Fluorescence dyes and their usefulness in yeast cell research

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Yeast cells have long been accepted as a model system for studying basic eukaryotic processes starting form cell division till cell death. One of the major reason for current advancement in yeast research is the easiness by which we can monitor intracellular events in real-time. Fluorescence dyes played pivotal roles in our understanding of intracellular molecular dynamics of yeast in a quantitative way. The present chapter summarises fluorescent dyes and their applications from budding yeast to fission yeast. Detailed studies have already shown the advantages and limitations of imaging of yeast intracellular events by those particular dyes. This chapter is intended to summarize these pros and cons and compare the usefulness of dyes with florescent protein tagging.

Keywords: Fluorescence; Yeast; Imaging; Fission; Budding; Microscopy

1. Introduction

Fluorescence imaging has taken pivotal role in elucidating the activity of individual cells, understand subcellular trafficking of signals especially in between organelles, and to realize how the function of individual organelle is regulated. Research in the field of designing fluorescence probes in past two decades have seen tectonic shifts which made it possible for us to follow signaling traffic, sense polar/ non-polar second messengers, report various enzyme activities etc. The probes have been applied with various degree of success across a vast range of model systems(1). One of the most extensively studied biological model system comprising of Yeasts. Starting from budding yeast (*Saccharomyces cerevisiae*), fission yeast (*Schizosaccharomyces pombe*) to pathogenic yeast models i.e *Candida sp.* Yeasts have long been considered as a model system due to availability of their genome sequences, easy to cultivate overcoming ethical and experimental constrains and also they represent a larger class of existing eukaryotes (2, 3). Yeast cells share a high degree of similarity in conserved molecular and cellular mechanisms with human cells (4). For example, CDC (cell division cycle) genes were discovered primarily in *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe* and their roles in controlling and synchronising cell division helped in understanding the complex mechanism of cell division in humans at a later point of time. A whole lot of proto-oncogenes, tumor suppressor genes, checkpoint genes came into light by using yeast as a model system (5). Apart from playing a fundamental role in cancer research, yeast has been widely used as a model system for neurodegenaration. Formation of intracellular protein aggregates, cellular toxicity brought about by misfolded proteins, oxidative stress and hallmarks of apoptosis have been quite accurately recapitulated in yeast (4, 6). These features are characteristics for various neurodegenarative diseases such as Huntington and Parkinsons diseases. After validation, Yeast can also be used to accelerate the identification of both novel therapeutic targets and compounds with therapeutic potential. The present chapter will summarize the fluorescent dyes used for determining various intracellular biological processes and acitivities in yeast. Organelle specific dyes will also be summarized in this chapter.

2. Determination of intracellular biochemical and signaling activities using fluorescent probes

Fluorescent probes have been successfully used for yeast to determine cellular viability, vitality, redox environment inside cells, lipid accumulation dynamics, intraceellular pH status etc. The present section provides a comprehensive literature review about fluorescence probes that are used to track diverse biochemical components and cellular activities.

2.1 Cell viability and vitality assessment

Measuring yeast cell viability becomes paramount in understading diverse stress responses. Cells subjected to physical, chemical or environmental stresses develop cyto or genotoxicity. Generally viability of cells are referred as percentage of live cells in whole population. On the otherside “cell vitality“ is reffered as an effect of the stress to alter the cellular ability to divide without necessarily causing death to the cell, thereby hampering the physiological capabilities. Apart from growth and CFU (colony forming unit) based methods which are ofcourse time consuming, viability and vitality measurements can be carried out using colorimetric and fluorescent dye based methods. One caterogry of dyes are basically anionic molecules under physiological pH, that are repulsed by negatively charged membranes of live cells. Only when the cell membranes are damaged, the dyes can penetrate and dead cells give colour or fluorescent signals. Dyes from this class include trypan blue or erythrosine B (colorimetric detection) and propidium iodide (PI) or ehidium bromide (fluorescence detection) (7-11). Another category of dyes can penetrate all kinds of cells (live or dead) and based on the metabolic status of cell they get modified. Methylene blue is one of many colorometricaly detectable dyes that get reduced by enzymes within live cells, thereby rendering it colorles whereas in dead cells it shows blue color

