluorescent stains and sensors be targeted to the organelle of interest. To date, many organelle-targeted fluorescent stains and sensors have been reported, and used to uncover new information about various processes that underpin health and disease. In order to move the field forward, it is important to identify robust strategies by which stains and sensors can be reliably targeted to each sub-cellular organelle. While this review focusses on the targeting of fluorescent cargo, the strategies described here are equally applicable to other classes of cargo, such as drug molecules.

The need for diverse targeting strategies

Our ability to observe and manipulate the biochemical processes that occur within live organelles has advanced in conjunction with the discovery of innovative organelle targeting strategies. Some notable advancements include the discovery of green fluorescent protein (GFP) in 1962, which was applied for protein labelling soon afterwards.[39] For the specific labelling of organelles, peptidic organelle targeting groups were incorporated onto genetically modified proteins. A handful of these short peptidic targeting groups were subsequently used for the targeting of small synthetic cargoes. The discovery of synthetic, small-molecule targeting groups such as triphenylphosphonium (TPP) further enabled the subcellular location of synthetic cargoes to be controlled.[1, 40] The sub-cellular targeting of synthetic cargo has wide-reaching implications: not only do targeted chemical sensors enable the environment within organelles to be analysed, but the targeting of drugs and bioactive species has enabled organelle function to be manipulated. More recently, targeting systems have diversified, enabling the conjugation of genetically encoded proteins with a variety of synthetic molecules including dyes, florescent sensors and synthetic targeting groups. This has enabled the mapping of protein location *via* conjugation of dyes, the analysis of microenvironments within organelles *via* the conjugation of probes to targeted proteins, and control of protein localisation and cell signalling *via* the conjugation of self-localising ligands.[41-43]

While numerous organelle targeted tools have been published in the literature, the majority of these tools apply only a handful of targeting groups. Developing new, robust organelle targeting strategies is difficult, which is confounded as cargoes themselves can vary greatly in their physical and chemical properties. Despite this, it is essential to develop robust organelle targeting strategies as fluorescent tools themselves are becoming more diverse, encompassing structures such as quantum dots, fluorescent proteins, nanoparticles, and small molecules. While organelle targeting is often not straight forward, it is advantageous to have diverse targeting strategies on hand to match the diverse range of cargoes. Successfully targeting new classes of cargo can involve some trial and error, and there is still much to be understood about optimising this process.

The general approaches to targeting differs based on whether the cargo is synthesised endogenously or exogenously. Endogenous cargo such as genetically engineered fluorescent proteins (FPs) can be localised by fusion of the FP to a signal peptide or a protein of interest in the target organelle. On the other hand, targeting groups for exogenous cargo (small molecules or nanoparticle-based fluorescent systems) are generally synthetic chemical moieties that can be appended onto the fluorophore, allowing localisation to the target organelle via passive or active transport mechanisms. These chemical groups may be small molecules themselves, or may be short peptide sequences.

This review aims to provide a comprehensive overview of the major organelle targeting strategies for both endogenous and exogenous fluorescent cargo. Due to advances in protein bio-conjugation, both protein and synthetic targeting groups are accessible to biologists and chemists alike, thus this review aims to cover both. We outline the specific challenges for targeting of each organelle type, and the various strategies that

have been employed to date, giving key examples. Ultimately, the application of diverse targeting strategies may achieve the final goal of broadening the availability of tools for the study of organelles, especially those that are less commonly investigated.

Targeting strategies for the nucleus

The nucleus is often called the "control centre" of the cell because it contains most of the genetic information. While virtually all cells contain the same DNA, different cells make different proteins because they exhibit varied gene expression. The synthesised proteins then act as enzymes, structural components, and signalling molecules that determine the function of the cell. Because of its central role in health and disease, the nucleus is a widely studied organelle, and many therapeutic agents target the nucleus. However, it is also one of the most difficult organelles to target because there are many mechanisms in place to prevent potential mutagens from reaching the DNA.[44] In order for molecules to enter the nucleus, they must pass through the nuclear envelope, a double membrane that surrounds the nucleus and connects to the endoplasmic reticulum. Small molecules and proteins diffuse freely into the nucleus through the nuclear pore complex (NPC), a large 30 nm-wide protein channel spanning across the inner and outer nuclear membrane.[45, 46] Larger proteins must be actively carried into the nucleus.

Targeting of endogenous cargo

Nuclear proteins of up to 40 kDa are made and assembled in the cytoplasm, and then imported through the nuclear pore complex in a fully folded state.[47] Nuclear proteins are distinguished from other proteins because they carry a short peptide identification tag called a nuclear localisation sequence (NLS). The NLS is usually rich in lysine and arginine. Most commonly, four to six basic amino acids occur in tandem to form a "monopartite" NLS $(Arg/Lys)_{4\text{-}6}$. In some cases, the basic residues are separated by other amino acids, forming a "bipartite" NLS with the sequence $(Arg/Lys)_2\text{-}X_{10\text{-}12}(Arg/Lys)_3$, where X can be any amino acid. There are also some nuclear localisation sequences that do not contain basic amino acids, but these are less common.[48]

Due to their small size, small FPs like GFP can to some extent translocate to the nucleus through the nuclear membrane pore. The attachment of an NLS to the FP can ensure selective nuclear uptake; this can also be accomplished by tagging with nuclear proteins such as histone 2B (see Table S1). It has also been demonstrated that endogenous proteins can be translocated after the addition of an exogenous self-labelled ligand. For example, Ishida *et al.* demonstrated that eDHFR-GFP was efficiently localised to the nucleus from the cytoplasm after subsequent binding to a Hoechst labelled trimethoprim (hoeTMP).[43]

Targeting of exogenous cargo

Some small molecules have been successfully targeted to the nucleus by attaching a nuclear localisation sequence. For example, numerous fluorophores have been targeted to the nucleus *via* highly cationic NLS peptides. For example, pep-NP1 is a naphthalimide-based H_2O_2 sensor decorated with an NLS (Figure 2A).[2] However, the use of a hydrophilic charged peptide reduces cell uptake, requiring cell permeabilisation, microinjection or long incubation times; the latter strategy may lead to targeting of lysosomes.[49, 50]

Polyvalent cations have also been explored for targeting the nucleus, as their highly positive charge leads to their association with the negatively charged DNA backbone. Polyamines such as spermidine, spermine and putrescene are naturally occurring polyamines that have roles in cell division and DNA condensation, and a synthetic analogue, polyethymeneimine, has been used successfully to target fluorescein isothiocyanate (FITC) to the nucleus (Figure 2B).[3] Due to the highly cationic and hydrophilic nature of this targeting group, the cells were permeabilized with digitonin in order to allow uptake of the dye.

Alternatively, cargo can also be targeted to the nucleus by attachment to major and minor groove binders. For example, Hoechst dyes are a class of blue fluorescent minor groove binders that have been used to stain DNA and thus the nucleus (Figure 2C).[8] A number of fluorophores have been targeted to the nucleus by attachment to a Hoechst tag, such as HoeSR, which contains a sulforhodamine fluorophore (Figure 2D).[9] Given the size of the Hoechst tag, there is also interest in using truncated versions of the targeting group. For example, CQPP contains a coumarin fluorophore tethered to a cationic quinolinium unit as a Hoechst mimic (Figure 2E), although this probe showed lipid droplet as well as nuclear accumulation.[25] A disadvantage of using minor groove binders is that they can distort the structure of DNA.

Another less commonly used minor groove binder is pyrrole polyamide which was used to target fluorescein in F-DisT (Figure 2F).[26] These smaller hydrophobic molecules are useful as targeting groups due to their higher membrane permeability (Table S2).

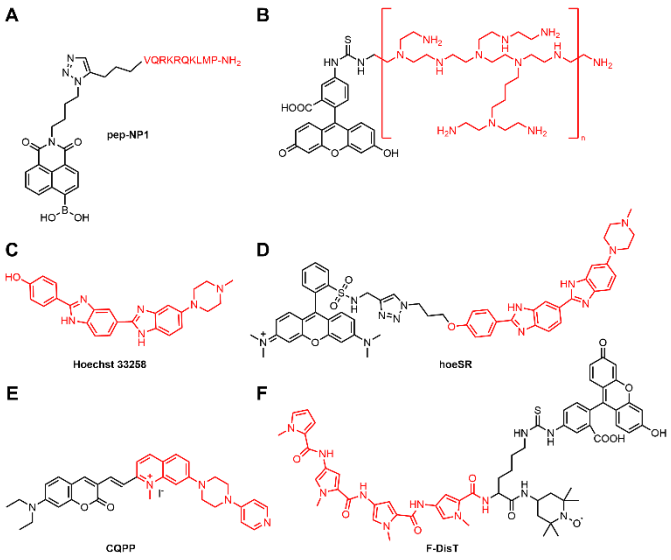

Figure 2. Structures of nuclear-targeted fluorophores, *with targeting group highlighted in red. (A) pep-NP1, the NLS50 sequence;[2] FITC tagged with polyethymeneimine;[3] (C) Hoechst 33258, DNA intercalator;[8] (D) Hoechst tag used in hoeSR;[9] (E) Hoechst mimic used in CQPP;[25] (F) polyamide polyamide used in F-DisT.[26]*

