

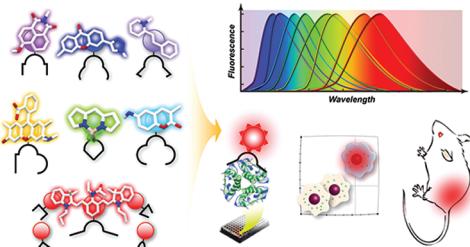
Combinatorial Strategies in Fluorescent Probe Development

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1. INTRODUCTION

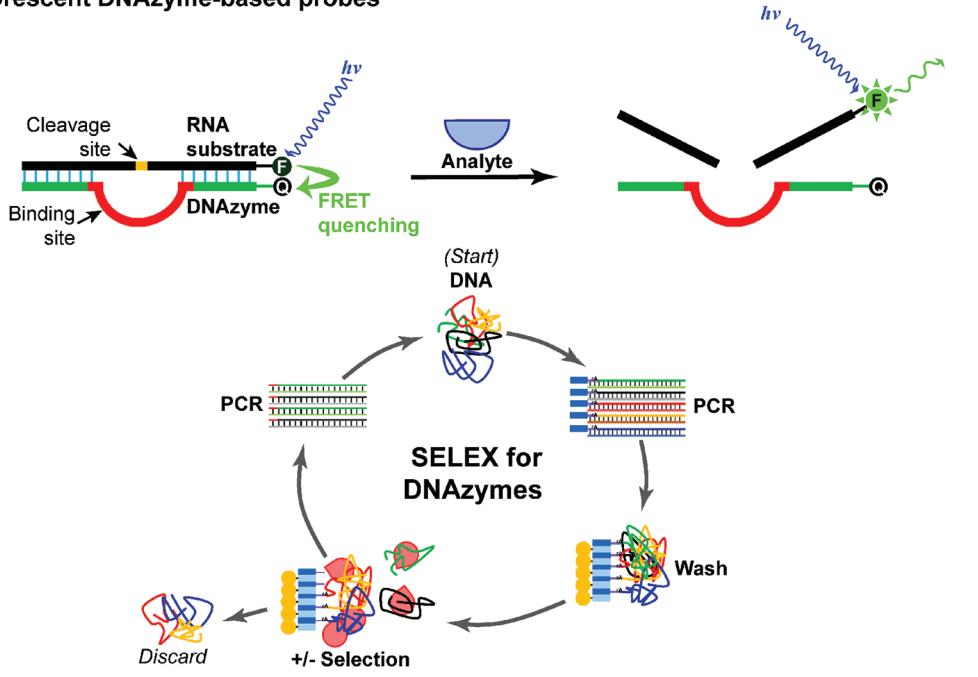
The need for understanding essential recognition events in chemistry and biology has directed considerable efforts toward the development of chemical probes. Fluorescent probes are attractive and versatile tools for both analytical sensing and optical imaging because of their high sensitivity, fast response time, and technical simplicity. However, the mechanisms that regulate the interaction between fluorescent probes and their targets are often poorly understood; hence, our ability to predict the structural requirements and design new probes using theoretical calculations is limited.¹ Combinatorial approaches, which were first exploited in drug discovery to obtain large numbers of structurally related drug candidates,² have recently boosted the generation and optimization of fluorescent probes, especially for complex scientific problems that remain indefinite at the molecular recognition level.³ Combinatorial strategies have become an actual alternative to rational design approaches and broadened the scope of fluorescent probes to targets that were not previously accessible (e.g., cell phenotypes, cell differentiation stages, and macrostructures).

Several reviews have described the performance of fluorescent probes with specific applications (e.g., intracellular pH indicators,⁴ sensors for reactive oxygen and nitrogen