(12). FDA (fluorescein diacetate) is one such fluorescent dye which can penetrate all kind of cells but only in live cells it gets hydrolyzed by intracellular esterases to fluorescein, thereby producing green fluorescence (13). A staining strategy combining both categories of dyes together has been shown to produce optimum results in yeast. Cells stained with PI and FDA together have been shown to give accurate assessment of viability. Here in this staining method dead cells show red (PI) whereas live cells show green fluorescence (FDA), thereby rendering easy visualisation (11). Among other fluorescent viability biomarkers carboxyfluorescein diacetate (cFDA), calcein AM and SYTO-13 are widely used for yeast cells. Viability studies using these dyes may include fluorescence quantification, microscopic observations, FACS or even Field-Flow Fractionation (14).

Cellular vitality analysis consider various aspects of physiological state of cells. This analysis can be broadly categorised in three parts. (1) Intracellular ATP contents based on luciferin assay (2) fluorescence based mitochondrial membrane potential assay (3) enzyme activity assay (15-18). Intracellular ATP content is closely related with metabolic activity of the cell. The most easy method to detect intracellular ATP, comprise of luminescence determination using BactTiter-Glo^{TM}. The principle of the assay is based on the reaction of luciferin with luciferase in presence of ATP, Mg^{2+} and Oxygen. Therefore more amount of intracellular ATP within cell will show more signal intensity (19). For determination of mitochondrial membrane potential and thereby respiratory activity within yeast cells, dyes like Rhodamine 123 and Rhodamine B are used. Rhodamine 123 is a cationic green-fluorescent dye which is readily incorporated by active mitochondria and rhodamine B is a hexyl ester red-fluorescent dye (16, 20). Based on the respiratory activity within the yeast cell, one can readily see the changes in mitochondrial network distribution as well as membrane potential when stained with any one of the dyes. Fluorescence based methods can also show the enzyme activity within the cells and thereby giving an idea of metabolic nature of the yeast. FUN-1 is a chlorinated cyanide dye which penetrates to the vacuole of the live cell and gives red fluorescent structures. Whereas in dead cells or metabolically dormant yeast cells the same dye gives cytoplasmic green fluorescence (18). Several other examples of dyes that can be used to determine the intracellular enzymatic acivity of different kinds of enzymes by either colorimetric or fluorescence methods are FDA for esterase enzymes, tetrazolium salts MTT for oxidoreductases, XTT or WST-8 for different redox enzymes etc. (17, 21, 22).

2.2 Intracellular pH assessment

One of the most crucial and fundamental physiological challenges of yeast cell is to maintain intracellular pH homeostasis during their life cycle. H^+ATPases *PMA1* and *PMA2* genes are pivotal in balancing both intra and extracellular pH. Fluorescence probes were long been used to analyse the pH changes within yeast cells. Initialy fluorescent compounds like quinine, chromotropic acid or FDA were found to be handy in analysing the intracellular pH of yeast cells. Depending on pH, chromotropic acid shows two different forms of fluorescence spectra. The transition between the acidic and basic forms can be used for pH measurement above the pH range of 3-7. It is suitable to analyze the ratio of fluorescence intensities measured at 360 and 430 nm (fluorescence maxima of acidic and basic forms, respectively) after excitation around 330-340 nm. Quinine can also be used by similar measurement methods due to its two different spectral patters in acidic and basic conditions. The ratio of fluorescence intensities at 380 and 450nm after excitation around 330-340nm allows the determination of pH over 3-8 (23). But the handicap using these two compounds lies with their inability to bind inside cells and their fluorescence depend solely on $[H^+]$ as well as high degree of efflux out of the cell (>50%). Quinine has an additive problem of it's susceptibility to Cl^-, rendering it less suitable for in vivo assessments. Fluorescin is one of the best compounds to ascertain intracellular pH changes, as it has been shown to detect pH changes as low as 0.01 pH units. High quantity of fluorescin gets retained inside cells mostly freely in cytoplasm making it a good dye to analyse any change in cytoplasmic pH (23). Membrane-permeable prefluorochrome, 5-(and-6)-carboxy SNARF-1, acetoxymethyl ester, acetate (termed as carboxy SNARF-1 AM acetate) is also an effective compound to analyse pH changes. They are hydrolyzed by intracellular esterases to their polar forms whose fluorescence depend on the pH (24, 25). The exitation and emmision kinetics are measured at 525 and 580 nm wavelengths. Other variations of SNARF fluorescent probe like SNARF-4F are routinely used for intracellular pH measurements of yeast presently (26). Pena et al. 1995 used another fluorescent coumpound called pyranine (8-hydroxy-1,3,6-pyrene-trisulfonic acid) ,which they introduced by electroporation in yeast (27). The compound was found to retain in cytoplam avoiding any vacuolar leakage and could measure very precise changes in internal pH. Provided the right fluorescence compound and staining method is selected, fluorescence detection can be the most reliable and easy method to judge even small changes in intracellular pH in yeast.

