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Methods of detection of β-galactosidase enzyme in living cells

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ABSTRACT

The application of β-galactosidase enzyme ranges from industrial use as probiotics to medically important application such as cancer detection. The irregular activities of β-galactosidase enzyme are directly related to the development of cancers. Identifying the location and expression levels of enzymes in cancer cells have considerable importance in early-stage cancer diagnosis and monitoring the efficacy of therapies. Most importantly, the knowledge of the efficient method of detection of β-galactosidase enzyme will help in the early-stage treatment of the disease. In this review paper, we provide an overview of recent advances in the detection methods of β-galactosidase enzyme in the living cells, including the detection strategies, and approaches in human beings, plants, and microorganisms such as bacteria. Further, we emphasized on the challenges and opportunities in this rapidly developing field of development of different biomarkers and fluorescent probes based on β-galactosidase enzyme. We found that previously used chromo-fluorogenic methods have been mostly replaced by the new molecular probes, although they have certain drawbacks. Upon comparing the different methods, it was found that near-infrared fluorescent probes are dominating the other detection methods.

1. Introduction

β-Galactosidase, a glycoside hydrolase enzyme, is one of the important enzymes for digestion of lactose. It is categorized in exoglycosidase family because it plays a vital role in human body in the elimination of galactose residues from different substrates like glycoproteins, gangliosides, and sphingolipids [1,2]. It also has been proved to be a crucial enzyme as it is used as a biomarker of cell senescence and primary ovarian cancer [3–7]. Besides, it has also been used in the gene expression [8], and transcriptional regulation [9]. The concentration of β-galactosidase, an important enzyme, must be maintained in the human body. The alteration of concentration of β-galactosidase has been associated with β-galactosialidosis, morquio B syndrome [10], and ovarian cancer [11,12]. Hence, it is significant to keep track of the concentration and activity changes of β-galactosidase *in situ* and thereby well-define the roles of β-galactosidase in associated diseases.

To monitor the β-galactosidase concentration *in vitro* and *in vivo,* many researchers are interested in developing highly selective and sensitive methods. Several researchers have adopted different methods for the effective detection of β-galactosidase. Some of the techniques that have been used in the past are bioluminescence [13], chemiluminescence [14], magnetic resonance (MR) [15,16], single photoemission computed tomography [17], positron emission tomography (PET) [18], colorimetric [19] and fluorogenic [20,21] approaches. These methods have different advantages and disadvantages. Therefore, there is a necessity of proper categorization of the methods used (Fig. 1). The methods like, MR, positron emission tomography, single photoemission computed tomography are hampered by high cost, laborious manipulation, modest sensitivity, and invalid monitoring of β-galactosidase enzyme. Moreover, these techniques have failed to achieve real-time *in situ* non-destructive detection of this enzyme in the biological system.

Among these techniques, fluorescent sensors [22] have been really popular owing to their convenience, high sensitivity, simple handling procedures, inexpensive instruments, and bioimaging ability. As of now, only a few fluorescent probes for β-galactosidase have been developed, with some of them applied to monitor the enzyme activity in living cells or in tissues [23–35]. However, most of the reported sensors are fabricated with traditional fluorophores, which suff ;ered from aggregation caused quenching (ACQ) of fluorescence in high concentration solutions or after they accumulated in cells, making the fluorescence emission much weaker as compared to that in solution [26]. Great interest to

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develop β-galactosidase probes without the ACQ eff ;ect for living cell or tumor tissue imaging has been perceived recently. Apart from this, most of the researchers are inclined in the development of fluorescent probes for monitoring of β-galactosidase activity due to the advantage of advanced sensitivity, less expensive instrumentation, convenient handling procedures, and convenience of bioimaging.

Herein, we have reviewed various methods used in the detection of β-galactosidase in the living cells. We have provided an overview of recent advances in the detection methods of β-galactosidase enzyme in the living cells, including the detection strategies, and approaches in human beings, plants and microorganisms as shown in Fig. 2. To our knowledge, there are not any recent reviews that have compared the different methods used in the detection of β-galactosidase in living cells. We specifically summarized recent advances in the development of enzyme detection methods. The main objective of this paper is to serve as a narrow footbridge by comparing the literary works on the different methods of β-galactosidase detection, critically analyze their reliability by showing the pros and cons of the predicted methods for the practical use. In the detection of β-galactosidase both *in vivo* and *in vitro*, we assessed the progress made in designing the molecular probes from the previously used chromo-fluorogenic probes. Most of the reported molecular probes are based on the technique of attaching the galactopyranoside residue to the specific signaling units. These probes have enriched properties as compared to the traditional technique. However, they lack proper cellular membrane permeability, have inappropriate excitation and emission wavelengths, and have diminished chemical stability. With all these information, we hope that this review paper will enable to enrich the knowledge to facilitate the monitoring of β-galactosidase activity within living cells or tissues and open the minds to the interested researcher to develop new advances in the detection of this enzyme.

2. Detection of β-galactosidase in human cells

2.1. Use of fluorescent probes

Inception of fluorescence imaging with a real-time and *in situ* manner has restructured the fields of tracing the dynamics and revealing the pathological and physiological functions of enzymes in biological systems [36]. Among the sensing methods, ratiometric fluorescence sensing has received particular attention as a technique with the potential to provide precise and quantitative analysis [37]. These techniques have been extensively used for β-galactosidase detection in human cells. β-galactosidase detection in human cells is often accomplished through the use of near infrared (NIR) fluorescent probes in which β-galactoside moiety gets cleaved and later transformed into a fluorescent compound (Fig. 3) [22]. These probes are typically used in the cancer cells (*e.g.* HeLa and OVCAR-3 cell lines), as they are the useful indicator of the irregular overexpression of certain genes, such as the LacZ operon. Fluorescent probes utilize β-galactosidase's enzymatic activity to cleave fluorescently active compounds from β-galactoside probes *in vivo*, making them an useful visualization tools for gene expression in living cells (Fig. 4) [26]. Along with *in vivo* visualization, fluorescent probes offer simplicity, cost effectiveness, high sensitivity, real-time detection of enzymatic activity, and a non-invasive means of disease diagnoses [22]. Many probes have been developed, and each aims to increase the efficacy of detection or to eliminate a specific problem inherent of fluorescent probes.

A common issue with fluorescent compounds, such as rhodol is their tendency towards aggregation-caused quenching (ACQ), a concentration-dependent phenomenon that limits a probe's utility. Peng et al. described a light-up probe, SA-β-Gal, that is active under aggregation induced emission (AIE) and excited-state intramolecular proton transfer (ESIPT) processes [26]. Through the cleavage of β-galactopyranoside blocking groups by β-galactosidase, the hydroxyl groups of the compound salicylaldehyde azine becomes fluorescently active at 545 nm, offering up to an 820-fold increase in fluorescence activity with a

