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Diversity Oriented Fluorescence Library Approach (DOFLA) for Live Cell Imaging Probe Development

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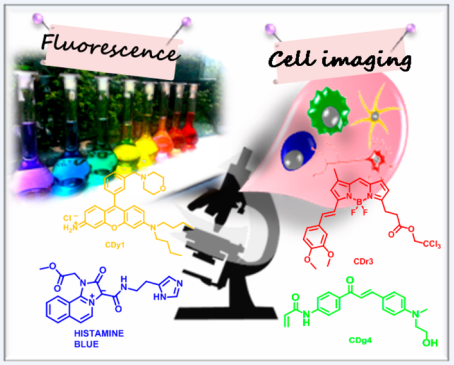
ABSTRACT: A cell is the smallest functional unit of life. All forms of life rely on cellular processes to maintain normal functions, and changes in cell function induced by metabolic disturbances, physicochemical damage, infection, or abnormal gene expression may cause disease. To understand basic biology and to develop therapeutics for diseases, researchers need to study live cells. Along with advances in fluorescence microscopy and *in vitro* cell culture, live-cell imaging has become an essential tool in modern biology for the study of molecular and cellular events. Although researchers have often used fluorescent proteins to visualize cell-type-specific markers, this method requires genetic manipulations, which may not be appropriate in nontransgenic cells. Immunodetection of cellular markers requires the use of xenogenic antibodies, which may not detect intracellular markers in live cells. One option for overcoming these problems is the use of fluorescent small molecules targeted to specific cell types, which can enter live cells and interact with molecules of interest.



We have used combinatorial chemistry to develop a large number of fluorescent small molecules as new imaging probes even without prior information about the probes' binding targets and mechanism, a strategy that we call the diversity oriented fluorescence library approach (DOFLA). We have used DOFLA to produce novel sensors and probes that detect a variety of biological and chemical molecules *in vivo* as well as *in vitro*.

In this Account, we describe a series of fluorescent small molecules developed using DOFLA that bind specifically to particular cell types. These molecules provide new ways to detect and isolate these cells. The fluorescent probes **CDy1**, **CDg4**, and **CDb8** tag embryonic stem cells and induced pluripotent stem cells but not fibroblasts or germ-line cells. **CDr3** binds to an intracellular neural stem cell marker, fatty acid binding protein 7, which allows researchers to separate neural stem cells from embryonic stems cells and more differentiated cells such as neurons and glia. In addition, we have developed **CDr10**, which distinguishes microglia from neurons and glia. **CDy2** stains myocytes much more brightly than myoblasts because of the increase in mitochondrial membrane potential during myogenesis. **GY** and **PiY** selectively stain α and β cells of pancreatic islets, respectively. Histamine Blue binds directly to histamine and stains basophils and macrophages containing high quantities of histamine. Glutathione Green allows researchers to measure the level of glutathione in cells and tissues by binding to glutathione and then triggering a hypsochromic shift. We have also developed a set of compounds that bind to cancer cells based on the cell type of origin and biocompatible surface-enhanced Raman spectroscopy (SERS) nanotags for cancer detection. In addition to discussing these new probes and their cell-type specificity, we also describe their applications in new assays, cell characterization, and pathology studies.

INTRODUCTION

For the identification of cell types, fluorescent proteins expressed by activation of cell-type specific promoters or fluorophore-conjugated antibodies that recognize cell type markers have been commonly used. Various fluorescent proteins whose emission wavelengths range from blue to near-infrared have been developed and applied to study the expression of cell-type specific marker genes in live cells.[1] However, this method cannot be applied for the analysis of nontransgenic cells or cells for clinical use. More than 300 cell surface markers have been identified to be serially named with the prefix of "Cluster of Designation (CD)" and used for identification of live cells by immunolabeling. Still, quite a few

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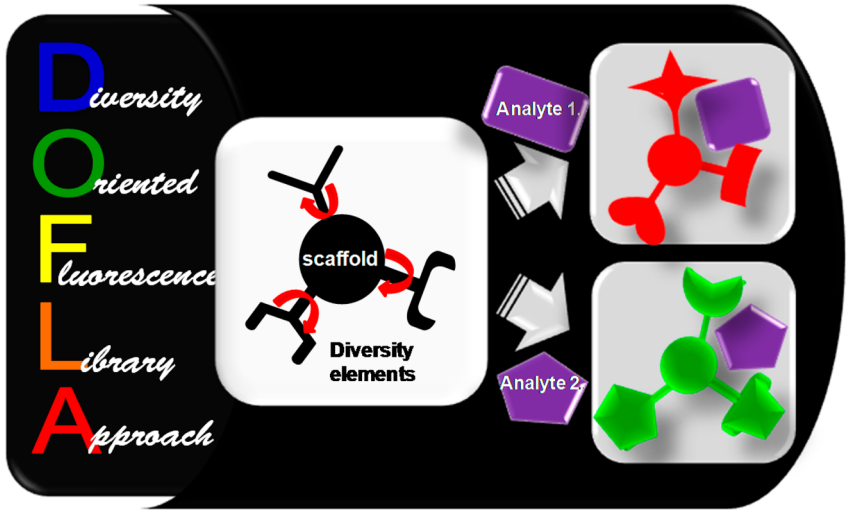

Figure 1. Diversity oriented fluorescence library approach (DOFLA). Combinatorial structural and spectral diversity increases the opportunity for discovering new probes.