Targeting strategies for the mitochondria

The mitochondria are the primary sites of energy production within the cell, responsible for cellular respiration and oxidative phosphorylation. They have a double set of phospholipid bilayers, each with distinctive properties; this contributes to the compartmentalisation of the mitochondria.[51] The outer mitochondrial membrane has very little membrane potential as it is porous to small ions and molecules through pore-forming proteins (porins), but larger molecules and proteins must be imported by translocases.[52] The inner membrane space between the inner and outer membranes thus contains similar ion and small molecule content to the cytosol, but different protein composition. On the other hand, the inner mitochondrial membrane is not porous, and transport through this membrane to the mitochondrial matrix is highly regulated by transport proteins. The inner membrane is also the location of oxidative phosphorylation and ATP synthesis, mediated by an electron transport chain and series of proton pumps, resulting in an electrochemical potential across the inner mitochondrial membrane as high as –180 mV.[52] The surface area of the inner membrane is expanded through cristae, accounting for the ubiquitous wrinkled fold and allowing for increased activity. These cristae extend deeply into the inner mitochondrial matrix, containing several biologically significant enzymes, including those involved in the citric acid cycle and electron transport chain.[52] It is also the location of mitochondrial genetic material, which codes for proteins involved in the electron transport chain.[53]

Targeting of endogenous cargo

While the mitochondria possess their own genetic material, this only codes for 13 proteins involved in the electron transport chain.[53] Most other mitochondrial proteins are encoded in the nucleus and synthesised in the cytosol as precursor proteins and then post-translationally localised to the mitochondria.[54] An N-terminal cleavable pre-sequence is the most common type of targeting signal for these precursor proteins. While the lengths can vary, it is typically a sequence of 20-50 amino acid residues in length, containing alternating hydrophobic and basic residues that form an amphipathic helix.[55] This pre-sequence interacts with the machinery of the various mitochondrial import receptors and enables the precursor protein to be transported across both mitochondrial membranes and is cleaved by the mitochondrial processing peptidase or other proteases after import. Variations on these types of pre-sequences can be found for sorting to the different mitochondrial sub-compartments. Fusing the FP gene to a mitochondrial targeting sequence can thus achieve localisation. The most commonly used targeting sequences are those of the cytochrome *c* oxidase subunits, located in the mitochondrial matrix.[56] It is also possible to use software to improve prediction of mitochondrial targeting sequences.[57] Notable examples of mitochondrial targeting of endogenous cargo are summarised in Table S3.

Targeting of exogenous cargo

As the inner mitochondrial membrane is highly negative, cationic molecules are attracted to and accumulate preferentially in the mitochondria.[58] Most mitochondrially-targeted probes take advantage of this property, with delocalised lipophilic cations (DLCs) predominating the literature. To diffuse through the cell and mitochondrial membranes, the charge is delocalised over the relatively large surface area of the lipophilic cations. Some inherently fluorescent dyes are cationic lipophilic heterocycles that accumulate in the mitochondria, such as rhodamines (e.g. Rhodamine 123, Figure 3A),[59] rosamines (e.g. MitoTracker Orange and MitoTracker Red, Figure 3B,C) and cyanines (e.g. MitoTracker Deep Red, Figure 3D). In recent years, novel classes of cationic dyes have been developed to add to the range of imaging colours and to improve spectral properties such as brightness and photostability.[60]

For non-cationic fluorophores, it is possible to covalently attach a cationic moiety. While several DLCs have been reported for this purpose,[61] the vast majority of examples use TPP as the mitochondrial targeting moiety. Beyond synthetic ease, the advantages of TPP include its amphiphilicity, stability and non-reactivity in biological systems and lack of spectral overlap in the visible and near-infrared regions.[40] TPP is a robust targeting group that has been successful in delivering a variety of fluorescent cargo, including small molecule fluorophores, quantum dots and nanostructures. For example, TPP has been used to target the naphthalimides-flavin conjugate NpFR2[10], the BODIPY-based peroxynitrite sensor Mito-A2[62] and fluorescein conjugate mitoFluo[63] (Figure 5A-C).

However, DLCs are wholly dependent on the maintenance of negative mitochondrial membrane potential and does not ensure retention in the mitochondria. Additional anchors are needed for retention; for example, the chloromethyl moiety in the MitoTracker probes is thiol reactive and attaches covalently to proteins.[64] At high concentrations, cationic targeting groups can also result in depolarisation of the membrane potential or disruption of the electron transport chain.[65]

More recently, TPP derivatives have been shown to enhance aspects of mitochondrial targeting and uptake. For example, Hu *et al.* synthesised a range of methyl-functionalised TPP derivatives, showing that substitution on the phenyl ring can lead to stronger binding to the mitochondrial membrane, leading to increase in mitochondrial uptake (Figure 5D).[12] On the other hand, Kulkarni *et al.* investigated substituents at the *para*-position of the phenyl ring of TPP that affected the electron density on the phosphorus atom.[22] A CF_3 substituent was found to decrease electron density and alleviated uncoupling activity compared to the unsubstituted TPP moiety, preventing depolarisation of the membrane without affecting delivery of cargo (Figure 5E).

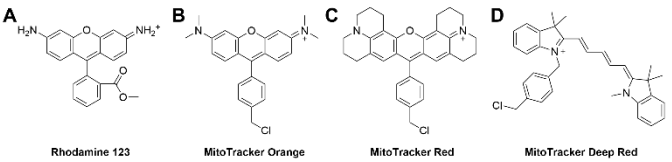

Figure 3. Structures of commercially available mitochondrial stains that are inherently fluorescent.(A) Rhodamine 123; (B), MitoTracker Orange, a rosamine; (C) MitoTracker Red, a rosamine; (D) MitoTracker Deep Red, a cyanine.

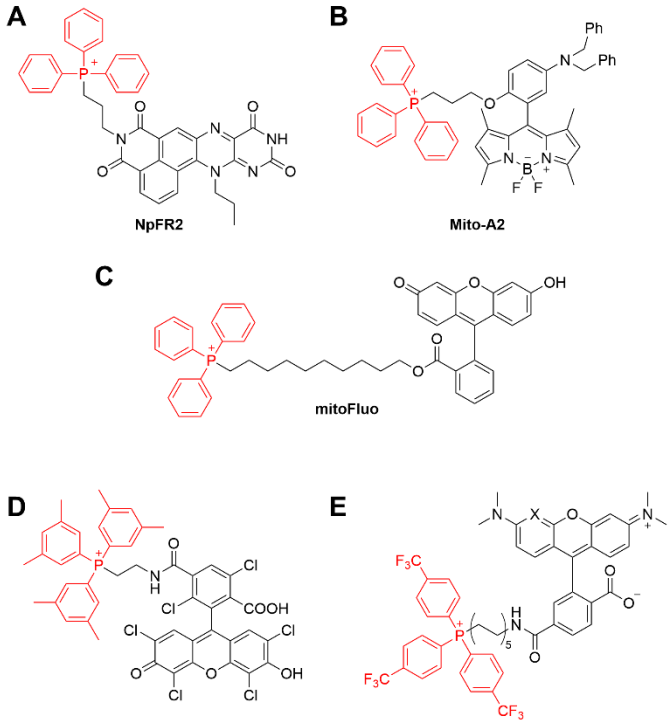

Figure 5. Structures of TPP- and functionalized TPP-derivatives that can be used as mitochondrial targeting groups, with targeting group highlighted in red. (A) TPP used to target a flavin derivative, NpFR2;[10] (B) methyl-functionalized TPP used to target hexachloro-fluorescein;[12] (C) CF_3 functionalized TPP used to target TAMRA.[22]

Synthetic mitochondria-targeted peptides (MPPs) present an alternative method for delivering cargo to the mitochondria.[21, 66, 67] These include Szeto-Schiller (SS) peptides, consisting of four alternating aromatic and cationic amino acids, known to penetrate the inner mitochondrial membrane.[13, 67, 68] For example, [aladan]SS-31 is a fluorescent analogue of an SS peptide, in which aladan, a polarity-sensitive fluorescent amino acid, replaces a phenylalanine (Figure 4A).[13] Other MPPs are also short peptide sequences (generally 4-8 amino acids), consisting of alternating cationic and hydrophobic residues, with the inclusion of D-arginine to prevent enzymatic cleavage.[21, 66] For example, the fluorophore thiazole orange has been successfully targeted to the mitochondria by conjugation to a range of tetrapeptides (Figure 4B).[21] A summary of key mitochondrial targeting strategies is provided in Table S4.

MPPs can be targeted to different mitochondrial sub-compartments, but the exact method of uptake has yet to be determined. Peptide bond formation allows the conjugation of a variety of cargo, particularly therapeutics. However, this is not a widely-used strategy for fluorescent probes and the successful delivery of fluorescent cargo is often used to validate the robustness of the MPP. Furthermore, most MPPs require long incubation times (> 1 h), precluding the adoption of this targeting group for sensors designed to detect analytes with short lifespans. However, conjugation with their original drug cargo may allow the formation of mitochondrially targeted theranostics.