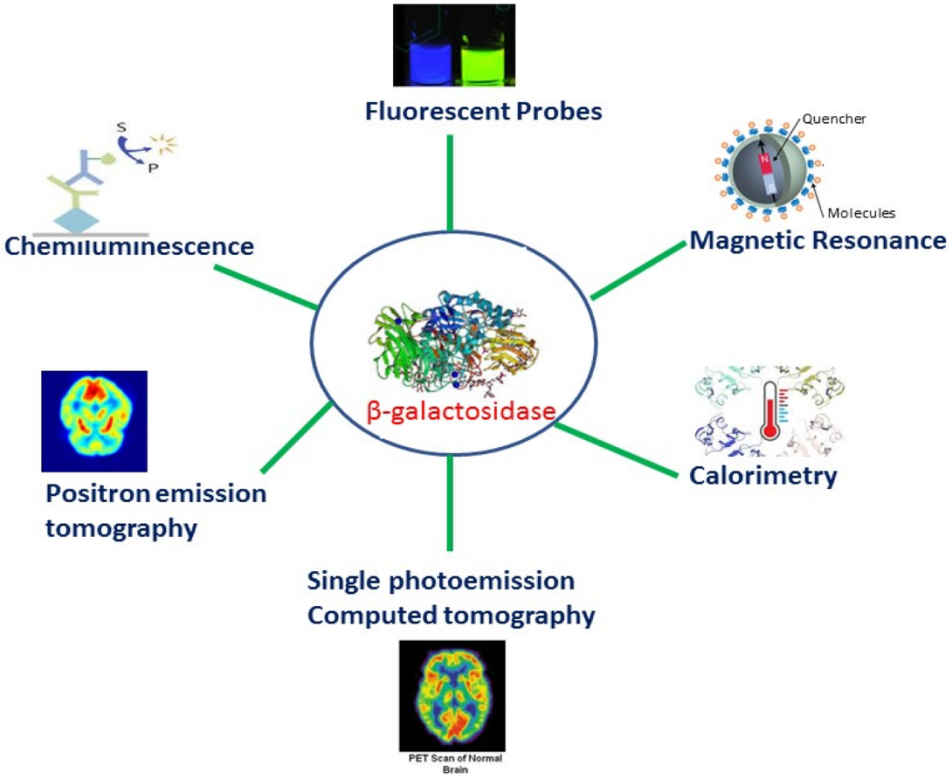

Fig. 1. Different techniques of detection of β-galactosidase enzyme.

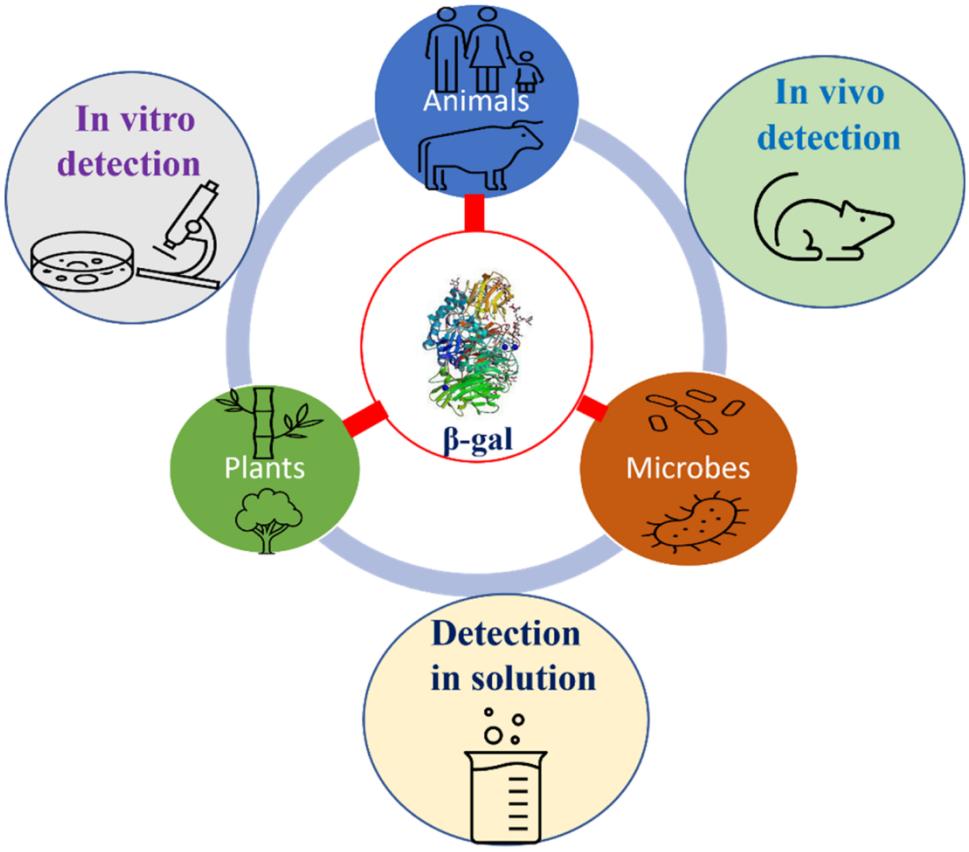

Fig. 2. Approaches used in the detection of β-galactosidase in animals, plants, and microbes.

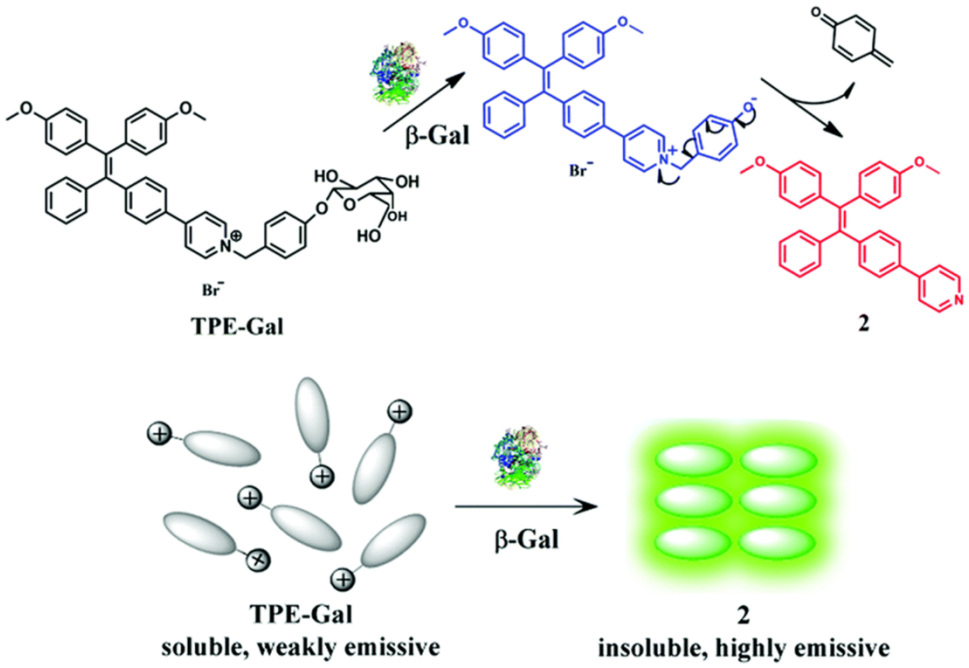

Fig. 3. Fluorescent probes that allow detection of β-galactosidase in living cells through the use of a cleavable β-galactoside moiety. Here, TPE-Gal is shown to undergo two transformations, one from β-galactosidase and the other from an intramolecular conversion, giving rise to fluorescently active compounds [22] (Reprinted with permission from Chem. Comm., RSC, 2017).

strong Stokes shift (190 nm) in AIE. This probe combined with these processes, allowed effective *in vitro* visualization of β-galactosidase in HeLa cells.

In a continuation of research described by Peng et al., Jiang et al. described the possibility of the use of ACQ avoiding probes based on tetraphenylethylene.[22] The developed probe, TPE-Gal, used the same

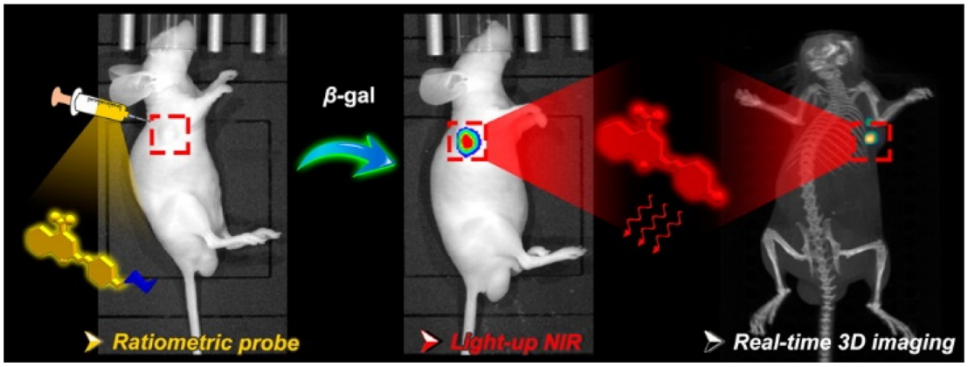

Fig. 4. Schematic representation of the utilization of *in vivo* fluorescent probes to monitor β-galactosidase activity in tumor-bearing nude mice [38] (Reprinted with permission from ACS – JACS, 2016).