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a) Fluorescent DNAzyme-based probes



b) Fluorescent aptamer-based probes

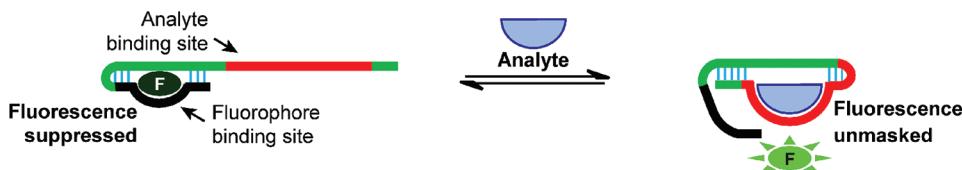


Figure 1. Target-oriented fluorescent nucleic acid libraries. (a) The catalytic action of DNAzymes is exploited to prepare sensors that emit fluorescence only after analyte binding and concomitant RNA cleavage. SELEX strategies improve the characteristics of the nucleic acid probes with several rounds of mutation and positive and negative selection to amplify the DNA sequences with affinity for the analyte; (b) label-free aptamer probes include a fluorophore-binding sequence that quenches its fluorescence in the absence of the target. Adapted with permission from ref 30. Copyright 2009 American Chemical Society.

species,^{5,6} metal sensors,^{7,8} diagnosis imaging tools^{9,10}) and mechanisms,¹¹ as well as their synthesis from particular chemical structures (e.g., peptides,¹² aptamers,¹³ biomolecular building blocks,¹⁴ sensing materials,¹⁵ and DNA-multichromophores¹⁶). In this article we will review the role that combinatorial and high-throughput strategies have played in the development of fluorescent probes during the last five years, mainly as a result of the expansion of combinatorial chemistry on fluorescent scaffolds and the major improvements in high-throughput screenings and imaging methodologies.

The first combinatorial approaches appeared with fluorescent libraries that derivatized molecular recognition elements (also known as receptors) and included a fluorescent readout to develop new probes for particular targets. In this review we refer to these libraries as **target-oriented fluorescent libraries**. Although the combinatorial derivatization of receptors has rendered a number of successful probes over the past few years, these approaches are limited to a few targets (e.g., metals, saccharides, anions, small peptides, or gases). In an effort to increase the speed and scope of probe development, combinatorial approaches have been recently complemented with a significant growth in **diversity-oriented fluorescent**

libraries. Diversity-oriented fluorescent libraries consist of combinatorially derived fluorescent molecules that are directly evaluated for multipurpose sensing applications. Fluorescent libraries with broad chemical diversity are synthesized and screened in a high-throughput manner to discover new probes for targets that might not be accessible with known receptors. To this end, the recent technical improvements in high-throughput imaging have been a key factor to expand the evaluation of fluorescent libraries from *in vitro* assays to more challenging systems, such as living cells or whole organisms. In this review we will discuss examples of fluorescent probes obtained from both target-oriented and diversity-oriented fluorescent libraries in the format of individual sensors and for the construction of differential arrays. Although combinatorial methodologies in fluorescent probe development are still young and important challenges (e.g., target identification) need to be addressed, the significance of the recent findings suggests that these methods may consolidate as an efficacious strategy for the discovery of new fluorescent probes.

2. TARGET-ORIENTED FLUORESCENT LIBRARIES

The conventional workflow in the preparation of target-oriented fluorescent libraries begins with the selection of a target analyte, followed by the search of receptors or binding elements and their attachment to reporters that transduce the molecular recognition event into a fluorescent signal by different mechanisms (e.g., fluorescence quenching, fluorescence resonance energy transfer (FRET), fluorescence anisotropy, photoinduced electron transfer (PeT), chelation-enhanced fluorescence, environment sensitivity, or metal-based fluorescence, among others).¹⁷ Occasionally, a receptor for the analyte is known (e.g., zinc finger domains for Zn²⁺ ions) and combinatorial approaches optimize the fluorescent reporter or the transduction mechanism. More commonly, the receptor responds to an entire class of analytes (e.g., boronic acids bind to saccharides and crown ethers interact with alkali and alkali metals) and libraries are constructed to develop an improved receptor that selectively binds to the analyte of interest. Finally, combinatorial techniques can partially overcome the limitations of rational design strategies when little or no information about the molecular recognition is available.³ Many fluorescent probes have been developed from target-oriented libraries, and we classified them into four groups according to their molecular structure (i.e., nucleic acids, peptides, small molecules, and polymers). In this section we will review the combinatorial chemistry behind the design and synthesis of target-oriented fluorescent libraries, first in the context of metal ion sensing and later with probes for saccharides, adenosine triphosphate (ATP), tripeptides, and volatile analytes.