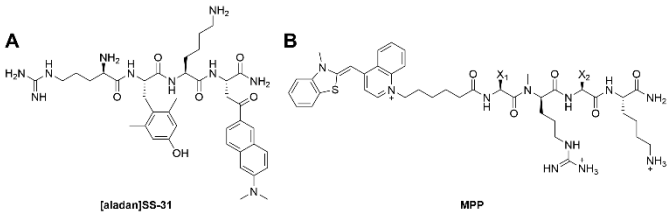

Figure 4. Structures of fluorescent analogs of MPPs.(A) [aladan]SS-31, a fluorescent analogue of SS peptide SS-31, D-Arg-Dmt-Lys-Aladan-NH_2, where DMT = 2',6'-dimethylTyr. Here, aladan is incorporated by substituting Phe.[13] (B) General structure of thiazole orange appended tetrapeptides, where X_1 and X_2 are hydrophobic amino acids.[21]

Targeting strategies for the lysosome

The lysosome is the central digestive organelle, with the most acidic environment amongst all organelles (pH 4-6)[69] and containing a variety of acid hydrolases that degrade biological macromolecules into low molecular weight biomolecules that can be released for use by the rest of the cell.[70] The lysosome is the primary centre of cellular catabolism and a centre for cell signalling, particularly in sensing nutrient availability. Lysosomes are highly heterogenous in distribution, number, size and morphology, adapting to nutrient availability and consumption; they are also heterogenous in luminal composition and pH.[71] These factors present difficulties in selectively targeting the lysosomes. For waste disposal, biomolecules come to the lysosome via either endocytosis or autophagy; both pathways involve a series of membrane-bound vesicles that can further complicate the selective targeting of organelles.

Targeting of endogenous cargo

Lysosomal proteins are generally categorised as either soluble acid hydrolases or transmembrane proteins.[72] Most hydrolases are localised to the lysosome *via* the mannose-6-phosphate pathway; after the initial synthesis of the precursor proteins in the cytoplasm, they are transported to the endoplasmic reticulum and then Golgi for processing, where a mannose-6-phosphate tag allows for recognition and trafficking to the lysosome. Transmembrane proteins are transported to endosomal/lysosomal compartments in a mannose-6-phosphate independent manner *via* short amino-acid based sorting signals. These are either tyrosine (YXXØ) or dileucine ([DE]XXXL[LI] or DXXLL) based motifs and are recognised by cytosolic adapter protein complexes or Golgi-localised, gamma adaptin ear-containing ARF binding (GGA) proteins.[72]

Fluorescent fusion proteins have been used to investigate the lysosome. The most common lysosomal markers include those in the lysosomal associated membrane protein LAMP class, especially LAMP1 and LAMP2, which account for 50% of all lysosomal membrane proteins (Table S5).[73] It is also possible to fuse FPs with lysosomal hydrolases,[74] but these may not be distributed evenly amongst all lysosomes due to varied nutrient availability. The main issue with using protein biomarkers for the lysosome is that other digestive vesicles may also be labelled as the proteins are transported through the fusion events leading to lysosome formation and maturation. Using

multiple markers attached to FPs of different colours (*e.g.* specific markers associated with only endosomes or autophagosomes) can help overcome this issue.[75, 76]

Targeting of exogenous cargo

Most small molecule lysosomal fluorescent sensors use a small, lipophilic tertiary amine as an anchor (Table S6). The major driver of uptake is through pH-partitioning.[77, 78] Molecules smaller than 1 kDa passively diffuse through the lysosomal membrane. Once within the lysosomal lumen, the amine becomes protonated by the acidic environment of the lysosome. The positively-charged species is then sequestered within the lumen, as charged species cannot pass through the organelle membrane (Figure 7). Accumulation of such species within the lysosome can be as high as several hundred-fold compared to the rest of the cell and incubation media.[78, 79] Most classes of neutral fluorophores have been successfully targeted to the lysosome *via* this strategy. The anchoring group varies, but the most commonly-used amines are morpholines (such as in the commercially available LysoSensor; Figure 6A[80]), dimethylamines (such as in the commercially available LysoTracker Red and Green; Figure 6B[80]) and diethylamines.[81] Less commonly-used amines include DAMP (*N*-(3-[(2,4-Dinitrophenyl)amino]propyl)-N-(3-aminopropyl)methylamine dihydrochloride)[82] and histamine (as in BODIPY FL histamine[80]).

A challenge with the use of lipophilic amines as targeting groups is that they are not inert substances. Their mechanism of action means that there will be some basification of the lysosomal environment, known as lysosomotropism.[77] Lysosomotropic agents are known to increase pH and alter the morphology and function of lysosomes; alkanisation can also lead to cell death. Thus, this type of probe may be unsuitable for long-term imaging. Another consideration is that lipophilic amines will stain all organelles of a certain acidic pH; thus, this strategy can also stain endosomes and may miss some less acidic lysosomes. The lipophilic amine must also be strategically placed, as it can to photoinduced electron transfer (PeT) based quenching in some fluorophores and pH environments.[83-85] While this property is desirable in pH probes, it must be controlled for in other sensors.

More recently, several novel lysosomal targeting groups have been reported, though most have not been robustly tested with a variety of fluorophores and their mechanisms of action are unknown. Considering the glycosylation in lysosomal membrane proteins, N-linked glycans have been reported as a lysosomal targeting group for rhodamine spirolactam derivatives (Figure 6C).[14, 86] Methylcarbitol was first reported by

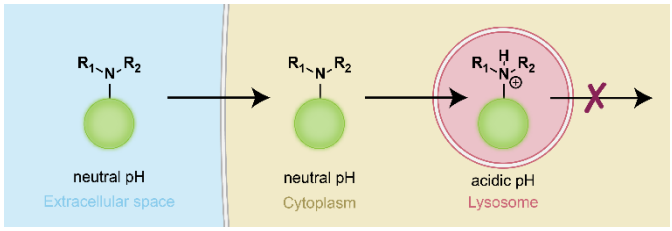

Figure 7. Mechanism of lysosomal uptake for lipophilic tertiary amines. At neutral pH, the probe is unprotonated and can freely diffuse through membranes, but once in the acidic lysosome, it is protonated and is effectively trapped.

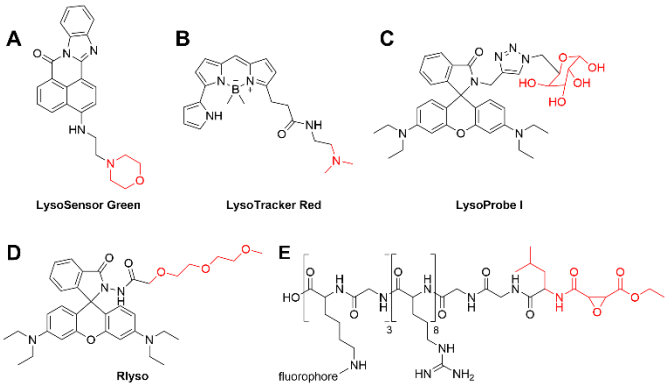

Figure 6. Structures of lysosomally-targeted fluorophores, with targeting group highlighted in red. (A) morphoine group used in LysoSensor Green; (B) dimethylamine used in LysoTracker Red; (C) N-linked glycans used in LysoProbe I, one of several rhodamine-based probes using this strategy;[14] (D) methylcarbitol used in Rlyso;[23] (E) general strategy used by Zhang and coworkers, with epoxysuccinyl group for covalent attachment to cysteine cathepsins.[24]

Peng and coworkers as a novel lysosome sensing group that does not appear to rely on pH partitioning and thus does not have an inherent lysosomotropic effect.[23, 87] While the uptake and retention mechanism of this targeting group remains unknown, it has been successfully used to achieve lysosomal targeting of rhodamine (Figure 6D)[23] and carbazole derivatives.[88]

Zhang and coworkers developed a lysosomal localisation strategy that involved the use of cysteine cathepsin proteases.[24, 89, 90] These are enzymes activated in low pH conditions and thus are almost exclusively found in the lysosome.[91] Their probes consisted of three main components: a fluorophore, a cell penetrating peptide (rRrRrRRR, r: D-Arg, R: L-Arg) to improve cell permeability and an epoxysuccinyl scaffold that could selectively form covalent bonds with cysteine cathepsins (Figure 6E). A variety of fluorescent small molecules were successfully delivered to the lysosome *via* this strategy, including Alexa Fluor 647, Atto 565 and Atto 488. It is also possible to selectively target specific cathepsins through the incorporation of a peptide specific for the active site of the protein.[92] However, these probes are generally designed to measure the activity of that specific cathepsin and are not suitable for general lysosomal targeting.

Large macromolecules cannot enter the cell *via* passive diffusion and must undergo endocytosis. Fluorescently labelled macromolecules, such as bovine serum albumin or dextran, can thus be used to track the progress of endocytosis in chase experiments. They have also been used as a measure of lysosomal localisation. Perhaps the best known of these is FITC-dextran,[93] though conjugates with many other fluorophores are commercially available. This technique is most useful for studying the rate and endpoints of endocytosis, but less useful for targeting specific organelles in the endocytic pathway.