β-galactopyranoside as SA-β-Gal, but employed the TPE moiety due to its simple structure and ease of synthesis and modification. This probe showed similar benefits as SA-β-Gal, in such a way that its cell permeability is high, showed a large Stokes shift (168 nm), high specificity and low cytotoxicity. This probe also allowed for the detection of β-galactosidase as low as 0.33 U mL^{-1}. Unlike with the SA-βGal probe, TPE-Gal was shown to be effective in ovarian carcinoma (OVCAR-3) cells as opposed to HeLa cells.

Other research into the detection of β-galactosidase in living cells has focused on the detection of the enzyme's activity *in vivo* using mouse models. Xenografted human cancer cells allowed researchers to study visualization techniques in whole systems which should indicate applicability to humans. The ability to detect enzyme activity is crucial to the development of cancer diagnostic techniques, but is difficult due to the complexity of living systems and the high auto-fluorescence of living cells [38,39]. Another *in vivo* probe was explored by Asanuma et al., showing that the probe HMRef-β-Gal could be used to detect in real-time and with the naked eye, to the cancerous metastases from seven human peritoneal ovarian cancer cell lines [40]. The effectiveness of the probe was derived from its intramolecular spirocyclic function and strong cell permeability. This probe offered a greater than 1400-fold increase in fluorescence. The rapidity of detection of this method, around five mins after being first administered, is promising for pre and possibly intra-operative malignancy detection. This technique offers great advantages over magnetic resonance imaging techniques.

At the same period, Zu and his co-workers developed a fluorescent probe to study *in vivo* and *in situ* β-galactosidase activity following the research on HMRef-βGal [25]. The work was based on the intramolecular charge transfer (ICT) mechanism for tracing β-galactosidase in living cells. Their research focused on real-time detection of β-galactosidase activity. Synthesis of the probe involved the grafting of a β-galactosidase activatable unit onto the DCM-OH (dicyanomethylene-4H-pyran) moiety. They claimed that this probe was the first developed to provide a real-time, high resolution, three-dimensional view of tumors in a human colorectal tumor mouse model. As per the data, their probe also showed effectiveness in ratiometric tracking (Fig. 5) of the overexpression of β-galactosidase in 293 T cells using the LacZ gene transfection method and OVCAR-3 cells. Han group also developed the similar probes [23]. Nevertheless, these probes were limited by their sensitivity and long-time response. Kong et al. developed fluorescent probe based on ICT-FRET (fluorescence resonance energy transfer) mechanism in which the response rate was faster as compared to the previous methods [41]. They also used OVCAR-3 cells in their experiment and tried to increase the signal output by using the synergetic mechanisms of ICT-FRET. The developed probe showed a fast response (< 20 s) as well as low detection limit (0.081 U.mL^{-1}).

Following the work done by Zu and co-workers, Kim et al. developed a probe for the detection of hepatocellular carcinoma (HepG2) *in vivo*, using a mouse model [39]. The probe was acted up by intracellular

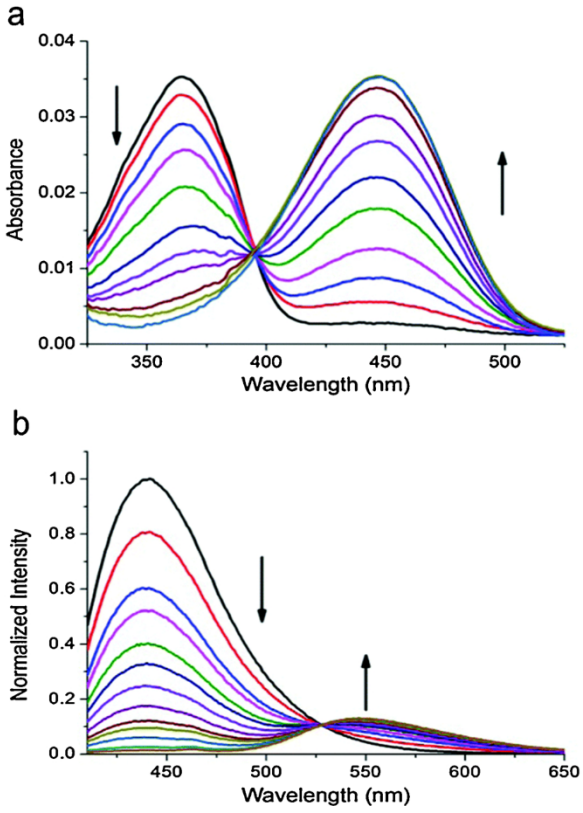

Fig. 5. Ratiometric tracking of enzymatic activity that uses two absorbance peaks and their changes in intensity over time to monitor the activity β-galactosidase. As β-galactosidase cleaves a fluorescent probe, the fluorescence of the cleaved fluorescently active moiety will increase as the fluorescence of the original probe will decrease [23]. (Reprinted with permission from RSC Chemical Communications, 2016).

β-galactosidase in a manner similar to previously described probes, but did so in cells that have the ligand ASPGR, allowing for a selective means of targeting hepatocellular carcinoma cells. This selective probe had a defect that caused a fluorescence emission shift, leading a ratiometric decrease in the fluorescence band at 615 nm, and promoting a novel band at 665 nm, manifesting the presence of β-galactosidase. Besides that, it also provided a light-up ratio of 10.7, showing a strong indication of enzymatic activity. Key characteristics of this probe included its ligand dependent specificity, insignificant cytotoxicity, and independence from autofluorescence.

In vitro studies into the detection of β-galactosidase in ovarian carcinoma cells have also been conducted. One such study specifically

targeted the lysosomal production of β-galactosidase [42]. Using a 4-propylmorpholine group to target lysosomes, and a 1,8-napthalamide fluorophore for imaging, the probe FC-βgal was found to be an effective means of detection. This probe caused a stark augmentation in fluorescence at 560 nm, providing a greater than 1000-fold increase in fluorescence intensity and a limit of detection of $4.0E^{-5}$ U. mL^{-1}. This probe was also found to be useful with two-photon confocal imaging.

Now-a-days, efforts concerning the detection of β-galactosidase have also focused on human diploid fibroblast cells, as pursued by Zhang et al. [43]. Their probe also used an NIR active compound connected to a β-galactoside, and was specifically designed for the detection of senescence-associated β-galactosidase. Senescence-associated β-galactosidase is synonymous with lysosomal β-galactosidase, thus indicating a difference in origin, not function. Their research employed the premature senescence model to induce a higher concentration of β-galactosidase and found that a strong fluorescent peak at 703 nm occurred as a function of the probe. The probe was found to be effective at detecting the β-galactosidase intrinsic to senescent cells, but the response rate was comparatively slow.

Very recently, Yang group has used a small molecular fluorescence dye with excimer emission property [44]. As the conventional small molecule fluorescent dyes like fluorescein, cyanine, rhodamine have a disadvantage of fluorescence quenching at higher concentration, they used a cyanovinylene dye. The fluorescence property of this dye is not quenched at higher concentration and is suitable in bioimaging. The developed probe showed low limit of detection (LOD) of 0.17 U. mL^{-1}. However, the selectivity and the response rate were comparatively low.