2.3 Determimination of intracellular redox balance

Fluorescent probes for sensing redox state inside the cells can be broadly classified into two categories. First is Small-molecule fluorescent probes and the second types are protein based fluorescent probes. One of the first usage of reaction based small molecule fluorescent probes came in the form of calcium probes developed by Roger Tsien, i,e Fura-2 and Indo-1 (28). Later on numerous fluorescence-based small-molecule probes with reaction centers have been developed in the field of redox signalling (29-32). Among redox species found inside cells the most common are H_2O_2, Peroxynitrite, hypochlorus acid (HClO) etc. Detection of these redox species can be done by fluorescent probes. The H_2O_2 responsive probes are plenty and they can be broadly grouped into four segments: (1) Boronate-based probes (e.g. TPE-DABA,

Mito H_2O_2, TPE-BO etc), (2) benzil-based probes (e.g. NBzF-BG), (3) chalcogenides-based probes (e.g. 2-Me-TeR) and (4) metal-mediated probe (e.g. ZP1Fe_2)(33). Peroxynitrite-responsive probes are categorized into three main groups: (1) addition-based probes (e.g. A2, Ds-DAB, MITO-CC etc), (2) oxidation-based probes (HKGreen-4, NP3, Cy-NTe etc), and other probes (e.g.5-Fluoroisatin)(33). HClO responsive probes are Mito-TP, MitoAR, HCTe etc. It is to be noted that although small-molecule fluorescent probes are easy to use but selectivity is an important draw back for the probes. For example DCFH, which was originally developed as an H_2O_2 detecting probe but the reaction for oxidation of DCFH to DCF involves multiple steps and other oxidants like oxidized glutathione, iron, nitric oxide (NO.) etc (34). Other small molecule probes are also susceptible to similar cross reactivity thereby can make the end results erroneous.

Compared with small-molecule fluorescent probes, protein based fluorescent probes are very few but they provide quite a few advantages. Two types of protein-based probes are widely used in redox biology, roGFPs and Hyper based sensors (35-37). roGFPs are used for measuring GSH redox potentials, whereas Hyper-based sensors are often used for measuring H_2O_2. The protein based probes are highly selective and their subcellular targeting is very precise thereby making them the probes of choice for many.