2.1. Principles and Applications. Case Study: Probes for Metals

2.1.1. Nucleic Acid Libraries. There is longstanding interest in sensors for metal ions due to their environmental and biological importance.^{18,19} The potential of combinatorial strategies in probe development is well-demonstrated with libraries derived from nucleic acids, because their immense modularity, compatibility with the polymerase chain reaction (PCR) and automated synthesis has led to collections of a very large size, generally up to 10¹⁴ components.

Following the procedures of Breaker and Joyce,²⁰ the group of Lu engineered libraries of catalytic DNA to prepare sensors for Pb²⁺ and Zn²⁺ ions.^{21–23} This methodology was later expanded to other metals and even different oxidation states (i.e., Fe²⁺ and Fe³⁺).²⁴ Their approach started with the generation of a combinatorial library of 10¹⁴ double-stranded DNA molecules with a random 40-nucleotide sequence flanked by conserved primer binding domains (Figure 1a). The authors employed the systematic evolution of ligands by exponential enrichment (SELEX), further detailed in section 4.2.1, which exploits the amenability of the PCR to screen large nucleic acid-based libraries against a particular analyte. After several rounds of mutation and selection, the sequences with high affinity for the analyte are amplified, leading to an improvement in the selectivity of the nucleic acid probes. The initial pool of DNAzymes (i.e., DNA molecules with catalytic action) was subjected to SELEX using an extended 5'-biotinylated primer with a single adenosine ribonucleotide. The PCR products were passed through a streptavidin affinity matrix to afford a library of 5'-biotinylated single-stranded DNAs embedded with an adenosine ribonucleotide. The library was then incubated with different metal ions to identify those sequences that, upon metal binding, cleaved at the labile adenosine ribonucleotide

site. Positive sequences were pooled for the next round of selection. The efficacy of the process was improved after incorporating a negative selection step, which removed sequences with high affinity for competing ions.²⁵ With a gradual stringency of the binding conditions (e.g., lower analyte concentration and shorter incubation times), the catalytic DNA sequences evolved into the best metal binder and a quencher: fluorophore pair (i.e., dabcyl and rhodamine, respectively) was appended to transduce the binding into a FRET mechanism.

Despite the powerful application of these DNAzyme probes, their irreversible sensing mechanism limited their widespread usage. Inspired by the work from Teramae's group,^{26,27} Lu and co-workers devised a new DNA sensor architecture from a label-free functional DNA (Figure 1b).²⁸ In this case, the selected DNA aptamer was coupled to an additional sequence that served as a binding site for the 2-amino-5,6,7-trimethyl-1,8-naphthyridine (ATMND) fluorophore. In the absence of the target, the binding site sequence was stabilized by different intermolecular forces and quenched the fluorescence of ATMND. Upon analyte binding, the aptamer underwent a structural change that resulted in the emission of fluorescence from ATMND. This approach constitutes a general platform for designing fluorescent sensors for metal ions (e.g., Pb²⁺, Hg²⁺, and UO₂²⁺) as well as for other biomolecules.^{29,30}

Other approaches have been oriented toward synthesizing probes mimicking the well-defined structure of DNA while expanding its molecular diversity and improving the chemical stability. The group of Kool recently prepared 4 096 fluorescence quenching metal ion sensors based on oligodeoxyfluoroside (ODF) structures.³¹ The authors linked deoxyribosides that had been modified with metal-binding ligands and hydrocarbon fluorophores in a phosphate-deoxyribose backbone so that the resulting DNA-like structure would promote direct π–π stacking interactions among fluorophores. The binding of different metal ions (i.e., Co²⁺, Ni²⁺, Cu²⁺, Hg²⁺, Pb²⁺, Ag⁺, Cr³⁺, and Fe³⁺) altered the interactions between fluorescent motifs and rendered unique changes in their emission profile, whose combination was used as a signaling mechanism for the identification of the metals.