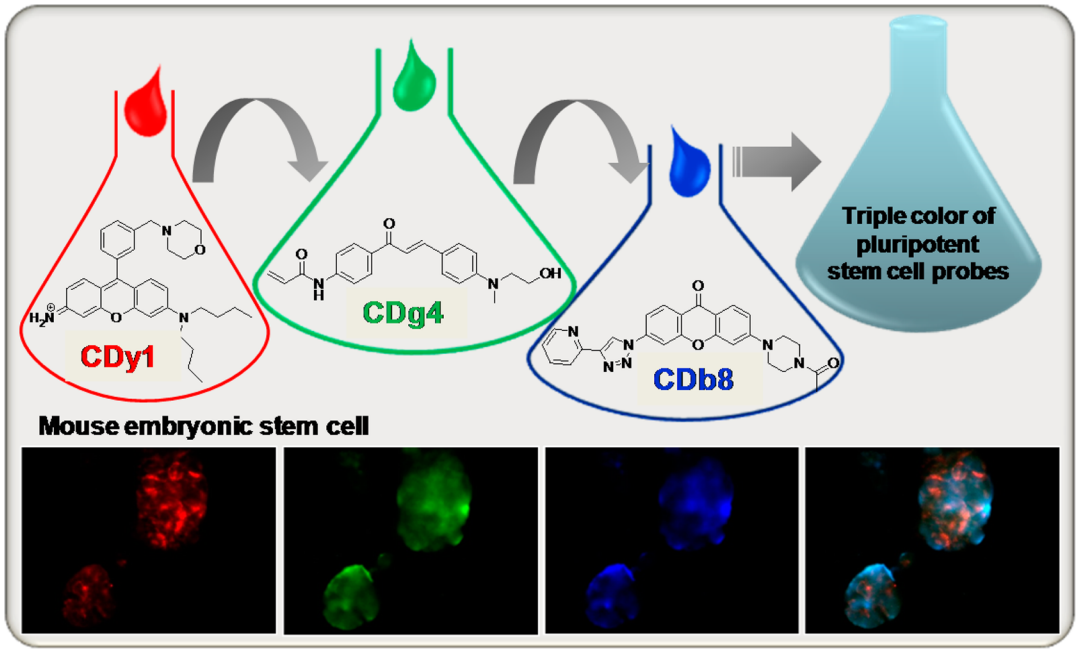

Figure 2. Triple color imaging of mESCs. Live mESCs were costained with **CDy1**, **CDb8**, and **CDg4**.

types of cells cannot be identified due to the limitation of CD antibodies to the detection of only cell surface markers leaving a variety of intracellular marker molecules undetectable in live cells. Compared with macromolecules such as antibodies, small molecules may enter live cells relatively freely. For drug discovery, usually more than a million small molecule compounds are screened to identify functional compounds. But for imaging probe development, such a large scale screening platform with small molecules has not been well established. Employing intrinsically fluorescent small molecules themselves as imaging probes offers advantages in screening by enabling visual detection of the probe–target interaction. Despite the potential of fluorescent small molecules to be developed as cell-type-specific imaging probes, the effort to synthesize a sufficient number of compounds possessing structural and chemical diversity had not been devoted. Therefore, we developed a diversity oriented fluorescence library (DOFL) composed of more than 10 000 intrinsically fluorescent small molecules and screened them in various types of live cells including stem cells, endocrine cells, muscle cells, immune cells, and cancer cells to develop cell-type-specific fluorescent chemical imaging probes.

■ DIVERSITY ORIENTED FLUORESCENCE LIBRARY

Small fluorescent molecules have been widely used as sensors and probes in the analysis of a variety of molecules with a great potential for live cell imaging.[2] The development of fluorescent sensors was initially approached by target-oriented synthesis, which requires domains for target recognition, tagging, and sensing. In this approach, the scope of sensor discovery is intrinsically limited by the identification of recognition domain, and the tagged sensing domain can change the activity and property of the probe. The DOFL approach (DOFLA) overcomes these limitations by systematic synthesis of small-

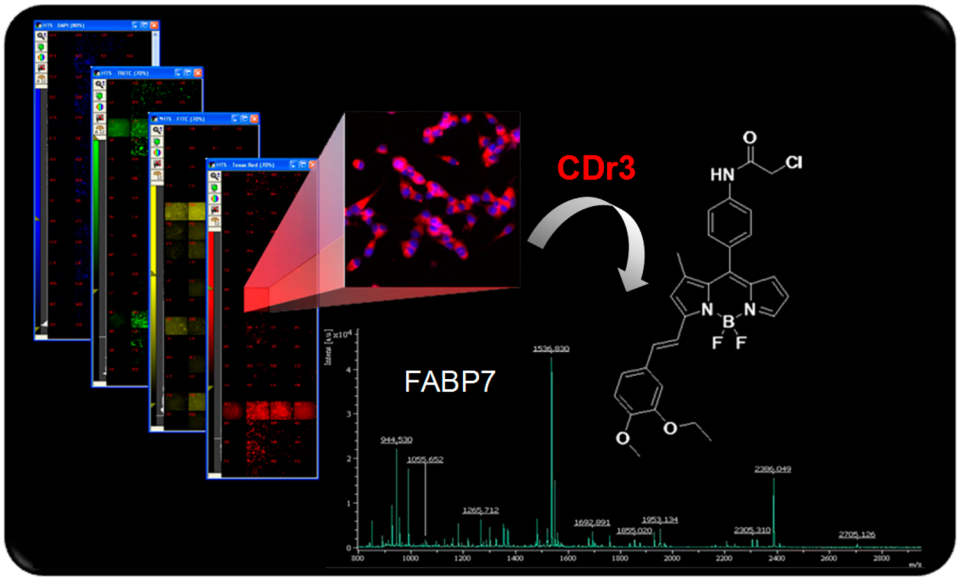

Figure 3. Neural stem cell specific fluorescent chemical probe **CDr3** was discovered by a high content screening of DOFL. The cellular binding target of **CDr3** was identified as FABP7 by mass spectrometry.

molecule fluorescent compounds, in which the target recognition domain and sensing domain are integrated, and screening them even without prior knowledge of target recognition (Figure 1).