Targeting strategies for endosomes

Endocytosis is the general term for the processes by which materials exogenous to the cell are internalised.[94] While there

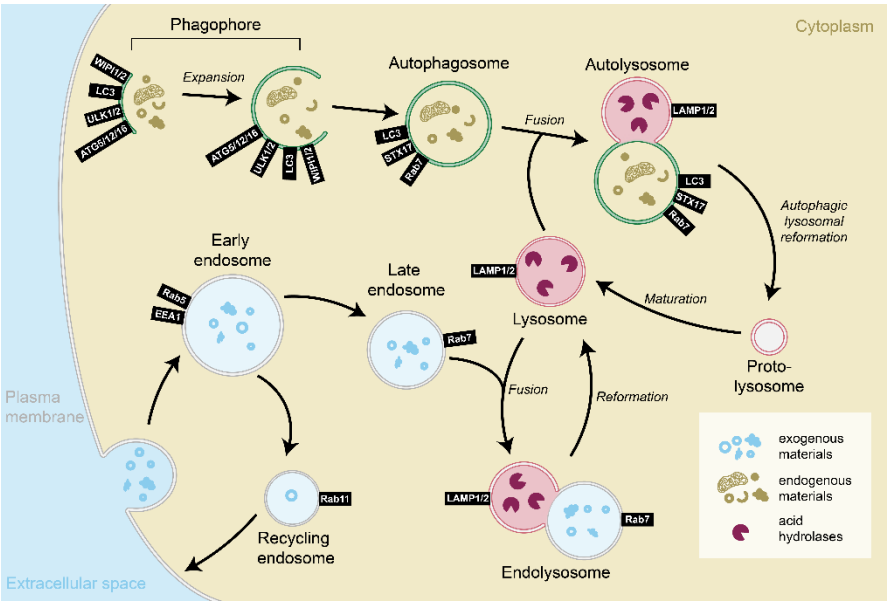

Figure 8. Schematic of the autophagic and endocytic pathways, and selected marker proteins for each compartment.

are substrate-specific endocytic pathways, most processes involve a section of the plasma membrane invaginating the foreign material. These sections bud into vesicles that then fuse with early endosomes. Here, some of the biomolecules are sorted for exocytosis, whilst others fuse or are transformed to late endosomes.[94] Late endosomes then fuse with the lysosome, completing the transfer of foreign biomolecules into the cell. Recycling endosomes are involved in trafficking cargo from early endosomes to the plasma membrane, or back to the Golgi apparatus.[95] Each type of endosome has a unique morphology and composition, including marker proteins (Figure 8). However, the characterisation of endosomal compartments remains challenging as endosomal compartments are fairly heterogenous and markers are not necessarily limited to one stage of the endocytic pathway.

Targeting of endogenous cargo

Rab GTPases are a class of proteins that are involved in the regulation of endocytosis and have been used extensively as endosomal markers with fluorescent fusion proteins (Table S7).[96, 97] Amongst this family, Rab5 functions as an early endosome marker, though it may also be found on the plasma membrane as it is involved in the fusion of the plasma membrane and early endosomes as well as vesicular trafficking.[96] Rab7 works downstream of Rab5, and is involved in trafficking to the late endosomes and lysosomes and is also involved in autophagy.[97, 98] Rab11 is associated with recycling endosomes. A range of other proteins can also be used as endosomal markers.[96] For early endosomes, EEA1 (early endosome antigen 1) is an effector of Rab5 and commonly used as a marker.[99] For recycling endosomes, transferrin receptor and Arf6 have also been used as markers.[100]

Targeting of exogenous cargo

Few robust small molecule targeting strategies exist for the endosomal pathway (Table S8). For early endosomes, Piazzolla *et al.* tested a range of weakly acidic benzyl substituted amines as potential targeting groups for fluorescent flipper probes (Figure 9A-B).[15] As for lysosomal targeting amines, they hypothesised that such probes would be retained in their cationic ammonium form. The study reported that cations with

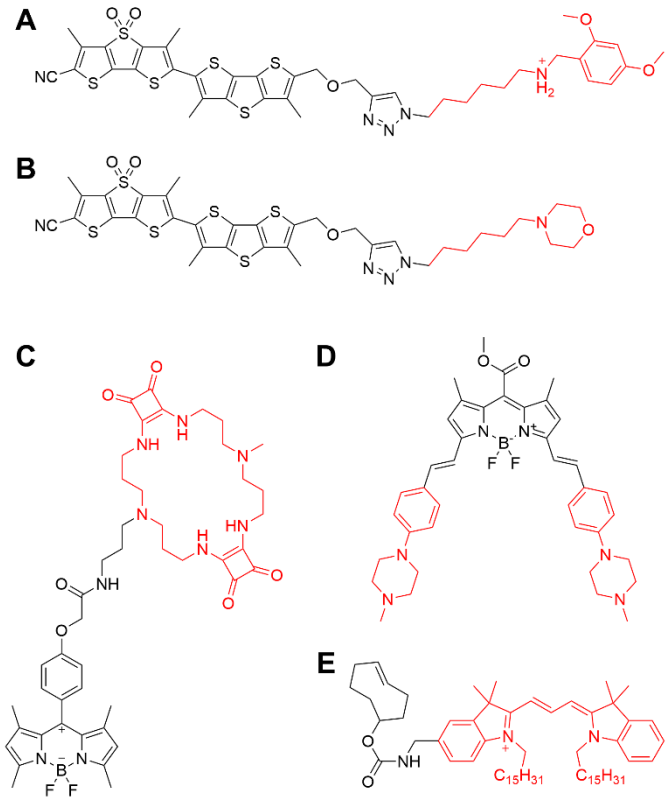

Figure 9. Structures of endosomally-targeted fluorophores, with targeting groups highlighted in red. (A) Weakly acidic benzyl substituted amines used to target early endosoems and (B) weakly acidic amines used to target late endosomes and lysosomes by Piazzolla et al.;[15] (C) macrocyclic di(cyclosquaramides) used by Sampedro et al. to target late endosomes;[16] (D) DiI_{16}(3) used in $DiIC_{16}$.TCO(1) that can later react with tetrazine reaction partner in late endosomes;[18] (E) (4-methyl-1-piperazinyl)phenyl-substituted BODIPY that can stain pH flux of late endosomes.[30]

higher pK_a values were retained in the less acidic early endosomes (Figure 9A, pK_a = 9.8) while those with lower pK_a values were retained in the more acidic late endosomes and lysosomes (Figure 9B, pK_a = 7.4).

Sampedro *et al.* developed a novel strategy for targeting late endosomes *via* macrocyclic di(cyclosquaramides).[16] These bind to phosphates on the outer cell membrane and are then internalised through the cell *via* receptor-mediated endocytosis. The authors demonstrated that this strategy could be applied to tagging both BODIPY (Figure 9C) and fluorescein.

Miao *et al.* used a modified version of the pH partitioning strategy – they synthesised a pH-responsive BODIPY based probe that could stain the change in pH from the late endosome to the lysosome (Figure 9D).[30] Variants of the fluorescent lipid membrane stain DiI with different hydrocarbon tail length were found to have different endocytic sorting; in particular, $DiIC_{16}(3)$, was found to be delivered to late endosomes (Figure 9E).[101] The late endosomal localisation of $DiIC_{16}(3)$ was used by Gupta *et al.* to develop analogues with a *trans*-cyclooctene moiety.[18] These could react *in cellulo* with a silicon-rhodamine dye with a tetrazine reaction partner. As the silicon-rhodamine dye is far more photostable than DiI, this also allowed for super-resolution imaging.

Targeting strategies for autophagic compartments

On the other hand, autophagy processes malfunctioning and obsolete endogenous material.[102] The major autophagic pathway, macroautophagy, involves the engulfing of target material by phagophores, vesicles containing hydrolytic enzymes.[102] This produces autophagosomes that later fuse with the lysosome, forming the autolysosome.[103] In some cases, autophagic lysosomal reformation provides a pathway for the generation of protolysosomes from autolysosomes, which can then mature into lysosomes (Figure 8).[103] While there are other autophagy processes, macroautophagy is the only pathway with a distinct set of intermediate vesicles, so the strategies below will discuss macroautophagy targeting only.

Targeting of endogenous cargo

Macroautophagy is associated with several autophagy-related (ATG) proteins, with each successive stage associated with different proteins.[104] Most ATG factors detach before or immediately after the membrane closes to become the autophagosome, limiting their utility as targeting vectors. A notable exception are the microtubule associated protein 1 light-chain 3 (LC3) and its homologs, which are retained in the enclosed autophagosomes.[104] The most common macroautophagosomal targeting of fluorescent proteins therefore involves LC3 (Table S9).[105] For example, a tandem fluorescent tagged analogue, mRFP-GFP-LC3, has been shown to be able to differentiate autophagosomes and autolysosomes due to the quenching of GFP in acidic environments.[106]

Another strategy for tagging macroautophagosomes is the use of syntaxin17 (STX17), which is recruited after membrane closure, such that mature autophagosomes contain STX17. STX17 fusion proteins have been reported with both GFP[107] and the Turquoise2 fluorescent protein.[108] It should be noted, however, that STX17 is not found exclusively in the autophagosomes so it cannot be used as a sole marker for them.