2.1.2. Peptide Libraries. The modularity of peptide structures and their automatable synthesis propelled the preparation of fluorescent peptide libraries for probe development. Peptides and proteins consist of a large pool of amino acids as building blocks, which results in libraries of a considerable size and generally with a broader chemical diversity than nucleic acids. Moreover, amino acids can be readily modified to bestow peptides with additional properties (e.g., fluorescence and binding affinity to specific analytes). One of the first examples of peptide libraries for sensor discovery was reported by the group of Imperiali, who developed a fluorescent probe for Zn²⁺ founded on the well-known zinc finger domain.³² The library of mutated peptides was constructed and modified with a dansyl fluorophore so their fluorescent signal could be detected upon Zn²⁺ binding. Subsequent efforts resulted in the development of smaller peptide sensors, containing between six and eight amino acids. A biased 19-member library integrating an unnatural amino acid based on the 8-hydroxy-5-(N,N-dimethylsulfonamido)-2-methylquinoline fluorophore enabled the application of these sensors to in vitro diagnostics of Zn²⁺.³³

A unique attraction of peptides is their potential to be used as protein tags. Imperiali and co-workers engineered lanthanide-binding tags (LBTs) as sequences of 17 amino acids that were

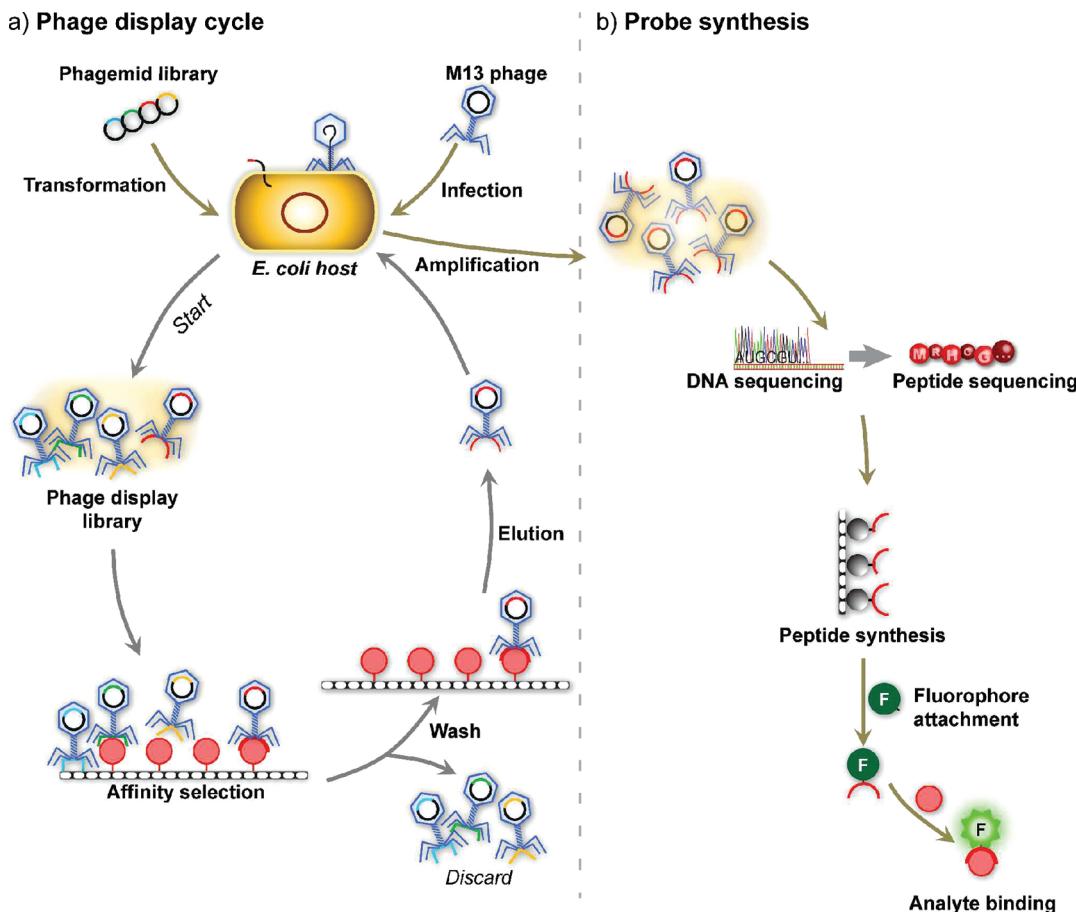


Figure 2. Phage-display peptide libraries. (a) Large peptide libraries are displayed in the surface of an infected or transformed host and integrated into an evolutionary process that includes various rounds of selection against the analytes; (b) consensus peptide sequences with high affinity for the targets are identified from the phage-display cycle to prepare the corresponding fluorescent peptide probes using peptide synthesis and derivatization with suitable labeling dyes.