DOFL compounds are generated by combinatorial modification of organic fluorescent scaffolds, where simple building blocks can be assembled with the aim of creating diverse compounds. The design and preparation of DOFL focuses on exploring the diverse chemical space directly around a fluorescent scaffold. Specific binding of fluorescent small molecules is readily detectable, and the target proteins can be tracked visibly during target identification processes by adding an affinity tag to the molecules. This approach provides some advantages in the development of bioimaging probes since it can be applied without knowledge about the target molecule and an additional reporter is not required for the visualization of the probes. Therefore, DOFLA depends entirely on the structural diversity of the fluorescence library empowered by unbiased evaluation of compounds to identify target-selective fluorescent probes. DOFL derived from fluorescence scaffolds such as rosamine, BODIPY, chalcone, and xanthone covers a very wide range of chemical space and fluorescence spectra (400−800 nm).

■ PLURIPOTENT STEM CELL PROBES

Pluripotent stem cells (PSCs) including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are able to differentiate into any type of cell. ESCs are prepared from the inner cell mass of blastocysts, while iPSCs are generated by reprogramming somatic cells. A large mass of PSCs can be continuously cultured and induced to differentiate into specific types of cells, which have been used as valuable tools to study developmental biology and human disease mechanisms.

To develop a PSC selective chemical imaging probe, Im et al.[3] screened 280 rosamine compounds in mouse ESCs and fibroblasts, which are used as feeder cells for culturing PSCs or as starting material for the generation of iPSCs. By analyzing fluorescence microscope images and flow cytometry data of the cells stained by the compounds, they identified a compound that stains mouse and human ESCs much more brightly than fibroblasts and named it compound of designation yellow 1 (**CDy1**). The PSC selectivity of **CDy1** was further confirmed by negative staining of ESCs differentiated into ecto-, meso-, and endoderm lineage cells and positive staining of fibroblasts reprogrammed into iPSCs. An interesting property of **CDy1** in iPSC detection is that it can distinguish cells at early stages of reprogramming even before the activation of endogenous PSC marker genes such as *Oct4* or *Nanog*. This allows for rapid screening to discover reprogramming enhancing factors even without the need for immunostaining or use of genetically modified cells that express fluorescent proteins. As a proof of concept study, Vendrell et al.[4] transfected fibroblasts with *Oct4*, *Sox2*, and *Klf4*, treated them with 240 hydroxamic acids and acquired fluorescence images of the cells undergoing reprogramming using **CDy1** on the seventh day after transfection. By image-based fluorescence quantification, they were able to identify a histone deacetylase inhibitor 1-26 that increases reprogramming efficiency 2.5-fold.

Compound of designation green 4 (**CDg4**) is another PSC probe discovered by Lee et al.[5] from a screening of 160 chalcone amide compounds produced by adding one amide and one amine on each side of the basic structure of chalcone as electron donors to enhance the fluorescence intensity. Unlike **CDy1**, which stains mitochondria inside the cell, **CDg4** signal is detected in the plasma membrane and around the ESC colony. In an *in vitro* fluorescence response assay against various biological analytes, a specific response of **CDg4** to glycogen was observed. When the ESCs were treated by amylase, which hydrolyses glycogen, the cells were not stained by **CDg4** confirming that the ESC selectivity of **CDg4** is due to the glycogen abundantly present in ESCs.

Options for different colors are frequently required in fluorescence imaging to stain a sample with more than one color. Ghosh et al.[6] synthesized 160 xanthone compounds by click chemistry on solid phase and screened them to develop a blue probe for ESC compound of designation blue 8 (**CDb8**). Depending on the need for different color and subcellular location of the dye, **CDy1**, **CDg4**, and **CDb8** can be effectively used for the detection of PSCs (Figure 2).

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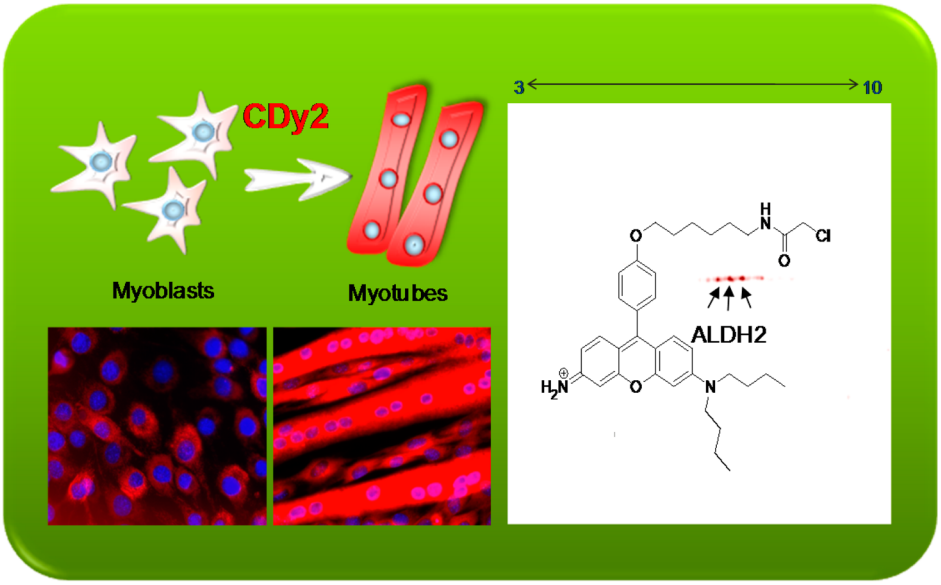

Figure 4. CDy2 selectivity on myocytes is driven by the increased mitochondrial membrane potential in differentiated myotubes. The fluorescence signal of **CDy2** bound to a mitochondrial protein ALDH2 was detected in 2D-PAGE. Adapted from ref 14 with permission from Wiley-VCH.

NEURAL STEM CELL PROBES

Neural stem cells (NSCs) differentiate into neurons, astrocytes, and oligodendrocytes to generate the nervous system not only during embryonic development but also throughout life. Since most neurodegenerative diseases are rarely curable by chemotherapy and primary human neuronal cells are difficult to obtain, NSCs differentiated from PSCs have emerged as promising tools for cell replacement therapy and *in vitro* experiments with human neurons. To develop a probe that distinguishes NSCs from PSCs, neurons, and glia, Yun et al.[7] screened 3160 DOFL compounds in mouse ESCs (E14), NSCs (NS5), astrocytes, and fibroblasts. A BODIPY derivative named compound of designation red 3 (**CDr3**) was finally identified as a probe for both human and mouse NSCs after confirmation of its NSC specificity in a mixed primary mouse brain cell culture as well as human cells differentiated from ESCs.