2.4 Detection of intracellular metabolite accumulation

Accumulation of diverse kinds of metabolites are commonly observed during life cycle of yeast cells. Among these metabolites certain metabolites provide intrinsic basal fluorescence in yeast. Pyridoxine, riboflavin, tryptophan etc are endogenous fluorophores in yeast cells that confer autofluorescence. Any staining method using external fluorescent dyes must consider intrinsic fluorescence of yeast cells (38). One of the common characteristic within yeast cell is the presence of lipid droplets. Neutral lipid droplets can be stained with fluorescent probe Nile Red (39). Intracellular red colored droplets of lipids can be detected by staining with Nile Red in various varieties of yeast starting from budding to fission yeasts (40). Apart from Nile Red, BODIPY 505/515 are used commonly to stain neutral lipid droplets in yeast (41). Like Lipid droplets, nucleic acids are readily detectable inside yeast cells by various fluorescent probes. Cyanine dyes have been shown to have specific affinity for nucleic acids. They can be grouped into cell permeable and impermeable Cyanine dyes. For staining the nucleic acids in live cells, cell permeable cyanine dyes are used. These dyes are trade named as SYTO® dyes. Although the SYTO® dyes can stain both intracellular DNA and RNA molecules, but SYTO12 and SYTO14 shows twice the amount of fluorescence when bound to RNA than DNA. On the other hand SYTO 16 shows double amount of fluorescence when bound with DNA than RNA. Apart from SYTO® dyes other commonly used dyes for nucleic acid staining in live cells include Acridine orange, DAPI, Dihydroethidium, Hoechst dyes etc. Metal availability inside the yeast cells can be measured using fluorescence detection methods. Wild-type strains of *S. cerevisiae* are known to excrete anthranilic acid and 3- hydroxyanthranilic acid in response to glucose. The excretion of anthranilic and 3-hydroxyanthranilic acids are correlated with the ferric-reducing capacity of the extracellular medium. Excretion during growth are much greater by cells cultured in iron-rich medium than by cells grown in iron-deficient medium. Thereby monitoring the fluorescence of 3- hydroxyanthranilic acid, intracellular levels of iron can be traced (42).

Apart from metabolites fluorescent probes have been developed to highlight the presence of various metal ions inside the cell. A 7-nitrobenz-2-oxa-1,3-diazole (NBD) derivative namely **probe 20** shows fluorescent properties for Zn^{2+} (43). The same group came up with another probe namely **probe 21** that showed change in fluorescent properties upon binding two different kinds of metal ions both *in vitro* or *in vivo*. When bound to Zn^{2+}, the probe showed green fluorescence and when bound to Cd^{2+} it gave blue fluorescence (44). Cyanine based fluorescent probe tris(2-pyridylmethyl)amine (TMPA) has been also shown to detect Zn^{2+} *in vivo* and in organisms (45). Rhodamine hydrazone derivatives .bearing either thiol or carboxylic acid could be successfully applied for fluorescence detection of intracellular Hg^{2+} in various organisms (46). Rhodamine derivative bearing boronic acid or Rhodamine-alkyne derivatives can be successfully used for detection of intracellular Cu^{2+} or Au^{3+} (47, 48). As most of these fluorescent probes are readily soluble small molecules by nature, they are likely to be very useful in detection of intracellular metal uptake in various yeast strains.

3. Detection of organelles using fluorescent probes

Detection and analysis of organeller dynamics is crucial in understanding effects on intracellular changes due to environmental and physical challenges that cells are constantly facing. For efficient detection and labelling of a organelle, the marker fluorescent probe must have a few common property like it's solubility in normal intracellular fluids, ready permeability of cell membrane, non toxic by property, high degree of intracellular retention etc. In this section we discuss about common fluorescent dyes that are in use for labelling yeast organelles:

3.1 Cell Wall

Cell wall is important in letting fungi to withstand environmental stresses, osmotic shocks as well as for interaction with adjacent cells. Apart from many cell wall active proteins, chitin and β1,3 glucan are important components of fungal