combinatorially evolved to bind Tb^{3+} with high affinity.³⁴ LBTs function like most peptide metal ion sensors and elicit a fluorescent response upon binding to Tb^{3+} . An advantage of LBTs over other lanthanide sensors is that they can be appended onto proteins without disrupting their native function. Starting from the EF-hand motif (i.e., a helix-loop-helix structural domain found in calcium-binding proteins), a prototype LBT sequence was identified from a split-and-mix library of 500 000 members.³⁵ Its evolution through a series of four, and later seven, iterative libraries increased the binding affinity for Tb^{3+} by 140-fold.³⁶

The exploitation of “biological” machinery for the construction of peptide libraries offers an interesting alternative to the split-and-mix chemical synthesis. Originally developed to study protein–ligand interactions,³⁷ the phage-display technology, further detailed in section 4.2.2, has been adapted to the preparation of fluorescent peptide probes (Figure 2). Through several rounds of positive and negative selections, consensus peptides with good binding affinities for specific metals are discovered. High-affinity fluorescent peptide probes are prepared later by derivatization with suitable labeling dyes.

Vreuls et al. prepared 12-mer libraries to discover fluorescent sensors for ZnO .³⁸ An initial library of $\sim 10^9$ virions was subjected to four rounds of positive selection against ZnO powder to identify a consensus peptide sequence from 30 phages. The peptide was then resynthesized with a fluorescein

tag to obtain a highly specific fluorescent peptide probe for ZnO . The authors demonstrated the generality of this approach with sensors for TiO_2 and stainless steel ($\text{Cr}-\text{Fe}-\text{Ni}$ alloy).

Targeting soluble metal ions with phage-display libraries has been hampered by the challenge of immobilizing the analytes on solid surfaces. Two recent approaches by the laboratories of Choe and Van Dorst have opened new possibilities for peptide libraries in metal sensing. In the former, the authors immobilized different metal ions (i.e., Pb^{2+} for positive selection and Cu^{2+} , Ni^{2+} , Co^{2+} , and Fe^{3+} for negative selection) on monolithic columns with iminodiacetic acid beads and discovered a 7-mer peptide with good selectivity for Pb^{2+} .³⁹ In the second approach, a nickel Sepharose resin was used to identify new 12-mer Ni^{2+} -binding peptides.⁴⁰ These advances may increase the use of phage-display libraries to develop fluorescent peptide probes for environmental and biological applications.

2.1.3. Small Molecule Libraries. The reduced and often simple architecture is the greatest advantage of small molecule probes. They hold an attractive potential as *in vivo* fluorescent probes because of their low molecular weight, high chemical stability, and relatively good cell permeability. On the other hand, because of their restricted modularity and lack of methods for automated synthesis, fluorescent libraries of small molecules are typically of a smaller size than those based on peptides or nucleic acids.

Most of the small molecule libraries focus on the derivatization of known recognition elements (e.g., crown ethers, polyamines, macrocyclic amines, pyridines, and acetates),⁴¹ and the discovery of new recognition elements remains challenging. To this end, one of the pioneer examples was reported by Sames and co-workers, who synthesized a library of 1470 triamine ionophores for environmental copper sensing (Figure 3a).⁴² The authors screened the library against