When the proteins extracted from **CDr3**-stained NSCs were separated by 2D gel electrophoresis and scanned on a fluorescence scanner, a fluorescent signal was detected allowing accurate excision of the protein spot. Fatty acid binding protein 7 (FABP7, also known as BLBP) was identified as the binding target of **CDr3** by mass spectrometry analysis, which was confirmed by specific staining of recombinant FABP7-expressing 293HEK cells by **CDr3** (Figure 3). FABP7 is a well-known intracellular marker of radial glial cells, which are a common intermediate NSC in the differentiation of PSCs into neuron and glia.[8] Deficiency of the protein causes a reduction of NSCs in the brain. However, NSCs stained with **CDr3** proliferate normally suggesting that **CDr3** does not affect the function of FABP7.

Primary NSCs have been isolated from brains using antibodies binding to CD133 (also known as prominin 1) or SSEA-1 (also known as Lewis X or CD15) or a fluorescent BODIPY-aminoacetaldehyde (BAAA, Aldefluor), which is hydrolyzed by aldehyde dehydrogenases to be retained inside the cell. BAAA was first designed to stain hematopoietic stem cells, which have been known to express high levels of aldehyde dehydrogenases,[9] but studies have shown that it also stains NSCs.[10] Analysis of the cells isolated based on the expression of CD133, SSEA-1, and aldehyde dehydrogenases by Obermair et al.[11] has shown the presence of heterogeneous NSC types in the brain. Leong et al.[12] further isolated **CDr3**-stained primary brain cells by FACS and characterized them to find that the **CDr3**-stained cells are NSCs that have phenotypes distinct from those isolated by CD133 or SSEA-1 antibodies or Aldefluor.

MYOCYTES PROBE

Skeletal muscle is formed by the fusion of myoblasts into multinucleated myotubes or myocytes during development. While the myocytes are terminally differentiated postmitotic cells, satellite cells remain to provide myoblasts for the regeneration of muscle in adulthood. Myocytes dedifferentiate into proliferative myoblasts in amphibians to regenerate lost tails and limbs. Therefore, understanding the mechanism of muscle cell state determination is expected to provide insights into the development of therapeutic approaches for muscle regeneration. To develop imaging probes for muscle cells in different differentiation states, Wagner et al.[13] screened 1606 DOFL compounds in undifferentiated and differentiated C2C12 mouse myoblast cell line to identify six rosamine (RS) compounds that stain myocytes more brightly than myoblasts. **RS-E26**, which distinguished myocytes from myoblasts with the biggest difference in fluorescence intensity, was chosen as the final hit probe for further application to a proof-of-concept screening of 84 kinase inhibitors. By staining the inhibitor-treated cells with **RS-E26** and measuring their fluorescence intensity, 17 compounds including known myogenesis inhibitors were identified. This result demonstrates that staining using fluorescent chemical probes can replace costly and time-consuming immunostaining of myocytes in basic research and drug screening for muscle regeneration. However, **RS-E26** easily loses the staining capability by illumination due to its photoinstability. By a rescreening of the rosamine library based on both myocyte selectivity and photostability, Kim et al.[14] identified new myocyte-selective compounds **RS-I25** and **RS-I31** and modified them to facilitate the identification of their binding targets. Despite the fact that the compounds

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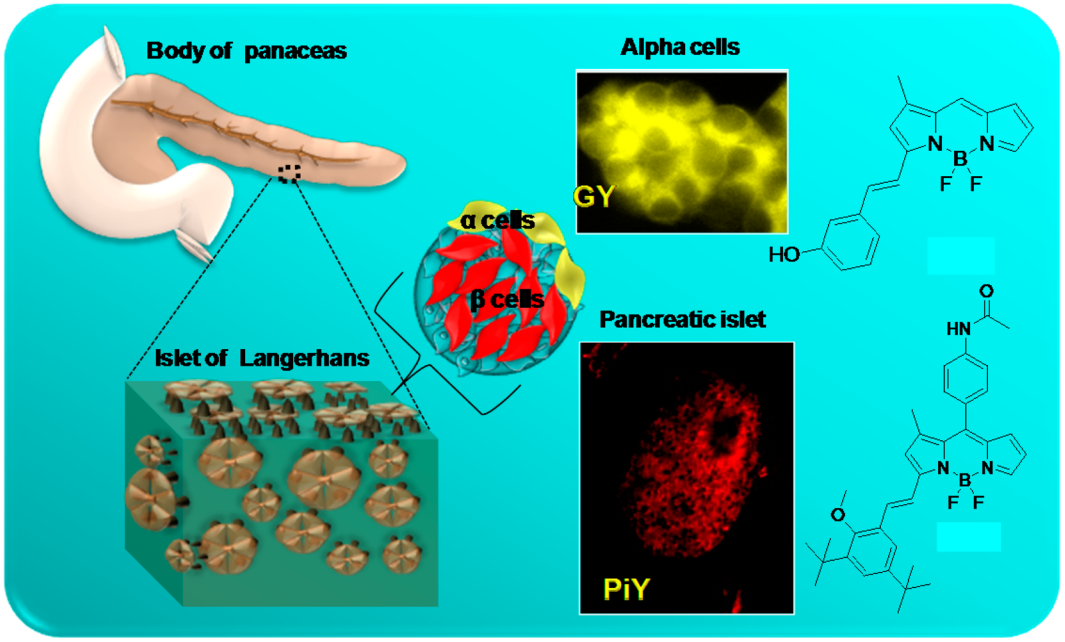

Figure 5. Glucagon probe **GY** selectively stains live α-cells (yellow), while pancreatic islet probe **PiY** visualizes the islets of Langerhans by binding to β-cells when infused into the bloodstream (red).