Targeting of exogenous cargo

The commercial dye Cyto-ID labels autophagic compartments and is known to be a cationic amphiphilic tracer.[109] However, no further information about mechanism or chemical structure is available, which could limit use. Iwashita *et al.* reported DALGreen and DAPgreen, with 1,8-naphthalimide as a fluorogenic scaffold (Figure 10A,B).[19] Both probes bear a terminal amino group in the 4-position, with DALGreen containing a piperazine moiety and DAPGreen an aminopentyl moiety. By comparison to analogous compounds, it was suggested that an amphiphilic, detergent like structure similar to intermembrane phospholipids allowed uptake in autophagic membranes. DAPgreen was found to be pH independent and stained all autophagic compartments. In contrast, DALGreen was found to stain autophagic flux, due to the pH sensitive piperazine providing fluorescent enhancement at acidic pH found in late-stage autophagy.

Similarly, other probes target chemical changes between different stages of autophagy, commonly for the transformation of the lysosome to the autolysosome (Table S10). These typically use traditional lysosomal targeting strategies and report a difference caused by autophagic flux. For example, Jiang *et al.* reported Lyso-OC (Figure 10C), a coumarin-based probe that could detect lysosomal polarity; autophagic flux could be detected as polarity changes after membrane fusion between lysosomes and autophagosomes.[29] pH fluctuation is another change that can be monitored after membrane fusion. Ning *et al.* reported Lyso-MPCB (Figure 10D), a ratiometric lysosomal pH sensor based on p-methoxyphenylacetylene-substituted carbazole that could monitor autophagic flux.[32]

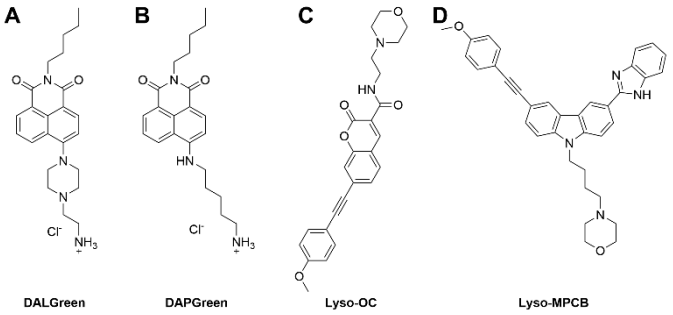

Figure 10. Structures of fluorescent probes targeted to autophagy-related compartments.(A) DALgreen and (B) DAPGreen contain an hydrophobic alkyl chain as imide substituent of both groups, with DALGreen containing an aminoethylpiperazine and DAPGreen an aminopentyl moiety in the 4-position.[19] (C) Lyso-OC[29] and (D) Lyso-MPCB[32] both contain lysosome-targeting morpholine, but are sensitive to changes in the flux from lysosome to autolysosome.

Targeting strategies for the peroxisomes

Many biological oxidation reactions occur in vesicles called peroxisomes, an organelle involved in many metabolic pathways. These include the oxidation and breakdown of fatty acids, as well as the metabolism of reactive oxygen and nitrogen species.[110] Hydrogen peroxide (H_2O_2) is a common by-product of cellular respiration, and high levels can cause toxicity.[111] To prevent accumulation of H2O2, peroxisomes contain large quantities of catalase, which catalyses the decomposition of H_2O_2.[112]

Targeting of endogenous cargo

Peroxisomal proteins such as catalase are imported after they have been fully translated and folded in the cytoplasm. Targeting is achieved by small peptide domains termed peroxisome targeting signals (PTS). Most peroxisomal proteins have a C-terminal PTS-1 tripeptide signal, of which the prototypic sequence is Ser-Lys-Leu (consensus sequence Ser/Cys/Ala-Lys/Arg/His-Leu).[113] A bipartite PTS-2 (consensus sequence Arg/Lys-Leu/Val/Ile-X_5-His/Gln-Leu/Ala) is used for a few peroxisomal matrix proteins and is usually found at the N-terminus, though it can also function internally. Alternatively, a less defined mPTS signal is used for membrane bound peroxisomal proteins.[114] Peroxisomal proteins are imported in a fully folded state, and it has been reported that the shape of the molecule does not affect import. Even proteins that bind to peroxisomal proteins can enter the peroxisome by a piggyback mechanism.[115] Fusion proteins can therefore be readily prepared, bearing either a PTS or a peroxisomal protein bound to a fluorescent protein. PTS-1 sequences Ser-Lys-Leu and Ala-Lys-Leu have been used to achieve peroxisomal targeting of GFP[116] as well as redox[94] and calcium-sensitive proteins (Table S11).[117, 118]

Targeting of exogenous cargo

The PTS peptides have proved sufficiently robust to transport not only proteins but also other molecules of all shapes and sizes to the peroxisomes (Table S12). For example, gold nanoparticles conjugated to the PTS-1 sequence Ala-Lys-Leu showed clear peroxisomal localisation.[119] Recently, fluorescein, BODIPY and a pH sensitive dye, SNAFL-2, have been delivered to peroxisomes using a PTS-1 tag (*Figure 11*).[119] To date, PTS-2 and m-PTS have not been a used as targeting groups for small molecules, and no non-peptide peroxisome targeting groups have yet been reported.

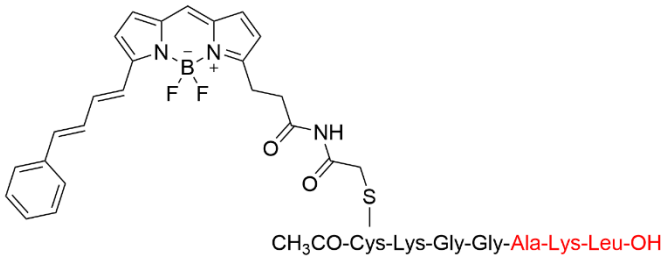

Figure 11. Structure of BODIPY tagged with C-terminal PTS-1 peptide.[1]

Targeting strategies for the endoplasmic reticulum

The endoplasmic reticulum (ER) is a large network of folded membrane found inside the cytoplasm. It links to the nuclear envelope, and buds off to form the Golgi apparatus and peroxisomes. Many membrane proteins and secreted proteins are made on the rough ER subunit, which contains bound ribosomes.[120] Ribosomes can either release newly made proteins into the cytosol or insert them into the rough ER, which is an oxidising environment that promotes disulfide bond formation and protein folding. In the lumen of the ER, proteins undergo post-translational modifications, like glycosylation or attachment of lipids. Cellular stressors such as oxidative stress can cause unfolding and aggregation of newly synthesised proteins. The unfolded protein response in the ER has therefore been widely studied.[121]

Targeting of endogenous cargo

Proteins that are targeted to the ER often have an *N*-terminal 30 amino-acid signal peptide, which is a hydrophobic alpha helix capped by a few basic residues.[122] As the signal peptide emerges from the ribosome, it is recognised by the signal receptor particle, which binds to the peptide and carries it to the translocon pore on the rough endoplasmic reticulum. The signal peptide then inserts into the membrane and the rest of the protein is pushed into the ER by the bound ribosome as it is being synthesised.[123] Some proteins can move from the ER to the Golgi apparatus since the Golgi apparatus is formed from vesicles that bud off from the ER. To prevent resident ER proteins from moving into the Golgi apparatus, they have a Lys-Asp-Glu-Leu retention signal. This signal is recognised by a receptor that is involved with retrograde transport from the Golgi to the endoplasmic reticulum.[124] It is possible to direct and retain FP cargo in the ER with a signal peptide and Lys-Asp-Glu-Leu retention sequence alone (Table S13).[125] The preprolactin signal sequence has been frequently used as the ER signal peptide.[126]

Targeting of exogenous cargo

It is challenging to mimic the natural processes that target proteins to the endoplasmic reticulum because signal sequences are long and bound ribosomes play a role. Despite this, there has been some success targeting the endoplasmic reticulum with the retention signal peptide alone (Table S14). For example, Pap and co-workers tagged a BODIPY with a seven amino acid peptide containing the Lys-Asp-Glu-Leu retention sequence, and observed clear ER localisation within 11 minutes of incubation[1] (Figure 12A). While this tagging strategy is synthetically accessible,[1] the generalisability of the approach needs to be further explored.