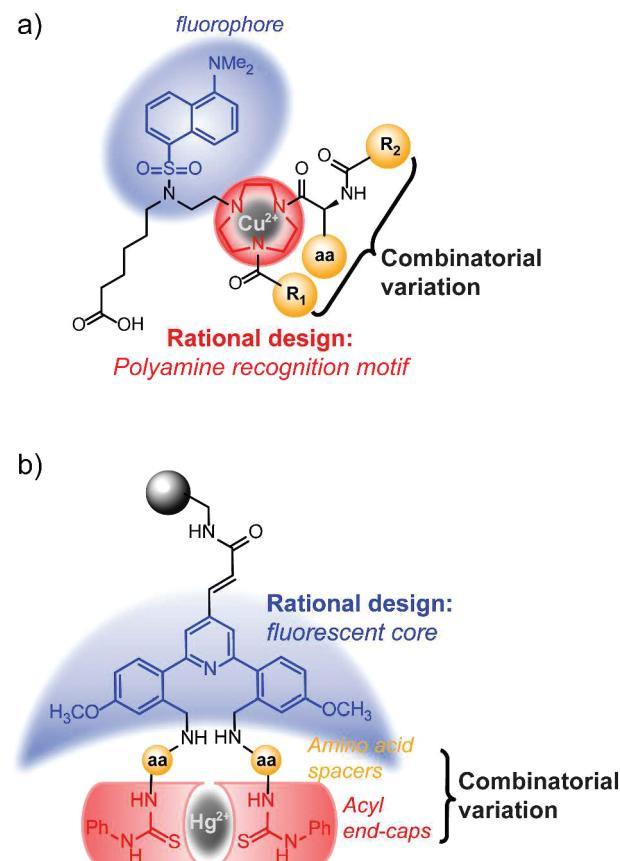


Figure 3. Target-oriented small molecule libraries. (a) Preparation of dansyl libraries based on the derivatization of polyamines as binding recognition motifs; (b) binding-induced restriction of fluorophore biaryl torsion as a novel signaling mechanism to develop fluorescent sensors for Hg²⁺.

aqueous copper solutions and prepared highly sensitive fluorescent dansyl sensors based on a newly discovered molecular entity. Rivero and co-workers similarly obtained fluorescence quenching probes for Cu²⁺ and Pb²⁺ from a small library of phosphine sulfides modified with anthracene fluorophores.^{43,44}

To achieve high sensitivity, probes that display a fluorescence enhancement after interacting with the analyte of interest are more desirable. Most of these probes rely on the PeT mechanism. They are generally constructed by attaching fluorophores (e.g., dansyl and anthracene) to receptor elements (e.g., short aliphatic amines and anilines) that behave as electron-donor groups and quench the emission of the fluorophore. Once the PeT probe interacts with the target, the ability of the receptor to donate an electron is diminished and a concomitant increase in fluorescence is observed. Therefore, PeT probes transduce the recognition of the target into a variation of the fluorescence emission.⁴⁵ The PeT

mechanism has been applied to screening on-bead libraries, where the recognition event is linked to a fluorescence increase of the “positive” beads that facilitates their isolation and characterization. On-bead screenings provide some advantages over other conventional assays, such as low consumption of reagent or continuous readout. Rivero and co-workers screened on-bead libraries of lariat ethers and benzamides (i.e., known receptors for alkali and alkali earth metal ions) that were derivatized with a dansyl fluorophore.^{46,47} A flow-injection analysis method enabled the isolation of the beads and the identification of the most selective probes.

While PeT probes are attractive in terms of reliability and the use of unsophisticated structures, there is a limited chemical diversity in the receptor elements that can be used.^{48,49} As an alternative to PeT, Mello and Finney conceived a novel signaling mechanism (binding-induced restriction of fluorophore biaryl torsion) to develop new fluorescent sensors for Hg²⁺ (Figure 3b).⁵⁰ The authors prepared a 198-member library based on a 2,6-diaryl-4-vinylpyridine core that was derivatized with two identical arms containing amino acids and acylating caps. The library was prepared on Argogel resin, which is compatible with both aqueous media and solid-phase organic synthesis. The authors screened the on-bead library against different metals and discovered a thiourea end-capped structure with a selective response to Hg²⁺ ions. In this case, the solid support facilitated the screening in aqueous media where the potential sensors may not be fully soluble. In subsequent studies, the authors developed second-generation libraries to improve the performance of the Hg²⁺ sensor and obtained new fluorescent probes for Ag⁺.⁵¹

2.1.4. Polymer Libraries. Fluorescent synthetic polymers constitute an excellent source for environmental sensing because they are stable under harsh environmental conditions and applicable to long-term monitoring experiments. Another unique feature of fluorescent polymer-based sensors is their preparation as solid materials or membranes, which can facilitate their integration into detection instruments and fiber optic systems. Fluorescent conjugated polymers may exhibit increased sensitivity (also known as amplification) because of their ability to behave as an efficient transport medium.^{52,53} While the sensitivity of monomer probes is mainly determined by the equilibrium constant between one receptor molecule and its target, fluorescent conjugated polymers display an amplified signal due to their collective system response.⁵⁴ Fluorescent polymer sensors are compatible with combinatorial chemistry because of the multiple variables (e.g., number, proportion, and chemical nature of building blocks, fluorescent dyes, and plasticizers) that define their properties.⁵⁵ However, the manipulation of these parameters is complicated even when sophisticated automation is used, and fluorescent polymer-based sensor libraries tend to be smaller than their above-mentioned counterparts (i.e., nucleic acids, peptides, and small molecules).