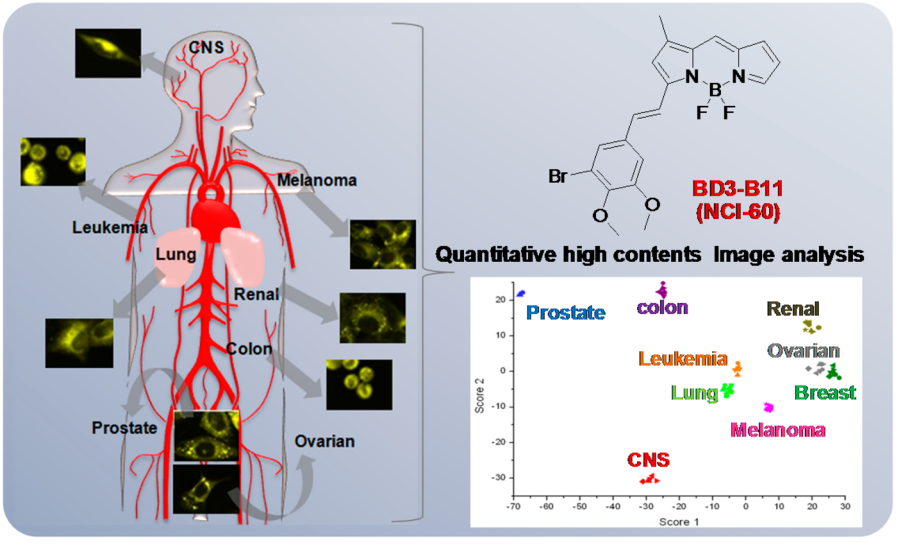

Figure 6. Identification of cancer cell-line origins using fluorescence image-based phonemic screening. Representative images of human cancer cell lines stained by **BD3-B11**. Linear discriminant analysis of the intensity kinetic profiles shows 60 cancer cell lines clustered according to the origin of cancer type.

localize in mitochondria, a cytoskeletal protein, tubulin, was identified as the target protein by *in vitro* affinity matrix pull-down assay. However, by the covalent labeling of the target in live cells with a cell-permeable thiol-reactive derivative compound of designation yellow 2 (**CDy2**), the target protein was identified as a mitochondrial protein aldehyde dehydrogenase (ALDH) 2 (Figure 4).

Interestingly, while **CDy2** fluorescence intensity is more than 2-fold stronger in myocytes than in myoblasts, the levels of ALDH2 are similar between both. When the mitochondrial membrane potential is disrupted by treatment with paraformaldehyde or a mitochondrial uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP), the myocytes are not as brightly stained by **CDy2** as healthy live myocytes. Mitochondrial membrane potential significantly increases during myogenesis, and this has been also demonstrated using C2C12 cell line.[15] These facts and results suggest that **CDy2** stains myocytes more strongly due to their higher mitochondrial membrane potential than that of myoblasts and then binds to ALDH2. Besides the development of a myocyte probe, this study shows that interactions between small molecules and proteins in live cells can be different from their interactions *in vitro*.

■ PANCREATIC ISLET CELL PROBES

α-Cells and β-cells in pancreatic islets of Langerhans produce peptide hormones glucagon and insulin, respectively. Destruction of the cells accompanied by perturbation of the balance between these hormones may result in metabolic disorders

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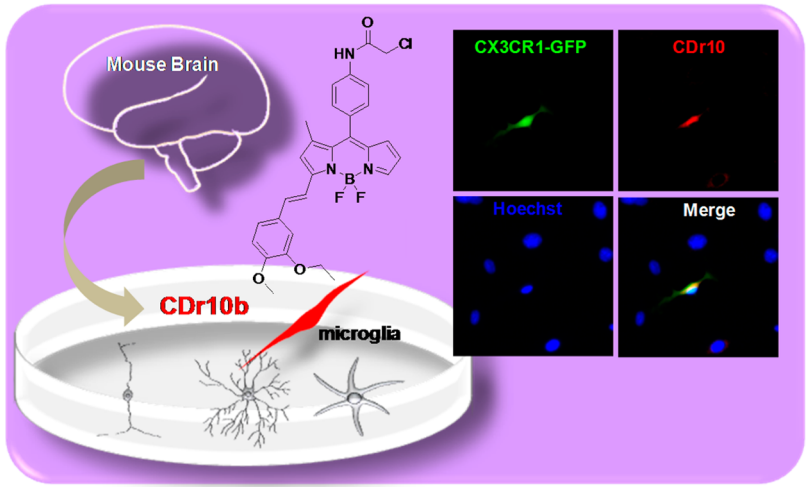

Figure 7. Microglia specific probe **CDr10b** (red) selectively stains a microglia cell expressing GFP (green) among mixed primary brain cells harvested from a CX3CR1-GFP transgenic mouse. Nuclei of all cells are shown by Hoechst 33342.

such as diabetes mellitus. While the levels of these hormones in the plasma or cell culture supernatant are readily measurable by biochemical assays, determining the structural integrity and mass of the live islet cells *in vivo* and *in vitro* is still challenging. By screening DOFL in AlphaTC1, BetaTC6, and other types of cells, Lee et al.[16] and Kang et al.[17] discovered BODIPY-based **BD-105** and **BDNCA-325**, which selectively stain α-cells and β-cells, respectively. In pancreas tissue sections, **BD-105** staining overlaps well with antiglucagon immunohistochemical staining but not with anti-insulin staining. Since the α-cell selectivity of **BD-105** appears to be attributed to its dramatic fluorescence increase in the presence of glucagon, the compound was named Glucagon Yellow (**GY**). The molecular mechanism of β-cell selectivity in **BDNCA-325** remains to be elucidated. The ultimate application of the pancreatic islet probes would be to measure the islet mass by a noninvasive imaging method. Since the population of β-cells in the mouse pancreas is much higher than that of α-cells, the authors attempted to systemically infuse the β-cell probe **BDNCA-325** and its derivatives for pancreatic islet labeling in live animals. The original **BDNCA-325** compound, which contains a thiol-reactive chloroacetyl group, did not go into the islets in various conditions tested. However, an acetyl derivative, which does not contain the chloroacetyl group, was shown to specifically stain the islets by necropsy examination. The β-cell selectivity of the acetyl derivative named pancreatic islet yellow (**PiY**) was confirmed by visualizing severely damaged islets in a diabetic mouse model induced by streptozotocin injection (Figure 5).