Alternatively, small molecules that bind tightly and specifically to endoplasmic reticulum proteins have proven to be feasible targeting groups. For example, glibenclamide-conjugated fluorophores are commercially available as endoplasmic reticulum tracking dyes, such as in ER-Tracker Red (Figure 12B). Glibenclamide is one of many sulfonylurea drugs that bind to the sulfonylurea receptor (SUR).[127] There are

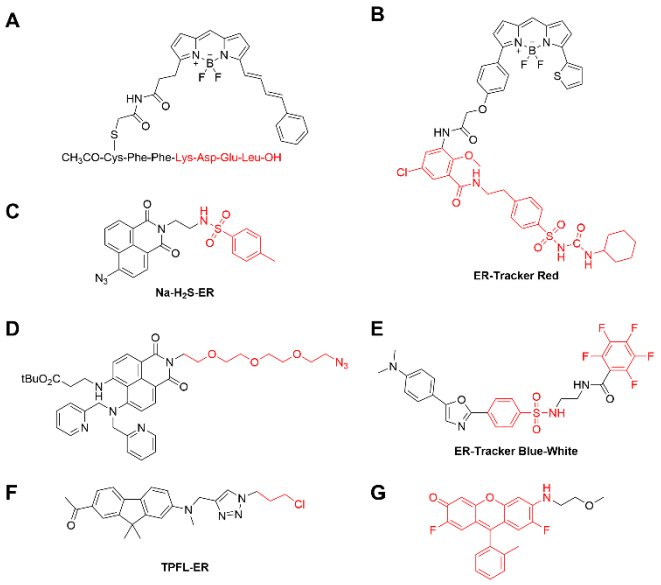

Figure 12. *Structures of ER-targeted fluorophores, with targeting group highlighted in red. (A) Pap et al. targeted a BODIPY with a Lys-Asp-Glu-Leu sequence;[1] (B) Glibenclamide is used by ER-Tracker Red, (C) Na-H_2S-ER[5] uses a sulfonamide. (C)*

different types of SURs that are expressed on various membranes depending on the type of the cell. In rat cardiomyocytes, SUR-2A and 2B were found in the mitochondria as well as the endoplasmic reticulum.[128] However in insulin secreting β-cells, SUR-2 and SUR-1are mainly found on the endoplasmic reticulum.[129]

Smaller sulfonamides have been recently used to target cargoes to the endoplasmic reticulum. For example, Na-H_2S-ER is a naphthalimide-based H_2S sensor that contains a methylsulfonamide targeting group (Figure 12C).[5] These are much more accessible compared to glibenclamide, but their discovery is relatively recent, and they have yet to be robustly tested with a range of fluorophores (Table S14).

The Kim group appended an ethylene glycol chain as an ER-targeted moiety, utilising the tendency of lipophilic or glycosylated compounds to localise in the ER.[130] They were able to successfully target a naphthalimide-based copper(II) sensor (Figure 12D). A similar strategy of long-chain lipophilic hydrocarbons was used by the same group to localise BODIPY-coumarin[131] and BODIPY-Nile Red[132] conjugates, allowing the detection of polarity and viscosity changes in the ER membrane.

Pentafluorophenyl (PFP) groups can react with thiols in ER proteins. A PFP moiety is present in the commercially available ER-Tracker Blue-White (Figure 12E), though this molecule also contains a sulfonamide.[80] Along with a long ethylene glycol linker, PFP has been used in ER membrane probes, including a Nile Red derivative[133] and mechanosensitive flipper molecule.[134] In some cases, PFP has been used without other targeting moieties,[135] but PFP has also been used to investigate other organelles, such as the activity of lipid droplet associated proteins.[136] Similarly, a propyl chloride moiety has been used to target fluorene[137] (Figure 12F) and Nile Red[133] derivatives to the ER, with the chlorine thought to bind to the chlorine pump in the ER.

Fluorinated variants of the fluorophore rhodol[138] have also been noted to localise in the ER and have been used in several probes (Figure 12G, Table S14); while no mechanism has been reported, hydrophobic amphipathic compounds are thought to preferentially associate with the cholesterol-poor ER membrane.

Another potential method for targeting small molecules to the endoplasmic reticulum is by targeting membranes, since the endoplasmic reticulum is the most extensive network of folded membrane in the cell. Targeting of the ER membrane has been achieved using lipids such as ceramide and hydrophobic dyes,[139, 140] but selectivity is more difficult to achieve as the Golgi apparatus and other membranes also tend to be stained.

Targeting strategies for the Golgi apparatus

The Golgi apparatus is found in all eukaryotic cells. It modifies proteins received from the rough ER and then sorts them into vesicles for delivery to other parts of the cell or for secretion. In the Golgi apparatus, proteins can be modified by glycosylation, sulfonation, phosphorylation or lipidation.[141] The Golgi apparatus is made up of flatted membrane sacs called cisternae, and vesicles constantly bud and fuse to cisternae, carrying cargo between its compartments. The sub cellular location varies but in mammalian cells the Golgi body is usually near the nucleus.[142]

Targeting of endogenous cargo

Mechanisms governing protein retention in the Golgi apparatus are poorly understood compared to other organelles. Because the Golgi is a dynamic organelle, it is believed that the retention of proteins in the Golgi depends on iterative cycles of anterograde and retrograde transport with the ER.[143] Golgi localisation can be influenced by factors including protein–protein interactions and the affinity of proteins for the lipid environment of Golgi membranes, as well as by the binding affinity of proteins for the COPI and COPII vesicle coat complexes. While Golgi localisation is not yet fully understood, there are relatively few examples of Golgi proteins that necessitate a signal peptide for steady-state localisation.[144] The signal peptide sequence from β-1,4-galactosyltransferase, a Golgi membrane-bound glycoprotein, has been used to direct FPs to the organelle (Table S15).[145, 146] It was shown that appending a 32-residue cytoplasmic domain from the trans-Golgi network integral membrane protein TGN38 conferred Golgi localisation to the surface integral membrane protein glycophorin A, and that the sequence Ser-X-Tyr-Gln-Arg-Leu alone was sufficient for significant localisation; this was confirmed using immunofluorescence microscopy.[147]

Targeting of exogenous cargo

As the Golgi apparatus plays a major role in lipidation,[148] it can be targeted using lipid-based tagging groups. For example, post-Golgi compartments are rich in sphingolipids such as ceramide, which is transported from the ER to the Golgi apparatus *via* the ceramide transfer protein.[149] Ceramide has been used to target a range of small molecule fluorophores to the Golgi apparatus. For example, C5-DMB-Cer is a BODIPY-based fluorophore that

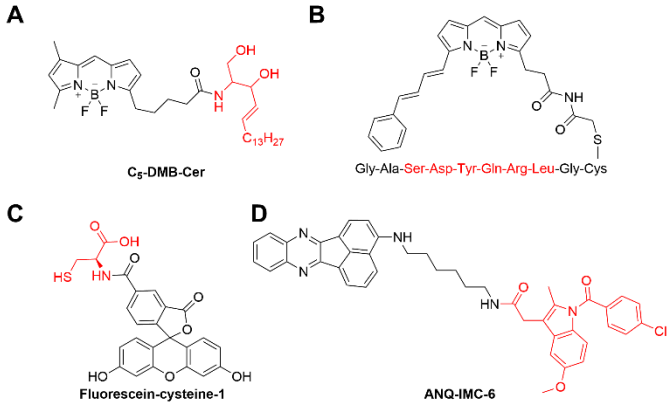

Figure 14. Structures of Golgi-targeted fluorophores, with targeting group highlighted in red.(A) Ceramide is used in C5-DMB-Cer (also known as BODIPY FL C5 ceramide);[17](B) BODIPY tagged with Ser-X-Tyr-Gln-Arg-Leu sequence;[1] (C) L-cysteine is used in fluorescein-cysteine-1;[27]; (C) indomethacine, a COX-2 inhibitor, is used in ANQ-IMC-6.[33]

shows high selectivity for the Golgi apparatus (Figure 13A; Table S16).[17]

The *trans* Golgi network has been successfully targeted using short peptide sequences. has also been some success targeting the trans Golgi network using peptides. The variant of the Ser-X-Tyr-Gln-Arg-Leu sequence was fused to BODIPY and found to localise in the *trans* Golgi network (Figure 13B).[1] Similarly, Li *et al.* used L-cysteine to target a variety of cargo. The authors took advantage of the propensity of Golgi apparatus localised proteins galactosyltransferase and protein kinase D to anchor *via* cysteine residues or cysteine rich domains.[27] They were able to achieve Golgi targeting of small molecules such as fluorescein (Figure 13C) and meso-tetra(4-carboxyphenyl)porphine, as well as nanomaterials including silica nanoparticles and quantum dots. While the mechanism has not been fully elucidated, the authors noted the necessity of free thiol groups for targeting and suggested they may bind to the sulfhydryl receptor site via formation of disulphide bonds.

It is also possible to take advantage of the overexpression of proteins in the Golgi apparatus. Zhang *et al.* reported ANQ-IMC-6, a acenaphtho[1,2-*b*]quinoxaline joined to indomethacine, a COX-2 inhibitor (Figure 13D).[33] As COX-2 is overexpressed in cancer cell lines and accumulates significantly in the Golgi apparatus, the conjugate showed good Golgi localisation in these cell lines.

Targeting strategies for lipid droplets

Lipid droplets are the major energy storage organelles, found in virtually every cell type. The lipid droplet contains a core of neutral lipids, mostly consisting of triacylglycerols and sterol esters. Its membrane consists of a phospholipid monolayer and proteins essential for structure and function.[150, 151] While lipid droplets were once considered inert cytoplasmic bodies, they are now known to dynamically interact with all major organelles.[152] The size and distribution of lipid droplets is diverse and dependent on cell line and nutrient availability.[153]

Targeting of endogenous cargo

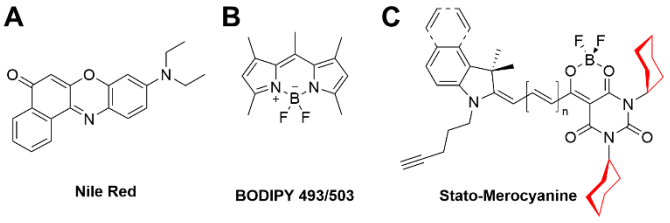

Figure 13. Structures of lipid droplet-targeted fluorophores, with targeting groups highlighted in red. (A) Nile Red and (B) BODIPY 493/503 are both examples of highly conjugated, lipophilic structures. (C) Two cyclohexyl rings are grafted onto the core of the Stato-Meracyanine structure.[6]

It is possible to target the proteins embedded in the exterior phospholipid monolayer of the lipid droplet. Perilipins (perilipin1-5) are the best studied of the lipid droplet-associated proteins, and perilipin-2 and perilipin-3 are ubiquitously expressed in mammalian cells.[154] However, even within a single cell, this expression can be heterogenous. A GFP-perilipin-3 fusion has been shown to useful in imaging the outer ring of the organelle (Table S17).[155] However, fusion FPs generated in this way cannot provide information on the lipid core that makes up the bulk interior of the organelle.