Wolfbeis and co-workers circumvented the need for complex instrumentation and prepared small collections of polymer probes for metals by limiting the variability of the library to the ion carrier (i.e., receptor element with ion binding affinity).⁵⁶ The other library components (e.g., fluorescent dyes, plasticizers, and anionic additives) were kept constant, and the entire library was manufactured in 96 microtiter well plates. The authors screened the library after polymerization by simply adding aqueous solutions of the ions and measuring the fluorescence spectra of the probes.

Dordick and co-workers prepared a collection of homo- and copolymers to discover new probes for environmentally related metal ions.⁵⁷ The authors combined five phenolic monomers (*p*-cresol, *p*-phenylphenol, *p*-methoxyphenol, *p*-hydroxyphenylacetic acid, and *p*-hydroxybenzoic acid) in a peroxidase-catalyzed oxidative polymerization and examined the fluorescent properties of the resulting polymers after incubation with aqueous solutions of metal ions (i.e., Fe³⁺, Cu²⁺, Co²⁺, and Ni²⁺). The changes in the intrinsic fluorescence of the polymers upon addition of the metals were employed as the signaling mechanism. Notably, the authors proved that the sensing capabilities of homo- and copolymers were significantly different and highly dependent on the chemical nature and the relative ratio of the monomers.

The introduction of recognition motifs in the side-chains of the monomers can be used to enhance the binding properties of polymer-based libraries for alkali metals.⁵⁸ The lab of Swager prepared a set of conjugated poly(*p*-phenylene ethynylene) (PPE) with crown ethers and studied the interactions between different chains. These experiments resulted in the discovery of an attenuation transduction mechanism based on the aggregation of conjugated polymers upon binding to K⁺ ions.⁵⁹

2.2. Fluorescent Libraries for Saccharides

The myriad of biological processes in which saccharides are involved (e.g., cell signaling, pathogenic infection, immune response, cancer metastasis, and cell adhesion) has driven an intense research toward the development of sensors for these biomolecules.^{60,61} However, the design of fluorescent probes with high specificity and affinity for saccharides remains challenging, partially due to their restricted functional group diversity, lack of ionic charges, and dynamic conformational states. In addition, the large number of hydroxyl groups in saccharides limits most recognition mechanisms to hydrogen bonding, which is often hampered by a strong competition from water molecules. Oligosaccharides and glycoproteins are particularly demanding because of the conformational flexibility of the former and the fact that combinatorial selection tends to identify protein segments rather than saccharides in the latter.

The synthesis of fluorescent combinatorial libraries to discover novel saccharide probes advanced significantly after the landmark discovery of Wulff and Shinkai and co-workers, who proved that boronic acids could form reversible cyclic boronate esters with sugar diols.^{62,63} Boronic acids (especially those with a low pK_a) have been incorporated as receptors in fluorescent libraries that rely on PeT as the signaling mechanism (Figure 4). In these cases, the binding to sugar diols leads to an increase in the acidity of the boron atom, which is transduced into a fluorescent signal.⁶⁴

The lab of Wang pursued the integration of fluorescent boronic acids into DNA libraries. The authors prepared PeT-based, monosaccharide-binding fluorescent boronic acids^{65–68} and derivatized them with terminal alkynes⁶⁹ and azides⁷⁰ to conjugate them to thymidine triphosphates (TTP) using click chemistry. These studies led to the development of boronic acid-labeled thymidine triphosphates (B-TTP) compatible with PCR⁷¹ and employed them to identify the glycosylation site of fibrinogen, a soluble plasma glycoprotein.⁷² The authors prepared a DNA library with 50 randomized positions flanked by two conserved primer regions and replaced natural nucleobases by B-TTPs. The library was subjected to 13 rounds of SELEX including some negative selection cycles to remove the sequences binding to nonglycan regions and