Although the fluorescent probe may not be used for *in vivo* imaging of human islets due to the very limited penetration depth of visible light in tissues, it can be modified to a nuclear imaging probe for deep tissue imaging by attaching a functional group or a single atomic radioisotope for MRI, PET, or SPECT. If the nuclear imaging probes selectively label β-cells, they will be extremely valuable tools for the diagnosis and prognosis of diabetes. Besides its potential medical application, **PiY** can also increase the efficiency of islet isolation from animals for basic research since **PiY**-stained islets are readily distinguishable under a fluorescence microscope.

CANCER CELL PROFILING PROBES

Genomic and proteomic analyses of cancer cells have revealed the vast degree of heterogeneity in cancer cells, which is related to clinical outcomes. It is expected that the types of cancer cells can be more accurately identified at the single cell level by combinatorial analyses of characterization data for more effective personalized medicine. The capability of a set of DOFL compounds to distinguish subtly different substances such as carbohydrates[18] or water from different geographical sources[19] by combinatorial analysis has been demonstrated. To apply this strategy to cancer cell classification by phenotypic characterization, Lee et al.[20] incubated 60 human cancer cell lines (NCI-60) originated from 9 tissues with 557 DOFL compounds and acquired fluorescence cell images 1, 24, and 48 h later. They analyzed fold change of the fluorescence intensity over time using linear discriminant analysis, which showed all 60 cell lines segregated depending on the origin of the cancer cells. By a forward stepwise variable selection algorithm, the number of probes to segregate the cells of same origin with 98% accuracy was reduced to 37. In addition, by individual data analysis, compound **RS-C3** was discovered to be specific for KM12 colon cancer cell line among the 60 cell lines, and the common specificity of the compounds **RS-E1**, **RS-E3**, and **RS-E7** for ACHN renal cancer cells and SW60 colon cancer was shown. Interestingly, the other seven renal cancer cell lines and six colon cell lines were not stained by the compounds. These results imply that the heterogeneous cancer cells even from a same origin can be phenotypically classified using DOFL (Figure 6).

While cancer cells can be observed *ex vivo* at high resolution by fluorescence microscopy, cancer detection *in vivo* using fluorescence imaging has been limited due to light scattering and autofluorescence. Surface-enhanced Raman spectroscopy (SERS) has been emerging as a sensitive detection method for biomolecules. Since each SERS nanotag has a unique spectral signature, the number of detectable molecules under a same excitation wavelength far exceeds the number distinguished by colors. Two important factors for SERS imaging are the Raman reporter molecule and the metallic nanoparticle that enhances the vibrational spectra of the reporter molecule. Maiti et al.[21,22] developed biocompatible SERS nanotags **B2LA**, **Cy3LA**, and **Cy5LA** with increased stability by attaching a triphenylmethine

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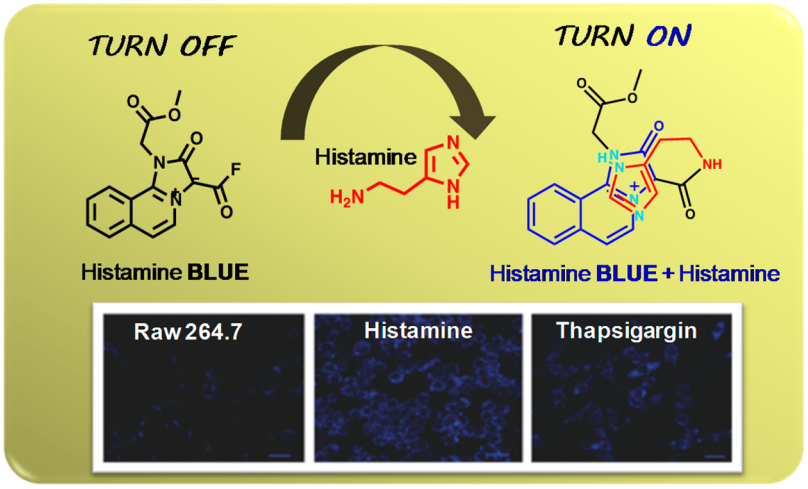

Figure 8. Fluorescence is turned on by the interaction of imidazoline ring of the Histamine Blue with the imidazole of histamine. Increased histamine in Raw264.7 cells by histamine uptake and the treatment of histamine synthesis inducer thapsigargin was visualized using Histamine Blue. Adapted from ref 28 with permission from The Royal Society of Chemistry.