2.2. Use of magnetic resonance

The detection of β-galactosidase can also be accomplished through the use of magnetic resonance imaging. Both hydrogen (^{1}H) and fluorine (^{19}F) isotopes are responsive to magnetic fields and allow the possibility of *in vivo* detection of enzymatic activity [45]. Using substrates with β-galactosides, β-galactosidase can be used to catalyze color-producing reactions that indicate the presence of this specific enzyme. Modifications to existing substrates can allow them to be used as detection methods for magnetic resonance imaging.

S-Gal is a standard reporter molecule in β-galactosidase staining methods. A reporter molecule has been developed through the testing of various S-Gal analogs [46]. The most successful of these analogs, C3-GD, operates through the separation of a β-galactopyranoside from an aglycone to form a paramagnetic chelate with Fe^{3+}. These chelates were shown to cause both T_1 (1.12 s) and T_2 (45 ms) shortening in MR images both *in vivo* (LacZ-transfected MCF7 breast tumors in mice) and *in vitro* (Fig. 6). Various other reporters were shown to work *in vitro* but were not selected for *in vivo* studies due to their poor water solubility. In a later experiment performed by Yu et al., a $^{1}H/^{19}F$ NMR reporter molecule was developed with similar chelate effects to show human xenografted tumors (PC3 prostate) in nude mice [47]. Out of their many developed

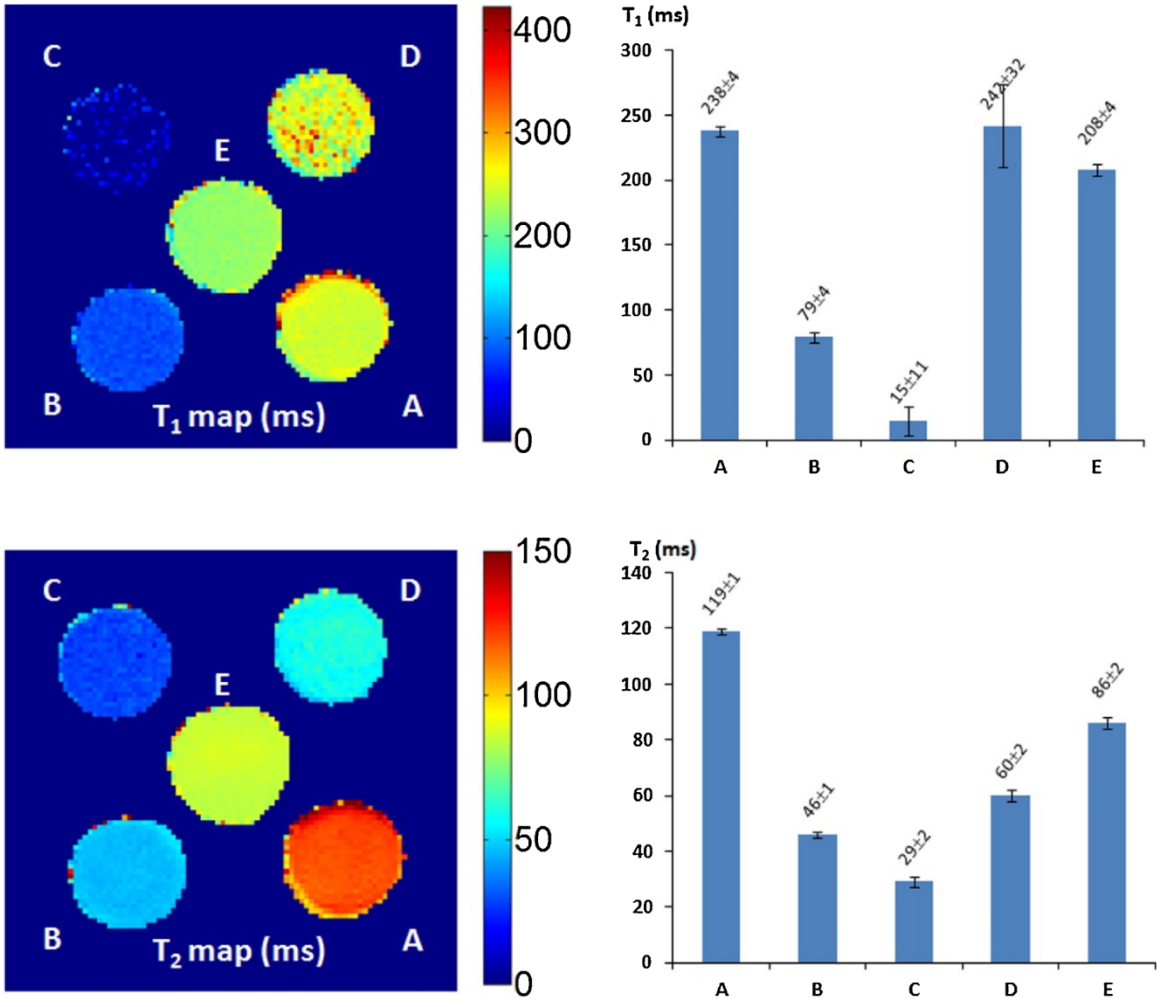

Fig. 6. Monitoring β-galactosidase with magnetic resonance techniques (^{1}HMRI pictured). Mapping of T_1 and T_2 relaxation processes can be used to show varying levels of enzymatic activity by monitoring the relaxation times inherent in magnetic resonance imaging techniques [48]. (Reprinted with permission from ACS Publication, 2013).

compounds, 13–15 showed $\Delta\partial_F$ values with the range 5.23~7.65 ppm. PC3 and MCF7 cells were used *in vitro* to show the reporter's efficacy.

This novel unnamed reporter improved upon previous reporters by incorporating ^{19}F into the reporter's structure but was found to be very slow acting and weakly permeating *in vitro,* and quick *in vivo* due to intratumoral injections. Fluorescence activated cells (FAC) was used in both studies and employed intratumoral injections for *in vivo* studies as a proof of the agents' abilities to contrast, as drug delivery was not the focus of the research. Chelation agents have also been explored due to tumors' that have naturally high abundance of ferric ions [48]. This work proposes a molecular platform that combines chelation based tumor treatment and imaging through ^{1}HMRI T_1 and T_2 relaxation mapping. Through the combination of a chemical reporter and chelating agent, the proposed platform would lead to tumor cell-cycle arrest by ferric ion depletion as well as the ability to visualize tumors *in vivo* through the use of paramagnetic chelate complexes.

MR imaging focusing on ^{19}F NMR has been of interest recently due to the need for highly sensitive and deep tissue reaching methods of β-galactosidase detection, which is impractically done with fluorescence techniques and has been reliably done with magnetic imaging methods [49,50]. Hu et al. have developed a low-weight (274.2 g.mol^{-1}) ^{19}F NMR probe for the detection of OVCAR-3, HeLa, and *E. coli* cells *in vitro* [51] (Fig. 7). These low-weight probes aid in cell permeability and avoid biological background noise. This probe named as FB-β-gal, is highly water soluble and capable of providing a unique, rapidly produced signal at a chemical shift of 124.8 ppm and a $\Delta\partial_F$ of -3.7 ppm, indicating a promising means of detection in future *in vivo* experiments.

Other fluorine-dependent magnetic resonance detection methods have been developed using various platform-integrated fluorinated antitumor nitrogen mustards by Yu et al. [52]. They described one of the developed probe for ^{19}F-MRS, 2-fluoro-4-[bis(2′-chloroethyl)amino] phenyl β-D- galactopyranoside. The probe was found was found very sensitive to β-galactosidase in PBS with the rate, ν = 738.4 μm.min^{-1}unit^{-1} and a Δδ response of ~4 ppm. This method was shown to be successful in the detection of β-galactosidase *in vitro* for LacZ transfected human MCF7 breast and PC3 prostate tumor cells. But the selectivity of the probe was not studied so far.