Targeting of exogenous cargo

Fluorescent stains are capable of visualising the lipid core of droplets in live cells (Table S18). Rather than a binding mechanism or targeting moiety, many lipid droplet stains undergo a solvatochromic shift and/or fluorescence turn on in non-polar, hydrophobic environments compared to the aqueous environment of the rest of the cell. These substances tend to have highly conjugated, lipophilic structures. There are a range of commonly-used commercial dyes that operate by this strategy. These include Nile Red (Figure 14),[156] a stain that has green fluorescence in the presence of neutral lipids like triglycerides and red fluorescence in phospholipids. It therefore exhibits some non-specific labelling, particularly that of intracellular membranes. Furthermore, its broad emission spectrum means cross-talk will occur with most green and red fluorophores, preventing multicolour imaging.[157] Another commercial stain is BODIPY 493/503 (Figure 14B); it and other BODIPY analogues stain neutral lipids with more selectivity than Nile Red, but may also stain membranes.[158]

To increase specificity in targeting lipid droplets over other lipophilic cell elements, Collot *et al.* developed StatoMerocyanines (Figure 14C),[6] a new class of merocyanine dyes. The authors grafted two cyclohexyl rings to the core to increase hydrophobicity and bulkiness while preventing quenching *via* π-stacking.[6, 159] This strategy was also used to increase the lipid droplet selectivity of a Nile Red fluorophore.[133]

Targeting strategies for the plasma membrane

The plasma membrane separates the cell from the outside environment. While the composition of individual membranes varies between different cell types, all membranes have several common components.[160] The backbone of the membrane is the phospholipid bilayer, comprising a range of phospholipids that

vary in their polar head groups and hydrocarbon tail lengths and saturation. Embedded within this bilayer are sterols, which can account for up to 40% of the total lipid content in a bilayer.[160] Cholesterol, for example, is necessary for the maintenance of membrane structure and fluidity. Proteins in the plasma membrane carry out diverse functions, including transport, cell signalling, structural support and enzymatic activity. They are broadly classified as integral membrane proteins permanently attached or embedded in the bilayer and peripheral membrane proteins temporarily bound to the lipid bilayer or integral proteins.

Targeting of endogenous cargo

Selective targeting of membrane-anchored fluorescent proteins can be challenging, as membrane structures undergo dynamic trafficking within the cell, such as in endocytic processes. Furthermore, some membrane proteins are not exclusively expressed in the plasma membrane but also in other intracellular membranes. A common strategy for attaching fluorescent proteins to the outer membrane is *via* palmitoylation. Palmitoylation is a common post-translational modification in membrane proteins; a palmitoylation signal can thus be included to a fusion protein to target it to the membrane. This strategy is employed with the commercially available CellLight™ Plasma Membrane-GFP, BacMan 2.0 reagent kit, which expresses GFP fused to the myristolyation/palmitoylation sequence from Lck tyrosine kinase, a sequence that is widely used for localisation[161] (Table S19). The palmitoylation sequence from the growth associated protein GAP43 has also been used.[162]

Targeting of exogenous cargo

The use of lipophilic fluorophores is not sufficient to achieve selectivity for the plasma membrane, as they may also stain intracellular membranes and lipid droplets. Instead, membrane localisation is usually achieved by creating fluorescently tagged lipids or lipid-like compounds to mimic the long hydrocarbon tails and polar head groups of membrane lipids (Table S20). In addition to lipophilicity, the incorporation of a positively charged group can aid retention in the negatively charged plasma membrane.

Membrane stains are often the fatty-acid or other alkyl chain derivatives of fluorophores. For example, DiI[163] is a di-alkyl indocarbocyanine dye (Figure 15A), while Laurdan[164] is the lauric acid derivative of the membrane dye Prodan (Figure 15B).[165] It is also possible to make fluorescent analogues of membrane lipids and follow their activity in the cell – for instance, the UV-emitting cholestatrienol (Figure 15C) is an analogue of cholesterol.[166]

Fatty acid appendages can also assist in minimising cellular uptake of probes intended for sensing events at or near the cell membrane. For example, P-IID, a fluorogenic probe for externalised phosphatidylserine, a marker of apoptosis, contains a stearate membrane anchor (Figure 15D).[7]

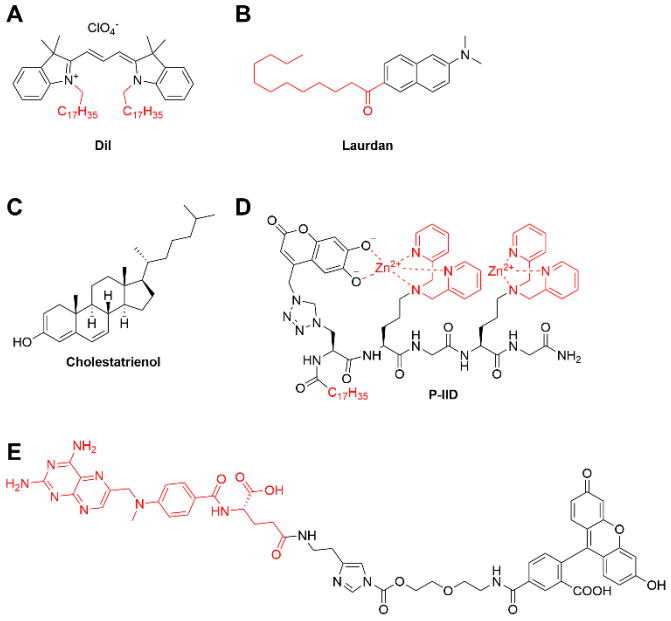

Figure 15. Structure of plasma membrane-targeted fluorophores. (A) DiI (or $DiI_{18}(3)$) and (B) Laurdan use long, hydrophobic alkyl chains. (C) Cholestatrienol is a fluorescent cholesterol analogue. (D) P-IID, with a stearate membrane anchor but the bis(zinc-dipicolylamine) only opens in the presence of phosphatidylserine.[7] (E) The affinity of the ligand methotrexate to folate receptors in the plasma membrane can be used for targeting.[20]

Targeting strategies for the cytoskeleton

The cytoskeleton of the cell is composed of fibres and filaments that function to provide structure and mechanical support, spatially organise the contents of the cytoplasm, and carry out functions including cell division and movement. The main fibres that make up the cytoskeleton are tubulin, actin and intermediate fibres.[167]

Tubulin is the largest fibre of the cytoskeleton, and is made up of α and β subunits. Tubulin is anchored to the centrosome, which is often near the nucleus, and grows towards the plasma membrane. Actin is the smallest type of cytoskeletal fibre, and is made up of actin protein monomers. In many cells, a network of actin is found beneath the plasma membrane and it functions to allow the cell to change shape and move. While actin and myosin are dynamic, focal adhesions and intermediate fibres are more static and function to provide mechanical strength, support, and some rigidity in cell shape.[168]

Targeting of endogenous cargo

Unlike other organelles, the cytoskeleton is not bound by any membranes, and so the protein components must be directly targeted. The most common method of targeting the cytoskeleton is to fuse a fluorescent protein directly to its protein components *e.g.* α/β tubulin or actin monomers (Table S21). The main problem associated with this method is that fluorescent proteins are quite large compared to the actin and tubulin monomers to which they are fused, and this may alter the behaviour of the native protein.[169] An alternative targeting method is *via* the use of microtubule binding domains, which

connect a protein of interest to tubulin fibres in a non-covalent manner.[170, 171]

Targeting of exogenous cargo

As for exogenous cargo, small molecule fluorophores can also be directly appended to tubulin or actin (Table S22). These chemically labelled proteins are commercially available and they overcome problems associated with large fluorescent protein labels, but the labelled proteins must be microinjected into the cell as they are not endogenously expressed. Furthermore, it is not straightforward to control the point of fluorophore attachment to the protein.[172, 173]

An alternative strategy for cytoskeletal targeting is to conjugate the cargo to drugs that bind components of the cytoskeleton. For example, SiR-tubulin contains a silicon rhodamine fluorophore tethered to a docetaxel derivative,