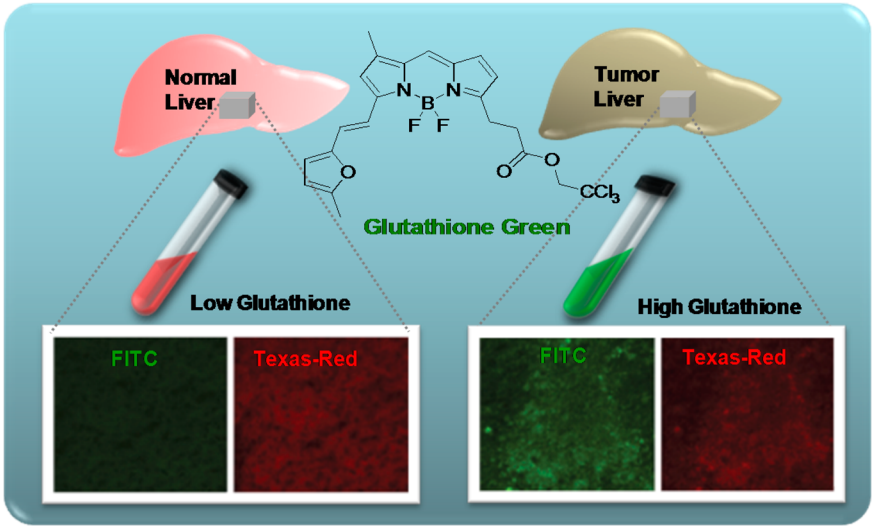

Figure 9. Ratiometric fluorescent dye for the detection of glutathione. Normal (left panels) and cancerous (right panels) liver tissues were stained with **Glutathione Green**. Higher GSH level in the cancerous tissue was detected by the spectral shift from red to green. Adapted from ref 29. with permission from The Royal Society of Chemistry.

or cyanine reporter molecule to a gold colloid using lipoic acid as a linker and demonstrated cancer marker detection using **B2LA** and **Cy3LA** conjugated to EGFR and HER2 antibodies. Samanta et al.[23] further extended this technology to develop **CyNAMLA-381−AuNP**, which can be detected by near-infrared with 12-fold higher sensitivity than commonly used 3,3′-diethylthiatricarbocyanine. With the development of Raman-based endoscopy,[24] these SERS nanotags could be used for real-time cancer detection during clinical endoscopy.

■ MICROGLIA PROBE

Microglia are the main immune cells residing in the central nervous system. They play important roles in normal structural development and functional maintenance of the brain but are overactivated to mediate inflammation in the brain. Due to their major involvement in neuroinflammation observed in patients with various neurological diseases, microglia have become a target for imaging and therapy. While radiochemicals such as PK-11195 and DAA1106, which selectively bind translocator protein 18 kDa (also known as peripheral benzodiazepine receptor), have been used for PET imaging of microglia,[25] *ex vivo* optical imaging of microglia has been conducted mostly using fluorescence proteins expressed by genetic manipulation or fluorophore-conjugated antibodies and lectin. To develop chemical imaging probes that specifically stain live microglia in heterogeneous populations of brain cells, Leong et al.[26] screened 5000 DOFL compounds derived from BODIPY, rosamine, xanthone, and chalcone scaffolds in primary neurons, astrocytes, and microglia. The final hit compound for microglia was identified from the BODIPY library and named compound of designation red 10b (**CDr10b**). Its specificity for microglia was confirmed by specific staining of CX3CR1-GFP positive cells by **CDr10b** in a mixture of whole brain cells (Figure 7).

While **CDr10b** has a chloroacetyl group that forms a covalent bond with certain proteins, a derivative **CDr10a** has an acetyl group instead. Microscopy and flow cytometry showed that **CDr10a** is as specific as **CDr10b** for microglia but washed

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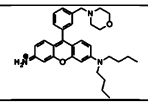
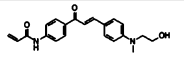
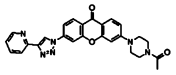
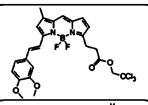
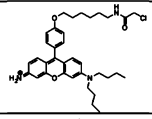
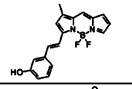
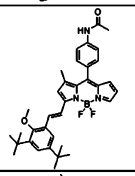
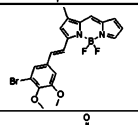
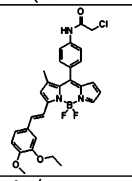
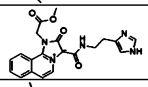
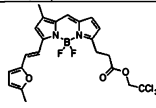
Table 1. Summary of Probes and Their Physical Properties with Target Information

Imaging Probes	Probe name	Chemical Class	Chemical Structure	Optical Wavelengths		Target
				λ_{ex}	λ_{em}	
PSCs	**CDy1**	Rosamine		535nm	570nm	mitochondria
	CDg4	Chalcone		430nm	560nm	glycogen
	CDb8	Xanthone		369nm	487nm	unknown
NSCs	**CDr3**	BODIPY		579nm	604nm	FABP7
Myocytes	**CDy2**	Rosamine		530nm	580nm	ALDH2
Pancreatic islet cells	**GY**	BODIPY		553nm	570nm	Glucagon
	PiY	BODIPY		558nm	585nm	unknown
Cancer Cell	**BD3-B11**	BODIPY		560nm	578nm	No specific target
Microglia	**CDr10b**	BODIPY		571nm	611nm	unknown
Histamine	**Histamine Blue**	Isoquinoline		346nm	417nm	histamine
Glutathione	**Glutathione Green**	BODIPY		565nm	585nm	glutathione

out much more rapidly. As the first fluorescent small molecules intrinsically specific for microglia, **CDr10a** and **CDr10b** will be useful tools for the transient and stable staining of live microglia, respectively.

HISTAMINE PROBE

Histamine is a biologically active amine synthesized by the decarboxylation of histidine. It is particularly abundant in mast cells and basophils and secreted by immune stimulation, prominently contributing to most allergic reactions. While histamine in biological samples has conventionally been detected by liquid chromatography, immunocytochemistry, and enzyme immunoassay, detecting it in live cells by optical imaging had not been possible until Seto et al.[27] developed a Nile Red derivative conjugated with an iminodiacetic acid−Ni^{2+} complex. Cu^{2+} or Ni^{2+} quenches fluorescence of the probe but is dissociated to make a complex with histamines turning on the fluorescence. Using the probe, they demonstrated histamine imaging in live cells in a mouse macrophage cell line RAW 264.7, which takes up and accumulates histamine inside cells. While the staining mechanism of Nile Red−iminodiacetic acid−Ni^{2+} is based on ligand exchange, another fluorescence probe, Histamine Blue, developed by Kielland et al.,[28] is turned on by direct binding to histamine (Figure 8).