Another study that used magnetic resonance imaging of β-galactosidase has focused on improving the technique's ability to show enzymatic activity *in vivo*, as the researchers claim that current *in vivo* fluorescent techniques provide poor spatial resolution and inability to visualize deep tissue [53]. Their technique involved a chemical exchange saturation transfer (CEST) MRI to avoid relaxation control and shift issues inherent in current methods. The method, catalyCEST MRI, has been reported to be effective both *in vivo* and *in vitro* when using diamagnetic CEST agents to detect and quantify glycoside hydrolase enzymes. In a follow-up UV–vis assay, the CEST agents for β-galactosidase were shown to provide a 3.8-fold increase in absorbance, indicating strong detection sensitivity. This work can be worthy for future *in vitro* validation and *in vivo* translation to establish the method as a reliable source of detection although the response rate of the probe was slow.

2.3. Other uncommon methods

There are some methods of detection of β-galactosidase which are not carried out frequently. For example, chemically initiated electron exchange luminescence (CIEEL) has been explored as a means of *in vivo* β-galactosidase detection. The process is accomplished through the use of metastable 1,2-dioxetane substrates, which are activated endogenously through β-galactosidase interactions [54]. This method provides better signal to noise ratios than fluorescence can offer due to chemical substrates as energy sources as opposed to the pharmacokinetic dependent mechanisms of fluorescent probes. This technique offers advantages of fluorescence, luminescence, and has shown to be effective in transfected colon cancer (HCT116) cells in mice.

Another uncommon method is the use of carbon dots for the detection of β-galactosidase. Carbon dots are newly synthesized nanoparticles which are biocompatible and photostable. These photostable nanoparticles have also been recently explored as a means of β-galactosidase detection. They are said to offer advantages over molecular fluorophores due to their high biocompatibility, modularity and stable light emission [55]. Through the use of β-cyclodextrin modified carbon quantum dots (β-CD-CQDs), a host-guest recognition and static quenching mechanism was developed for specific enzyme targeting and detection. This method was shown to provide high-quality and real-time visualization of *in vitro* ovarian cancer cells with a limit of detection of 0.06 U mL^{-1} (OVCAR3). However, the toxicity of the carbon dots has not been studied in this research.

Colorimetric staining assays are a classic form of β-galactosidase detection. There have, however, concerns with the efficacy of standard staining methods due to their lack of specificity in certain cases [56]. It has been claimed that a marker, EdU, a thymidine analog, may be used to mark cell proliferation. Other stains check for SAHF and add-in stains for other senescence associated protein markers that may help to limit confusion with standard SA-βgal (senescence-associated) staining assays both *in vivo* and *in vitro*.

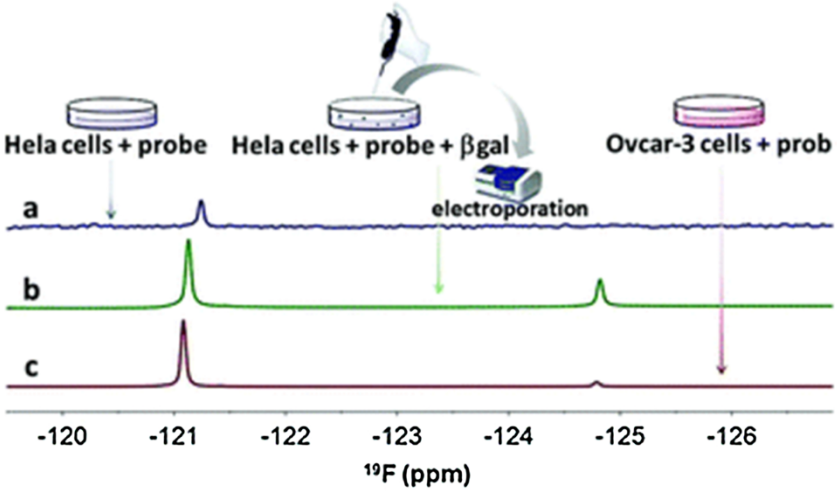

Fig. 7. The use ^{19}FNMR probe peaks and chemical shifts to monitor changes in the chemical composition of compounds. Fluorine probes use the enzymatic activity of β-galactosidase to create novel peaks as a function of β-galactosidase (b,c) activity, allowing a means to distinguish the presence or absence (a) of the enzyme of interest [51]. (Reprinted with permission from RSC Publication, 2017).

Shlush et al. addressed another issue with colorimetric assays through research on the detection of senescence associated β-galactosidase [57]. Current assays are unspecific in the extent of gene expression, preventing differentiation between weakly and strongly β-galactosidase expressing cells. This required the reseachers to develop a cell staining intensity (CSI) quantitative method based on arbitraty intensity values related to a ratio of pixel colors in digitally processed images. *E. coli* when analyzed with cyclic voltammetry provides a limit of detection of 5 ng mL^{-1}, while differential pulse voltammetry provided an limit of detection (LOD) of 1 ng. mL^{-1}. For *A. oryzae*, an LOD using cyclic voltammetry was found to be 0.06 ng mL^{-1}, while DPV had an LOD of 8 pg. mL^{-1}.

Recently, metal-to-ligand charge-transfer (MLCT) has been developed as a method of β-galactosidase detection. Through the enzymatic hydrolysis of p-aminophenyl-β-D-galactopyranoside, Fe^{3+} may be reduced to Fe^{2+}, which in turn complexes with a BPDS ligand to create an Fe(BPDS)34- reporter [58]. This reaction causes an intense clear to red transition and the development of a strong absorption peak at 535 nm. This reaction was tested with complex human serum samples and carcinoembryogenic antigen and was found to be effective in both cases providing a detection limit of 1.69 mU mL^{-1}. When integrated with the sandwich immunoassay of carcinoembryonic antigen, a detection limit of 1.16 ng mL^{-1} was observed. In this work, the selectivity and the response rate of the probe have not been discussed so far.

3. Detection of β-galactosidase in bacterial cells

3.1. Fluorescence techniques

Fluorometric techniques are also considered useful for the visualization of β-galactosidase activity in bacterial cells. A fluorescent probe, AcGQCy7 (acetylated GQCy7), was utilized to permeate cells in more effective manner [21]. Hydrolysis of this compound by endogenous β-galactosidase allows visualization of endogenous enzyme activity. This probe served to solve the common issue of diffusion of probes across cell membranes that reduce the specificity of targeting compounds. The probe produced a thiol-dependent signal at 560 nm but suffered from NIR disappearance issues that reduced the applicability of the probe to animal studies. However, it did provide a 20-fold increase in fluorescence when in the presence of the thiol cysteine.

Further study into fluorometric assays of bacterial cells have been conducted to improve β-galactosidase detection in gram-positive (*L. helveticus*) bacterial cells, as other existing assays had been ineffective with this category of bacteria [59]. This task was accomplished through the use of lactose activation mediums for cell growth, followed by the use of a β-galactosidase substrate, 4-methylumbelliferyl β-d-galactopyranoside (MUG). With the use of a plate reader to obtain fluorescence signals from the cultured bacteria, both gram-positive and probiotic cells were assayed. The detection limit of this method was relatively poor.

Chlorophenol red-β-galactopyanoside (CPRG) is a commonly used substrate for colorimetric assays. Specifically, this substrate has been improved due to the large fluorescence response caused by liberated chlorophenol red's (CPR) complexation with poly-L-arginine (pR) solution due to the sulphonate group of CPR [60]. Developed probe, CPR-pR, was intended to be good alternative to the commonly used substrate MUG (associated with short-wavelength dyes). This substrate provided a 70-fold enhancement of fluorescence and a limit of detection of 0.005 U mL^{-1} to 0.0004 U mL^{-1}, depending on incubation time. This long-wavelength dye avoids UV interferences associated with commonly used short-wavelength dyes, and has been demonstrated effective with *E. coli*. Apart from this, advancements in gram-negative bacterial fluorescent probes have been made through the development of a ratiometric, two-photon probe called NI-β-Gal based on a 1,8-naphthalamide platform. The probe established a 680-fold enhancement of fluorescence in *in vitro E. coli* cells and was confirmed to be capable of *in vivo* detection in stable mice tumors. This probe also provided large Stokes shifts (good for *in vivo* imaging) and low cytotoxicity, indicating promising *in vivo* uses in the future.