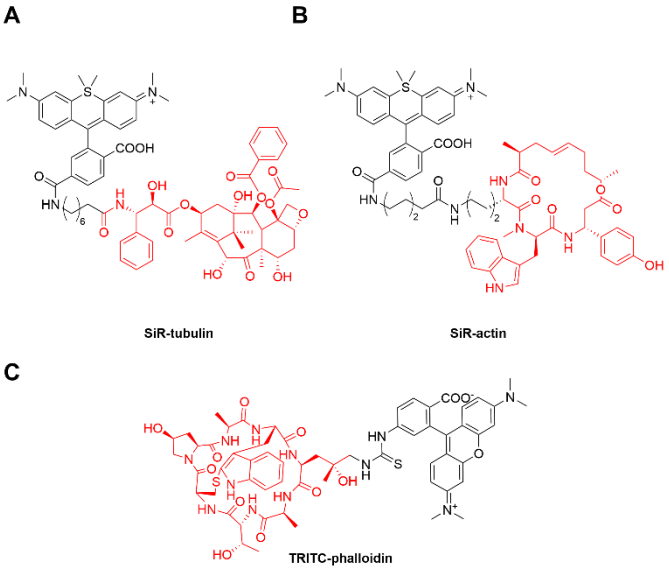

Figure 16. Structures of fluorescent probes for the cytoskeleton, targeting groups highlighted in red. (A) SiR-tubulin uses a docetaxel derivative to target tubulin, while (B) SiR-actin uses jasplakinolide to target actin.[4] (C) TRITC conjugated to phalloidin targets actin.[11]

which binds to microtubules (Figure 16A).[4] An analogue reported at the same time, SiR-actin, contains jasplakinolide as a targeting group for actin (Figure 16B). Phalloidin, a naturally-derived toxin, can also be used for actin targeting, as in TRITC-phalloidin (Figure 16C). The drawback of this method, however, is that the targeting group can affect the dynamics of microtubule formation and dissociation.

Targeting strategies for the cytosol

The cytosol of the cell is the viscous liquid matrix that surrounds the organelles and fills the interior of the cell.[174] The term is often, incorrectly, confused with the cytoplasm, which is defined as all the cell contents enclosed by the plasma membrane, excluding the nucleus. The chemical environment of the cytosol is known to be distinct from those of the organelles and extracellular spaces. While the cytosol was once considered a simple solution of molecules, it is now known to be a crowded environment with a high degree of organisation that participates in multiple cellular functions, including metabolism and transport.[175]

Targeting of exogenous cargo

For fluorescent proteins that are expressed endogenously, a specific targeting sequence is not necessarily required to ensure cytosolic targeting. Virtually all proteins begin translation in the cytosol and will remain there without a sequence directing trafficking to a subcellular target. For example, GFP shows cytosolic localisation without the addition of targeting sequences.[176] It should also be noted that without further targeting sequences, small proteins like GFP can to some extent translocate to the nucleus, as the nuclear membrane pore allows proteins < 40 kDa to enter *via* passive diffusion. The nuclear export sequence (NES), a short sequence containing four hydrophobic residues, can be appended onto proteins, preventing nuclear uptake and thus ensuring cytosolic localisation (Table S23).[177]

Targeting of endogenous cargo

Small molecules (<1 kDa) can passively diffuse through the plasma membrane and enter the cell. If there is a diffuse staining pattern, the dye is generally deemed cytoplasmic or cytosolic. In general, no targeting strategies are applied to achieve cytosolic localisation: rather, it is often hypothesised that the absence of a targeting group will achieve this aim. The observation of apparently cytosolic localisation must be treated with some caution, as incubation time and concentration of the probe may affect localisation. Some targeting groups may require a longer incubation time for uptake to the desired organelle to occur, and therefore at shorter timepoints can give cytosolic staining patterns. Similarly, high dosages may mask actual localisation. As such, it can be useful to carry out dose and incubation time studies on novel probes. Another factor to consider is retention of the fluorophore in the cytosol; without an anchor, the dye may diffuse from the cell, a factor that should be considered in experimental design.

Discussion

Trends in organelle targeting

For the design of organelle-targeted fluorescent sensors, it is essential to select targeting groups that are minimally disruptive to the biological environment and do not interfere with the sensing of the analyte. To this end, new targeting strategies are constantly emerging. It is essential to understand their mechanisms of action, and compatibility with a range of cargo in order to build new imaging tools.

An important factor to consider is the tendence of some fluorophores themselves to localise in a specific organelle, which can complicate efforts to target them elsewhere. For example, lipophilic cations like rhodamine will localise in the mitochondria, while lipophilic BODIPYs will tend to accumulate in lipid droplets.[178] A further confounding factor is the tendency of some fluorophores to display varying brightness in different environments, such as the solvatochromic or fluorogenic behaviour of lipid stains in non-polar environments, or the

sensitivity of some fluorophores to photoinduced electron transfer quenching in different pH environments.[179, 180] As these effects can give the impression of successful targeting, it is important to validate targeting groups with non-responsive fluorophores as controls. Lastly, use of the same targeting group for colocalisation experiments can lead to lower selectivity as certain targeting pathways become saturated.

Ultimately, understanding molecular interactions at the sub-cellular level underpins the rational design of novel organelle targeting strategies. For endogenous cargo, there are two common targeting strategies involving fusion proteins:

1) Append a known signal peptide or targeting sequence *e.g*. the nuclear targeting sequence tags a protein for nuclear import, the Lys-Asp-Glu-Leu sequence allows retention in the endoplasmic reticulum.
2) Append a protein known to localise to the organelle of interest *e.g*. LAMP1 is a native lysosomal membrane protein.

For exogenous cargo, there are four common strategies for small molecule targeting groups:

1) Passive targeting groups that rely on diffusion and non-covalent interactions with the local chemical environment, where the environment must be maintained. *e.g.* lipophilic cations diffuse to the negatively charged mitochondrial membrane, lipophilic amines diffuse to the acidic lysosomes and are trapped after protonation.
2) Targeting groups that are retained *via* binding interactions, *e.g.* Docetaxel binds to microtubules, glibenclamide binds to the sulfonylurea receptor in the endoplasmic reticulum, jasplakinolide binds to actin, and Hoescht binds to DNA.
3) Small peptide targeting sequences recognised by the native protein sorting machinery, *e.g.* The PTS-1 tripeptide (A/S-K-L) achieves peroxisome targeting, whilst the signal peptide (K/H-D-E-L) achieves this for endoplasmic reticulum targeting.
4) Appendage of biomolecules that are themselves targeted through cell sorting mechanisms, *e.g.* ceramide has been used to target the Golgi apparatus.

A fifth strategy for the targeting of small molecules bridges the exogenous and endogenous labelling strategies and involves the use of self-labelling proteins (SLPs). A detailed description of this methodology is beyond the scope of this review. In short, a SLP is an engineered enzyme that reacts covalently to a specific substrate group. The SLP can be directed to a subcellular location via fusion with a signal peptide or protein of interest, while the substrate can be attached to an exogenous fluorophore. Incubating the exogenous substrate with cells expressing the SLP allows for targeted labelling. HaloTag,[181] SNAP-Tag[182] and CLIP-tag[183] are examples of SLP tags in common use.

It should be noted that achieving localisation does not necessarily result in long-term retention; this is most common for passive targeting groups. For example, lipophilic cations can depolarise the mitochondrial membrane potential, and a moiety that provides covalent attachment is required for long term anchoring. Furthermore, targeting groups are not necessarily inert and can affect the biochemistry of the organelle. For example, DLCs can depolarise the negative mitochondrial membrane potential,[184] and the fusion of an FP with a native protein can affect the behaviour of the latter. While this may not be detrimental for one type of experiment, it can be fatal flaw in other experiments. Having a range of targeting groups, and understanding their mechanisms thus aids experimental design.

The quest for new organelle targeting strategies

For each organelle type, the holy grail is a robust targeting group that can reliably deliver cargo, regardless of its properties. The quest to develop new organelle targeting strategies is ongoing, and to meet this end, it is important to identify where new targeting groups can be found.

For the targeting of proteins, the development of new targeting strategies is dependent on the understanding of the localisation of native proteins and any signal peptide sequence they may have. As such, finding new targeting groups is potentially more straightforward, as the cell contains a multitude of proteins in each organelle, and each can be used as a possible candidate for targeting. This could provide cellular information on an even more localised level than the organelle.

Compared to the number of native proteins and signal peptides, there are fewer small molecule targeting groups. While novel strategies are emerging, it can take time before they have been robustly tested. This poses a problem, as the availability of robust tags can shape the direction of research. The organelles that have several robust targeting groups (*e.g.* mitochondria, lysosome) are well studied, whilst the organelles with few available targeting strategies (*e.g.* ER, Golgi apparatus and peroxisomes) are less well understood. The discovery and validation of more targeting strategies is therefore essential to shaping the direction of organelle research.

Many targeting groups now used for fluorophores originally came from the development of targeted therapeutics. For example, the TPP mitochondrial targeting group was first used for development of mitochondrial antioxidants.[185] This could be a potential source for new targeting groups, and even an avenue for theranostic probes that allow both diagnosis and treatment.

In summary, there are a plethora of methods for targeting both endogenous and exogenous cargo to specific sub-cellular locations. These strategies have enabled the development of highly selective fluorescent markers and sensors that have already proved invaluable in elucidating the structure and function of cells with organelle resolution. The further development of robust targeting strategies will further enable an understanding of the intricacies of sub-cellular changes that underpin health and disease.

Author Contributions

Conceptualization, J.L., K.Y. and E.J.N. Writing – original draft preparation, J.L and K.Y. Writing – review and editing, E.J.N.

Conflicts of interest

There are no conflicts to declare.

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