cell wall (49). One of the most widely used stains for staining fungal cell wall is calcoflour white. It stains the external chitin component of fungal cell wall. Chitin serves not only as an important component of yeast cell wall, but it is one of the major components of 'Bud Scar" in mother yeast cell during cytokinesis, hence can be used for cell polarity assesment. Calcoflour gives an extreemely strong fluorescence in very small concentrations during it's usage (50-53). Although calcoflour white M2H is widely used for fluorescent studies but it fades fast and it requires a non-standered laser beam for optimum results during confocal imaging (54). Another problem associated with calcoflour is it's functioning as cell wall disruptor, thereby it can cause inhibition of yeast growth when used in higher concentrations. Although *Saccharomyce*s is susceptible to inhibition with calcoflour, but fission yeast are resistant to any inhibition by calcoflour (55). Trypan blue also can be used to stain of yeast cell wall. It is very similar in it's binding pattern with chitin and glucan derivatives(53).The only deference is their respective exitation and emmision wavelengths. Hoch et al, 2005 also reported about two other fluorescence molecules namely Solophenyl Flavine 7GFE 500 and Pontamine Fast Scarlet 4B. Unlike calcoflour both these dyes donot fade quickly when exited with selective wavelength; however like calcoflour they are easy to use and can selectively stain fungal cell wall, bud scars and septum (54). Comparative fluorescent intensities of all three fluorescent dyes (Calcoflour, Solophenyl Flavine and Pontamine Fast Scarlet) were more in live fungal cells compared with formaldehyde fixed cells (54). For staining β1,3 glucan derivatives of fungal cell wall; the stain of choice is Aniline Blue (56). It can be used for both live and fixed cell staining using UV filtes for exitation purposes. Aniline is effective in staining wide range of yeast species including fission yeast (57). Another important fungal cell wall component is Mannoprotein. It can be stained with Concanavalin A, fluorescein isothiocyanate-conjugated (FITC-ConA). Concanavanin A is a mannose containing lectin binding probe, that has been used effectively in probing cell surface receptors in various organisms. For staining both live and fixed yeast cells, FITC-ConA can be used effectively (50). Depending upon the purpose of the experiment, a variety of fluorescent probes are available for using in deciphering diverse components of yeast cell wall.

3.2 Vacuoles and vesicles

Vacuoles are the largest compartments in yeast (58). The basic functions of yeast vacuoles include degradation of cell components, storage of ions and metabolites and various aspects of cellular homeostasis (59-61). Apart from that, vacuoles are instrumental in regulating morphological plasticity and niche-specific adaptation in certain yeasts (62). As vacuoles are the largest compartment of yeast cells, they can be visualised quite clearly using microscopical techniques such as phase contrast and differential interference contrast (DIC) microscopy (58). But to understand vacuolar dynamics and morphogenic transitions by changing vacuolar shapes and sizes, vacuole specific dyes are necessarry. Most of the vacuolar fluorescence markers take advantage of the intrinsic acidity of the vacuole. Chloromethyl coumarin-derivatives are non fluorescent by nature. By reaching vacuolar lumen, in acidic environment they undergo a chemical reaction generating a fluorescent derivative. This category of vacuole markers include CMAC (7-amino-4-chloromethylcoumarin) and CMFDA (5-chloromethylfluorescein diacetate) (63). Similarly, another set of fluorescent vacuole markers based on 6-carboxyfluorescein diacetate (CFDA) and its derivatives (CDCFDA, 5-[and 6-] carboxy-2′, 7′-dichlorofluorescein diacetate, cDFFDA, and Oregon Green 488 carboxylic acid diacetate) are widely used to visualise vacuoles in a range of fungi such as *C. albicans* and *S. cerevisiae* (64,65). They are similar as CMAC, and are colourless non-polar compounds which readily diffuse across cell membranes. Once within the vacuole the compounds are hydrolysed by intracellular esterases to produce a fluorescent product that is polar and thereby unable to re-enter the vacuolar membrane by diffusion. Apart from these widely used fluorescent probes which stain vacuolar lumen, the most widely used vacuolar membrane marker is FM4-64 {N-[triethylammoniumpropyl]- 4-[p-diethylaminophenylhexatrienyl] pyridium dibromide}(66,67). FM4-64 is a lipophilic styryl compound that does not permeate cell membranes. Instead it inserts into the plasma membrane, and becomes fluorescent upon doing so. Simultaneously it is taken up by the cell via endocytosis, staining compartments of the endocytic pathway, and ultimately reaches vacuolar membrane. Therefore this compound can be also used to stain and track endocytic vesicles in time-lapse live cell fluorescent microscopy (67). Compared with CMAC and CMFDA; FM4-64 is much more photostable. FM4-64 has played pivotal role in microscopic observation of internal invaginations within the vacuolar compartments in *Saccharomyces* and to track Multi vesicular body formation (MVB) (68).