3.2. Other methods

Electrochemical methods of detection have also been developed to test their efficacy *versus* standard β-galactosidase assays (Fig. 8). One such assay states that β-galactosidase activity can be detected rapidly in both whole and lysed cells, providing an alternative to the standard spectroscopic methods of detection [61]. The method involved the use of a 4-aminophenyl β–D-galactopyranoside (PAPG) substrate, which upon entering the cell releases a redox reactive molecule that is quantifiable through cyclic voltammetry. The use of a biosensor bacterium that converts the signaling function of AI-2 to electronic signals, combined with the aforementioned enzyme-substrate system, allowed the detection of β-galactosidase *in situ* in a manner comparable to standard assay methods.

A novel 4-methoxyphenyl-β-galactopyranoside (4-MPGal) electrochemical substrate for bacterial (*E. coli* and *A. oryzae*) β-galactosidase has been developed [62]. This development was similarly inspired by limitations inherent in optical measurements and the substrate was found highly sensitive and specific in the detection of β-galactosidase in the two bacterial cells of interest, and also allowed the real-time detection of β-galactosidase activity *in vivo*.

Other research efforts have focused on reducing the complexity of existing assay methods, aiming for a faster and safer alternative to traditional methods [63,64]. Schaefer et al. determined how to reduce the complexity of the standard Miller Assay by reducing handling time by 90 % 64]. This was accomplished by decreasing the number of liquid handling steps necessary due to the interferences of certain organic solvents such as chloroform. The resultant method required one step and offered rapid cell permeabilization with no significant loss of effectiveness. Toulouse et al. has also recently developed a method to avoid chloroform [65]. Using *V. cholera,* their method using two detergents, CTAB and sodium deoxycholate, demonstrated a chloroform-free means of β-galactosidase assay. This technique resulted in 2.5 times increase in LacZ activities as compared with the traditional assay.

4. β-Galactosidase detection in embryos

The coding protein of β-galactosidase, LacZ, can be inserted at a genetic locus coding for a gene of interest [66]. The promoters of this gene will also drive the expression of β-galactosidase, allowing a means of monitoring the activity of a gene through β-galactosidase as a proxy which has been useful for monitoring embryonic regulatory genes to monitor temporal changes in their expression (Fig. 9).

Staining with X-gal/FeCN is a standard method of determining the presence and activity of β-galactosidase [67]. This method is analogous to staining with S-gal/TNBT and has lower background noise, but has lower sensitivity. Existing claims state that an X-gal/NBT (nitroblue tetrazolium) method that replaces standard KCN/FeCN salts with tetrazolium salts is the best detection method, with S-gal/NBT only being necessary when time constraints are of high priority [68]. Although, the most common LacZ staining methods used are X-gal/FeCN staining and Sgal/TNBT staining, a serious limitation of both of these methods is that they are not effective when the LacZ gene is expressed at a low level.

Researchers have attempted to remedy this problem. They have established a new staining protocol which combines both methods. Recently, Shen et al. determined that a succession of X-gal/FeCN followed by an extra chromogenic S-gal/TNBT step that would allow for the detection of β-galactosidase driven by weak promoters in mouse embryos [69]. This method showed 100 % accuracy for correct staining, indicating a high level of reliability. They have claimed that their method is better for visualizing lowly expressed genes and it has low

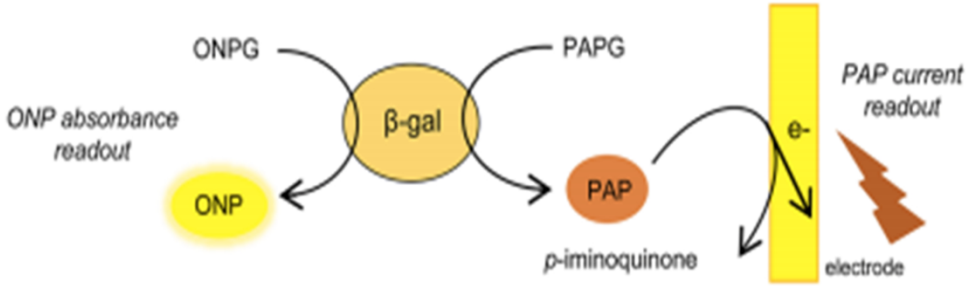

Fig. 8. Difference of electrochemical and fluorescence techniques for the utilization of substrates to obtain a measurable signal. Standard fluorescence methods (left) utilize substrates, such as ONPG, that have fluorescently active groups and are cleaved by β-galactosidase. Electrochemical substrates, such as PAPG, are acted upon similarly, but have electrochemically active redox reactive moietys [61]. (Reprinted with permission from ACS Publication, 2015).

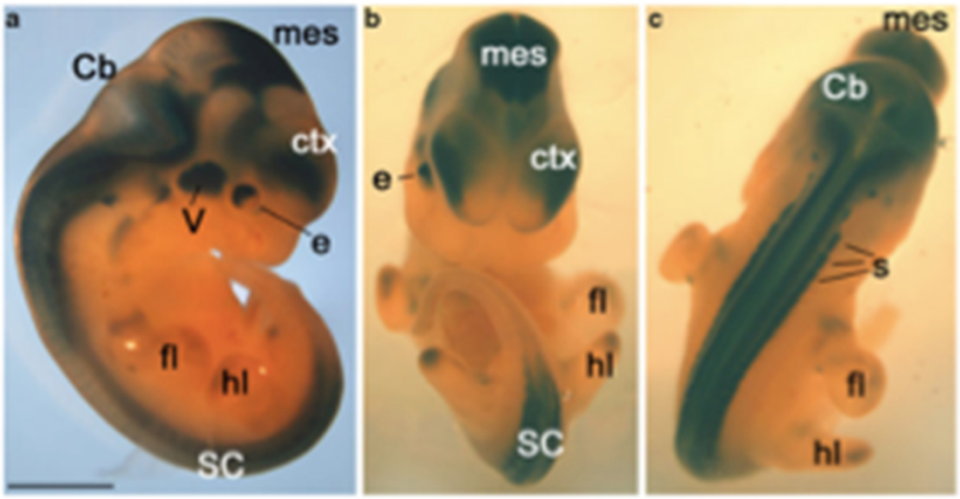

Fig. 9. *In vivo* imaging of embryonic β-galactosidase using the standard X-gal staining method to monitor the temporal changes in their expression [66]. (Reprinted with permission from Springer, 2013).

background noise with high sensitivity. Moreover, this method builds on the original development of the S-gal/TNBT detection method in a successful effort to detect β-galactosidase in early stage mouse embryos (Fig. 10) while avoiding the false readings given by the established X-gal method [70].

β-galactosidase detection in human embryos (oocytes) is of importance for fertility research purposes and has been studied by Edwards et al. [71]. They found that β-galactosidase is detectable in transcriptionally active oocytes and embryos through the use of fluorescein-di-galactoside (FDG) staining and confocal microscopy.