Histamine Blue was not designed for the purpose of histamine imaging but was discovered from a screening of mesoionic fluorescence compounds in a broad panel of biomolecules. The binding between Histamine Blue and histamine appears to be through the primary amine, but the other bioamines such as dopamine, epinephrine, or serotonin do not increase its fluorescence. Histamine imaging was performed in a live basophilic cell line, RBL-2H3, as well as RAW 264.7, and the authors further demonstrated that quantitative comparison of histamine in live cells is attainable using the probe.

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GLUTATHIONE PROBE

Glutathione (GSH), a thiol-containing tripeptide, plays a pivotal role in the detoxification of reactive oxygen species and regulation of cellular redox status. Disturbance of GSH homeostasis is implicated in the pathogenesis of a variety of diseases including neurodegeneration, inflammation, and cancer. To develop a new fluorescence probe for GSH, Zhai et al.[29] screened 5120 DOFL compounds against GSH. Among the hit compounds that responded to GSH were nine BODIPY compounds that changed color from red to green. They commonly have a furan ring suggesting that the small functional group, furan ring, is likely to be a binding site for GSH. The brightest one among nine compounds was named Glutathione Green ($\lambda_{ex}/\lambda_{em}$ = 565/585 nm), which shows a significant hypsochromic shift ($\lambda_{ex}/\lambda_{em}$ = 512/522 nm) in response to GSH but not to the oxidized form of glutathione (GSSG). The increased and decreased levels of GSH in live cells by a GSH inducing agent α-lipoic acid and a thiol blocker *N*-methylmaleimide were well visualized by a ratiometric analysis of the images taken from Glutathione Green-stained cells. The applicability of Glutathione Green to show increased GSH in tumor and intercellular heterogeneity of GSH among the cancer cells was demonstrated using a cryopreserved rat liver tumor section (Figure 9).

Despite the intratumor heterogeneity of GSH content and its association with drug resistance of cancer cells,[30] histological analysis of GSH content has been technically challenging due to the small size of GSH and the difficulty of producing a free GSH specific antibody. Glutathione Green can be applied to a broad range of research, as a convenient tool for a fluorescence image-based analysis of GSH content in cells and tissue.

SUMMARY AND PERSPECTIVES

All the probes described in this Account are summarized with their structures, optical properties, and molecular targets in Table 1. In terms of optical properties, isoquinoline and xanthone are in the blue range, chalcone is in the green, and rosamine dyes are usually in yellow to orange. BODIPY dyes span a broad range from green to red depending on the conjugated aromatic group's electronic structure. According to the dye classes, there are trends for their cellular localization and cell permeability. Rosamine dyes are usually excellent in cell permeability and tend to localize mainly in mitochondria, as observed in both **CDy1** and **CDy2**. The reactive group of **CDy2** allowed us to identify the target protein as ALDH2, a mitochondrial protein. Chalcone dyes show poor cell permeability, and the molecular target of **CDg4** was identified as glycogen present in the exctracellular matrix. Histamine Blue reacts with intracellular histamine and stays long in the cytoplasm of basophiles. BODIPY dyes have usually been considered to bind nonspecifically to vesicles because they are neutral and hydrophobic. However, we observed that BODIPY dyes are a rich source of various optical properties and biological responses depending on the auxiliary group around the BODIPY core and also through the conjugation. The cellular responses to the BODIPY dyes are broad from neural stem cells, pancreatic α-cells and β-cells, and microglia. The molecular targets also span a broad range from proteins such as FABP7 and glucagon to small metabolites such as glutathione.

It is noteworthy that a small change in dye structure dramatically changes the kinetics of the dye in the cell. In the conventional SAR (structure−activity relationships) of medicinal chemistry, small structural changes usually do not result in obvious functional change of small molecules *in vitro*, while their cellular effects are more variable. The fluorescence property of the dyes makes it possible to monitor the subtle SPR (structure−property relationships) of the dyes and their cellular uptake and release. Our next goal would be to systematically analyze the SPR and generate a prediction rule for small molecules' behavior in cells.

Along with the phenomenal development of fluorescence-based optical imaging technology in recent years, more various and specific fluorescent probes are needed for basic science and clinical applications. In addition to the probes introduced herein more cell-type-specific imaging probes are expected to be developed in the future by DOFLA.

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Notes

The authors declare no competing financial interest.

Biographies

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Nam-Young Kang received her Ph.D. in Biotechnology from Dong-A University, Korea, in 2007. She has been working to develop live cell imaging probes since 2008 as a research fellow at the National University of Singapore and currently as a research scientist at Singapore Bioimaging Consortium.

Sung-Jin Park received his Ph.D. in Anatomy from the Catholic University of Korea in 2003. He worked for the development and evaluation of drug delivery systems for radiosensitization at ASAN medical center, Korea. In 2009, he joined the Singapore Bioimaging Consortium.

Hyung-Ho Ha received his Ph.D. degree in Medicinal Chemistry from Seoul National University in 2008 and joined the Medicinal Chemistry Program of Life Science Institute, National University of Singapore, as a postdoctoral fellow. In 2011, he was appointed as an assistant professor at the College of Pharmacy of Sunchon National University, Korea, where he currently serves as the department chair of Medicinal Chemistry.

Yun Kyung Kim graduated with Ph.D. in Chemistry from New York University in 2009 under the supervision of Professor Young-Tae Chang, working on the development of bioimaging probes. At present, she is a senior research scientist at Korea Institute of Science and Technology (KIST) working on neuro-degenerative disorders and brain imaging.

Jun-Seok Lee received his Ph.D. from New York University in 2009 under supervision of Prof. Young-Tae Chang, working on diversity-oriented optical sensor development. In 2010, he joined Korea Institute of Science and Technology (KIST) as an research scientist and was promoted to a senior research scientist in 2012. He received TJ Park Science Fellowship in chemistry.

Young-Tae Chang received his Ph.D. in Chemistry from POSTECH, Korea, in 1997. He did his postdoctoral work with Prof. Peter Schultz

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