Small vesicles carry important functions inside the cells like carrying enzymes (both exocytic and endocytic vesicles), ions, nutrients etc (69, 70). But unlike vacuole staining dyes, the number of vesicle targeting dyes are quite low. As mentioned in earlier section, FM4-64 can be used to target endocytic vesicles. Previously, FITC-Dextran was used for the same purpose, i.e. tracking endocytic vesicles (71). Zinquin has been used in fluorescence microscopy to visualise zinc-rich vesicular compartment in yeast (72). Similarly Dithizone staining can be used for normal light microscopic detection of Zinc rich vesicles in yeast (70). In summary it can be said that although fluorescence dyes have been useful in tracking vacuolar morphology or endocytic trafficking, further noble compounds are necessary to selectively track different small vesicles destined for secretory purposes.

3.3 Mitochondria

In yeast, majority of the cellular ATP is generated within Mitochondria. Cellular respiratory electron-transport chain (ETC) and the ATP synthase complex, both are located at the mitochondrial inner membrane. In mitochondria, the

energy released by the transfer of electrons is used to pump out H+. Therefore, H+ electrochemical potential is formed by a proton gradient and an electrical gradient which is instrumental for ATP synthesis. Rhodamine 123 is one of the most widely used mitochondrial fluorescent dye to assess mitochondrial respiratory function and measure mitochondrial effux pump (73-76). Different concentrations of Rhodamine123 are required to stain yeast cells optimally between glucose-grown cells and early or late-exponential phase cells due to the well known shift from fermentative to respiratory metabolism(77). DiOC6(3) is another green-fluorescing dye which was used as the primary tool in early studies of yeast mitochondrial motility (78). It is a member of the carbocyanine class of compound, which is characterized by high-intensity fluorescence and photostability (79). DASPMI, also called 4-Di-1-ASP® (Molecular Probes) or 2-Di-1-ASP® (Sigma Chemical Co., St.Louis, MO), is a lipophilic styryl dye which is used in studying yeast mitochondrial morphology, including the screening of mutants (80). The most recent addition in mitochondria staining dyes are MitoTracker. The major highlight of this dye is that they remain associated with mitochondria even after fixation with paraformaldehyde. This can be useful in immunofluorescence experiment during colocalisation studies with specific antigens. MitoTracker dyes are available in a variety of colors (green, orange, and red), hence making it useful for colocalisation studies either with GFP taged target proteins in live cell or mutiple target protein specific antibodies using immunofluorescence (81).

Another set of dyes used in staining mitochondria are DNA binding dyes. Mitochondria carries it's own DNA, therefore making it susceptable to staining with DNA binding dyes. The most widely used DNA staining fluorescent dye for yeast is DAPI (4',6'-diamidino-2-phenylindole). DAPI is a strong nuclear and mitochondria marker and it's staining pattern doesnot depend on the metabolic state of mitochondria. Another advantage of using mitochondrial DNA marker is that it can be used for both live and fixed cells.The handicap of DAPI is that it stains nuclear as well as mitochondrial DNA so that mitochondria close to the nucleus can not be differentiated. A second disadvantage arises during prolonged visualization. Excitation of DAPI requires UV lamp, which is toxic to ceils; this toxicity is increased in the presence of the dye (80,81). SYTO 18® is a nucleic acid-binding dye, developed by Molecular Probes, Inc. (Eugene, OR), which is reported to stain yeast mitochondria. The main advantage of this DNA binding dye is that, it's signal can be captured using FITC filter during microscopy, hence avoiding damage due to prolong incubation in UV light (81).

3.4 Nucleus

Yeast cell nucleus needs to be stained with fluorescent dyes to study cell division, nuclear dynamic and movements, arrangement of chromatin etc. In older times conventional nuclear staining methods Giemsa, acetic orcein, acridine orange were used for yeast(82-84). These methods took several hours and multiple manipulations even when modified to study only the position of the nucleus rather than its internal structures (85). Slater ML used a compound called mithramycin which was easy to use and could be used to study different phases of mitosis during cell cycle progression using fluorescence imaging(86).