5. Methods of detection of β-galactosidase in mouse cells

As β-Galactosidase enzyme is encoded by lacZ gene, it is usually detected by using X-gal staining. Doing so a blue indole precipitate is formed that makes easy to detect visually. In the mouse, the bacterial lacZ gene is frequently used as a reporter in a many mouse transgenic experiment. So, many assays have been developed that provide a more sensitive and faster staining reaction than the traditional β-galactosidase assay in mouse embryos. β-galactosidase detection in hard tissues (also known as calcified tissue) is impractical due to laborious demineralization steps needed under standard protocols (*e.g.*, X-gal/FeCN). Using an (*Anxa5*)-*lacZ* reporter mouse model (specifically the periodontal ligament), Shimada et al. showed that fixation of hard tissues in 100 % acetone could be used to stain and analyze whole adult hard tissues [72]. The work by these researchers confirms expedited staining process for the use of difficult to stain tissues.

Kasper et al. described the protocol for the isolation of β-galactosidase expressing murine epidermal cells from genetically engineered mice [73]. Using fluorescein di-β-D-galactopyranoside (FDG) as a probe for β-galactosidase, keratinocytes may be detected and separated using fluorescence-activated cell sorting (FACS). Micro-chip capillary method has also been employed to measure the activity of β-galactosidase [74]. This method was based on the measurement of the activity of β-galactosidase enzyme (often used as a marker for coliforms) by the measurement of 4-aminophenol, which is produced upon hydrolysis of the substrate 4-aminophenyl-β-D-galactopyranoside (PAPG) by β-galactosidase. The detection limit was found to be 10 CFU mL^{-1} and has been considered higher than some other methods.

6. Methods of detection of β-galactosidase in plants

β-galactosidase plays an important role degradation of fruit [75]. It occurs due to the cell wall expansion mechanism assisted by this enzyme. It has also been found that the activity of β-gal positively correlates with the maturity of different fruits [76–79]. As β-galactosidase plays a crucial role in plant growth and development, it is necessary to survey the expression profiles. However, few works have been reported in the detection of β-galactosidase in the plants. Very recently, Sun and co-workers have developed a fluorescent probe for the ratiometric detection of β-galactosidase in fruit [80]. The probe uses benzothiophene-naphthalene as the fluorophore and β-galactosides as the reaction group. The probe detected β-galactosidase activity in the range 0–1.0 U. mL^{-1}, with a limit of detection of 0.025 U. mL^{-1}. The authors do not mention about the selectivity of the probe. Nevertheless, this method is relatively better than the other methods as they have high

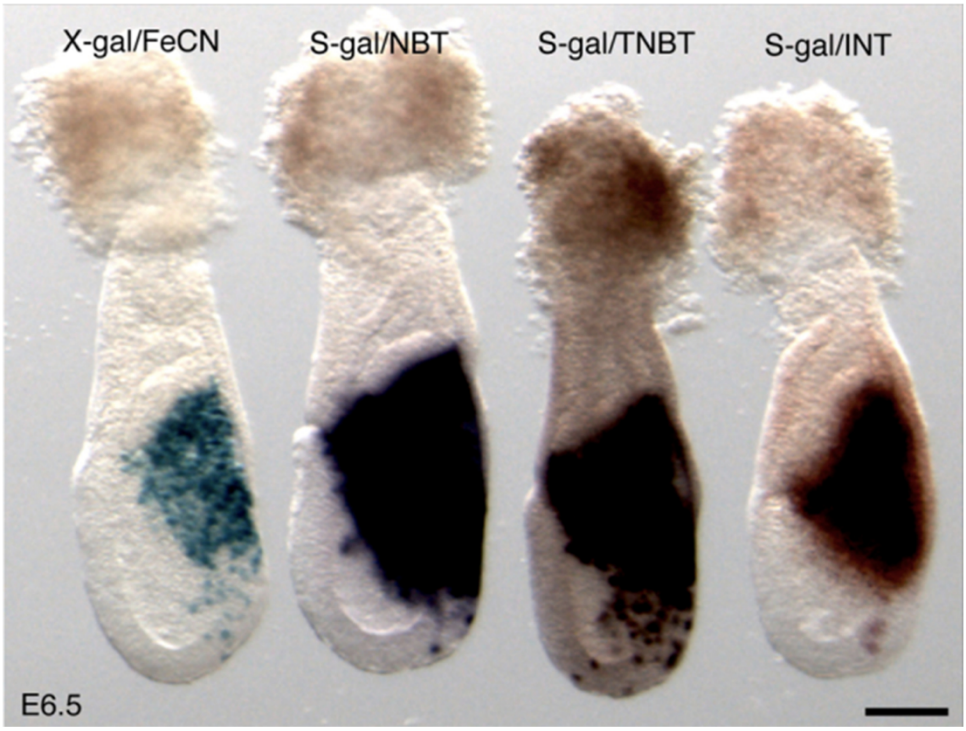

Fig. 10. Staining comparison of mouse embryos between various tetrazolium salts/S-gal and the standard X-gal/FeCN staining method. (Reprinted with permission, ACS Publications, 2016) [70].

precision and accuracy of detection levels. The mechanism of sensing process is depicted in Fig. 11.

7. Comparison of different methods with higher performance

Despite the significant advances in the design of fluorescent β-galactosidase probes, limited reports concerning the real-time monitoring and detection of early to middle stages of cellular senescence has been considered. There are many researches in the detection of activity of β-galactosidase. Most of the techniques that are put forward have average performance. It is utmost important to compare different methods with the most effective performance. Table 1 compares the best methods with performance that are used so far. *In vivo* detection of β-galactosidase activity based on systemic administration of reporter molecules has been achieved using a tandem approach based on bioluminescence of Lugal (6-o-β-galactopyranosyl-luciferin) following intraperitoneal (IP) administration [81]. However, this approach required doubly transfected cells, whereby β-gal (lacZ expression) releases luciferin, which becomes a substrate for luciferase. The most widely used approach currently exploits fluorescence to detect a 50 nm shift accompanying β-gal activated cleavage of DDAOG (7-hydroxy-9H-(1, 3-dichloro-9,9-dimethylacridin-2-one-7-yl) β-D-galactopyranoside) revealing β-galactosidase activity in stably transfected human tumors in mice following IV administration [35] but this method could not be used to detect LacZ expression *in vivo*.

Recently, the use of two photon fluorescent probes in the detection of β-galactosidase was developed [82]. Besides the living cells, this probe was used in the selective detection of β-galactosidase in the tissues. The tumor tissue slices of thickness 400 mm were treated with one photon and two photons to know the properties. The use of two-photon fluorescence microscopy has the superlative advantages of being used for 3D fluorescence imaging in thick biological tissues because of the characteristics of local volume excitation. The two-photon probe was able to sense the depth of penetration 150 μm in the tissue. Although, the probe has the advantages of rapid response, great fluorescence enhancement and superior specificity but lacks the high selectivity required in the detection of the enzyme in the living cells.

8. Near-infraed (NIR) fluorescent probes are dominating the other detection methods

From the past, different methods for detecting β-galactosidase such as colorimetry, electrochemistry, single photon emission computed tomography and positron emission tomography have been used. But these methods are not appropriate for real-time, in-situ and nondestructive use in the biological systems. So, different fluorescent assays were

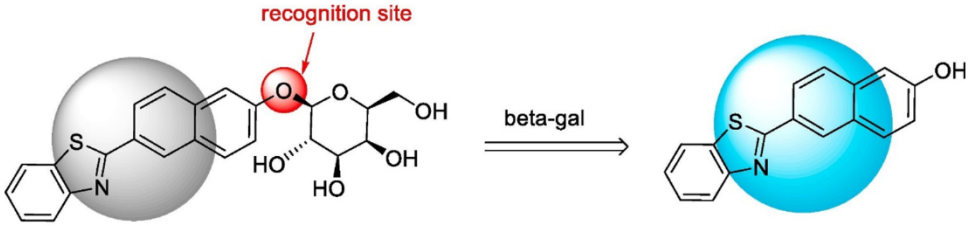

Fig. 11. Mechanism of detection of β-galactosidase. The sensing process mechanism of the probe (left) for β-galactosidase utilizes a hydrolysis reaction of β-galactosides to generate compound (right) (Reprinted with permission, Elsevier, 2020) [80].

Table 1
Comparison of different methods of β-galactosidase detection.

SN	Type of Probe	Characteristics	Detection Level	Uses	Ref.
1	Fluorescent probes (acetylated form of GQCy7)	1.Intracellular imaging 2.Prolonged retention of the intracellular fluorescence signal	GQCy7 produced a 110-fold increase in fluorescence with a peak at 680 nm in the presence of β-galactosidase.	Visualization of β-Gal activity in living cells for longer than 3 days	[21]
2	TPE-Gal fluorescent probe	Aggregated Induced Emission (AIE) responsive mechanism	0.33 U/mL	Imaging endogenous β-galactosidase activity in OVCAR-3 cells.	[22]
3	Near infrared fluorescent probe	High photostability, and pH independency; Km= 60.07 μM	1.7×10^{-4} U. mL^{-1}	Real time *in vivo* bioimaging of β-galactosidase activity in colorectal tumor-bearing nude mice	[25]
4	Bioluminescent probe: sequential reporter-enzyme luminescence (SRL)	Enzyme retains full activity outside of cells; detection of extracellular β-gal activity	LacZ-luc cells generated strong luminescent signals at Lugal concentrations as low as 10 μg/mL	Use of firefly luciferase (FLuc) to generate light.	[81]
5	Far red fluorescent imaging probe	Uses DDAOG, a conjugate of β-galactoside and 7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one) (DDAO)	β-gal-expressing 9 L gliomas are readily detectable	*In vivo* real-time imaging of β-galactosidase activity	[35]
6	Fluorescent probe	ICT-FRET based mechanism	0.081 U. mL^{-1}	Detection of β-galactosidase *in vitro* and *ex vivo*	[41]
7	Near-infrared fluorescent probe	Hemicyanine- D-galactose conjugation; $K_m = 3.6$ μM	0.057 nM	Detect β-galactosidase in living cells	[43]
8	Optical imaging *via* chemiluminescence probe	β-gal activity monitored in human transfected MCF7-lacZ tumors in mice	Tumors expressing β-gal were detected in short time	Non-invasive reporter techniques to assay lacZ gene expression *in vivo*	[14]
9	Microchip capillary electrophoresis (MCE) combined with laser-induced fluorescence (LIF)	The enzyme activity is detected by measuring 4-aminophenol (produced upon hydrolysis of the substrate 4-aminophenyl-β-D-galactopyranoside (PAPG) by β-gal	10 Colony forming unit (CFU). mL^{-1}	Detection of *E. coli* with high sensitivity in the samples.	[74]
10	Two photon fluorescent probes	Uses xanthene-1-one (GCTPOC) as a two-photon chromophore	Depth of penetration of the probe in the tissue was 150 μm	Employed for living cells and tissues	[82]
11	Calorimetry	1. β-Galactosidase-tannin molecular interaction were evaluated 2. Tannic acid reduces the affinity of β-galactosidase for its substrate	0.33 μmol L^{-1} tannic acid caused a decrease in the ΔH and ΔG from -842.8 ± 39 to -566.2 ± 59 kJ mol^{-1} respectively	Relates the use dairy products consumed with tannin-rich food	[83]

regarded as a valuable method detection of enzymes because of their noninvasiveness, high sensitivity and real-time imaging capability [84]. These fluorescent probes are also useful for on-site sensing and long-term tracking of specific biomarkers is particularly desirable for the early detection of diseases.

Nonetheless, these fluorescent probes exhibit emission in the ultraviolet or visible range, and do not circumvent the autofluorescence interference of bio-systems. So, their application *in vivo* imaging has been limited. The increasing use of near-infrared (NIR) probes effectively compensates for this deficiency. Now-a-days, for the imaging of tissues *in vivo*, fluorochromes whose absorption and emission maxima lie in the near-infrared (NIR) region (650–900 nm) are preferred because this wavelength region offers minimal interference from biomolecules, low autofluorescence, and good tissue penetration, as well as low phototoxicity to cells. Similarly, long-wavelength emitting probes minimize tissue background fluorescence (interference from biomolecules), favors low autofluorescence and light scattering during fluorescence imaging, providing more accurate imaging in living systems. That is why, developing NIR-emission fluorescent probe with high selectivity and noninvasive imaging of β-gal in biological systems is of great significance.

In this perspective, Li and co-workers designed a probe called DXM-βgal that consisted of a NIR fluorophore (2-((6-hydroxy-2,3-dihydro-1H-xanthen-4-yl)methylene)malononitrile (DXM-OH) and a specific recognition group of β-gal (β-d-galactopyranoside) for the visualization of endogenous β-galactosidase activity in living cells (human ovarian cancer cells) and zebrafish with a turn-on near-infrared fluorescent probe [85]. The probe detected B-galactosidase with high selectivity and a low limit of detection (2.92×10^{-4} U mL^{-1}). This limit of detection was higher than the data presented by Gu et al[25] where both groups used NIR probes. Further, there was no mention about the significance of use of whole zebrafish rather than targeting a specific organ of the zebrafish in their study. The pitfalls of this wholistic study were not mentioned at all.

Nagano and co-workers also used near-infrared fluorescence probes for enzymes based on binding affinity modulation of squarylium dye [86]. In their research work, they used NIR fluorescence probes utilizing dye–protein interaction as a trigger for fluorescence enhancement. Although the idea was new, but the effectiveness of the probe was jeopardized as the important properties like selectivity, sensitivity and limit of detection data was not presented.

Although some other methods like use of enzyme-activatable aggregation-induced emission (AIE) probes (that allow for on-site sensing of endogenous β-gal activity in living cells) are also used to overcome the drawbacks of fluorescent probes [87] but the risks linked to the denaturation of enzyme have not been addressed so far. Very recently another near-infrared (NIR) fluorescent probe which was based on dicyanoisophorone was developed [88]. This method had comparatively higher limit of detection 3.2×10^{-3} U than other similar probes based on organic molecules.

9. Conclusion

β-galactosidase enzyme has been known widely for its catalyzing nature in the digestion of lactose and it's use in the biosensing. Besides, it has also been used as a biomarker for senescence and primary ovarian cancer. Effective detection of this crucial enzyme is very important. There are many techniques used in the detection of the enzyme in human cells, plants and microbes. In this review paper, we pointed out the cons and pros of the methods used and compared the effectiveness of these different techniques. Among the different methods of detection of this enzyme, NIR fluorescent probes have been very effective. Fluorescent method of detection has been successful as the enzymatic hydrolysis produces a blue dimerized chromophore for fluorescent obervation.

Inspite of various benefits of the NIR platforms, major challenges are yet to be overcome. Specifically, high sensitivity is required for the accurate detection of the enzyme at the early stage of the cancer. Moreover, the chemical and photochemical properties of the probes such as toxicity, water solubility, quantum yield, and photostability are yet to be improved. One of the alternatives to minimize these drawbacks is the use of two-photon fluorophores in the design of the probes. The two-photon fluorophore probes use long wavelength light, which are comparatively safer to the tissues and have more penetrating power. Further, these probes have higher sensitivity and are better in *in vivo* tracking exploitation. However, sufficient study has not been conducted in this area. Besides this, we suggest the use of nanoparticles in the development of probes. The nanoparticles such as carbon dots, being benign and highly photostable, can be used to conjugate with the fluorophores. This helps in the controlled release of the fluorophores in the cells that overexpress β-galactosidase and minimizes the side effects to the greater extent. Finally, we hope that researchers will benefit from the review paper and with suggested improvements, the probes could be utilized in clinical samples in the near future.

Declaration of Competing Interest

The authors report no declarations of interest.

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