In present millenium, a lot of nucleus specific counterstains have been tried on yeast cells with high degree of specificitry and selectivity. DAPI, as mentioned earlier is possibly the most widely used nuclear marker dye. Hoechst 33342 dye is also one such dye that stains nucleus of both live and fixed cell samples (87). Hoechst is an effective dye for both flowcytometric applications as well as microscopic observations of chromatin content. Dimeric cyanide dyes have been useful in labelling mitotic chromosome movements in live cells. One of the widely used cyanide dye is YOYO-1. This dye was found to be stable during microscopy of yeast (88). Apart from these POPO-3, HCS (High content screening) Nuclearmusk stins ere found handy in staiing nucleus of both live and fixed cells.

3.5 Other organelles

The ER-Golgi aparatus occupy important position in secretory potein trafficking pathway in eukaryotes. Proteins are transferred from the ER to early or cis-Golgi compartments in a process that involves the formation and fusion of small transport vesicles (89). $DiOC_6$ stains ER network of yeast cells when used at higher concentrations (90). It also stains mitochondria as mentioned earlier.Another Dapoxyl family dye known as ER-Tracker Blue-White DPX (Molecular Probes) is very highly selective and photostable. Its fluorescence is highly environment sensitive i.e. with increasing solvent polarity, the fluorescence maximum shifts to longer wavelengths (91). For labelling Golgi, ceramide based probes are quite regularly used. NBD C6-ceramide (Invitrogen®) and BODIPY FL C5-ceramide (Invitrogen®), both of which can be used with FITC filter sets, are selective stains for the Golgi apparatus (92). NBD C6-ceramide has been used extensively for labelling Golgi in both live and fixed cells. Important cellular compartment, especially for research in the field of autophagy or phagocytosis is lysosome. Lysosome specific dyes are plenty in market. The LysoTracker probes (Invitrogen®) are available in several fluorescent conjugates, making them suitable for multicolor applications. Very recent a dye named Lyso-NA, a naphthalimide-based fluorescent probe, was synthesized. This dye was found to possess high specificity towards lysosome, superior photostability, and an appreciable tolerance to microenvironmental change (93). But application of this dye needs to be tested among a wide range of fungi before calling them useful for lysosomal tracking in a wide variety of yeasts.hh

4. Comparision of fluorescence dyes with fluorescent tagged proeins

Usage of fluorescent dyes and fluorescent proteins have their indivisual advantages and disadvantages. User need to first recognise the expeimental goals before they start searching about different probes. Both fluorescent dyes and tagged proteins can also be used together if experiment demands. One of the major advantages of using fluorescent dyes is it's ready availibility and easyness of handling thereby saving precious time during research. On the other hand fluorescent protein needs to be fused to the target protein for labelling specific compartments. This fusion can be achieved by molecular cloning or recombination methods, therfore making the entire experiment time consuming. Another benefit of dye is it guarantees labelling of organelles or enzymes. But with GFP, YFP or RFP fusion proteins the labelling of organelle also depends on proper folding of the fusion proteins, which in some cases cannot be achieved due to various reasons. Cellular toxicity is attached with both the methods. Hence viability and vitality assesments are needed during the course of experiment. Dyes also present advantage during usage because it's concentration can be manipulated during experiments but expression levels of fusion proteins are hard to control. They can be controlled if the construct is getting expressed under an inducible promoter but that too not as accurately as one can control dye concentration. The major disadvatages of dyes compared with fusion proteins are their relatively low stability under physiological conditions, low retainment inside the cells (because cells flux out considerable amount of dyes). Another disadvantage of dyes compared with tagged proteins are their restricted usage for microscopy or flowcytometric analysis. Not a lot of dyes are in use that can be used for FRET (Fluorescence resonance energy transfer) or FRAP (Fluorescence recovery after photobleaching) applications. But fusions proteins are widely used for studying protein protein interactions or half-life measurements using FRET and FRAP methods.

Overall it can be summarized that both fluorescent tagged protein or fluorescence dyes have been instrumental tools in our understanding physiological processes, organeller inheritence and viability assesments in diverse varieties of yeast cell.

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