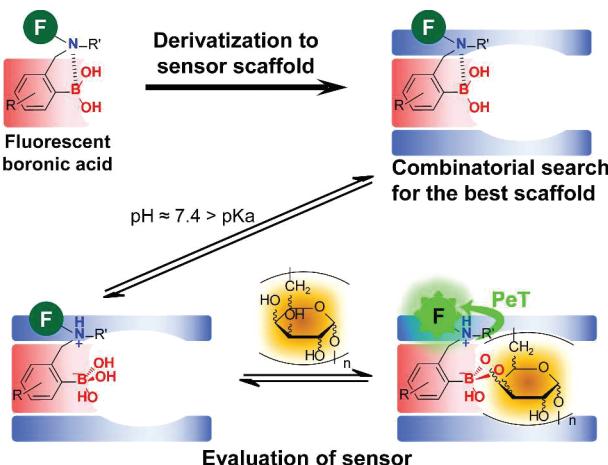


Figure 4. PeT-based fluorescent saccharide probes. Combinatorial libraries of PeT probes based on fluorescent boronic acids are designed so that the binding of boronic acids to saccharides leads to an increase in the acidity of the boron atom and a concomitant fluorescence emission.

eventually led to aptamers that recognized the glycosylation site of the protein. Similar strategies have been used to obtain nucleic acid fluorescent sensors for glycolipids.⁷³

Hall and co-workers developed an alternative strategy using a library of anthracene-capped polyamines modified with aryl boronic acids to discover new probes for disaccharides.⁷⁴ After the evaluation of the library in neutral aqueous media, the authors concluded that the modifications in the spacers between amines had a significant effect on the stability of the complexes and observed that higher binding affinities were obtained with electronically poor arylboronic acids. Furthermore, the chemical synthesis using automated IRORI MiniKans (i.e., radio frequency encoded microreactors for combinatorial synthesis⁷⁵) opened up the possibility of expanding the molecular diversity to large collections of compounds. Polymer-based structures have been also described for saccharide sensing albeit with limited success. Patterson, Smith, and Taylor prepared libraries of potential sialic acid sensors by combining PeT-based boronic acids with a poly(allylamine) scaffold.⁷⁶ The authors used an iterative process to optimize the selectivity and developed sensors with a preferential binding to sialic acid when compared to fructose and glucose.

Despite the remarkable progress during the past few years, boronic-acid based fluorescent probes are still limited in their practical use. The recognition of saccharides with boronic acid receptors is pH-dependent, which involves adjusting the pH of the samples or considering its effect when the pH cannot be easily altered (e.g., blood sensing).⁷⁷ Single-walled carbon nanotubes⁷⁸ and fluorescent DNA probes obtained by SELEX are promising tools but are restricted to *in vitro* applications due to their cell membrane impermeability.⁷⁹ On the other hand, cell-permeable small molecules typically suffer from a lack of selectivity, which might be overcome with the development of novel chiral scaffolds.⁸⁰

Less common are the approaches that target saccharides without employing boronic acids. Sugimoto et al. reported the synthesis of pentapeptides consisting of two aromatic terminal amino acids (tryptophan was always included as the fluorophore) flanking a variable tripeptide region.⁸¹ The authors envisioned that the sandwich interaction between the

aromatic side-chains of the terminal amino acids and the hydrophobic regions of the monosaccharide would mimic a pocket excluding water molecules and enable the recognition of the saccharide via hydrogen bonding. After screening a combinatorial split-and-mix library of 62 000 pentapeptides, the authors identified a fluorescent sensor of D-erythrose.

Nitz and co-workers designed an array of self-assembly fluorescent receptors based on a small collection of modified cyclodextrins and fluorescent derivatives of lithocholic acid (LCA).⁸² The authors made use of the stable inclusion complexes between cyclodextrins and LCA to prepare supramolecular receptors capable of differentiating heparin and common potential saccharide contaminants.

This work exemplifies the demand of new carbohydrate-recognition motifs that can bind strongly and reversibly to saccharides while overcoming the shortcomings of boronic acids. The design of new structures, such as the just-mentioned cyclodextrins or the aptamers developed by the group of Anslyn,⁸³ and their adaptation to pattern sensing^{84–86} are promising approaches for the future combinatorial development of fluorescent probes for saccharides.

2.3. Fluorescent Libraries for ATP and Peptides

In view of the versatility of fluorescent probes as tools for understanding complex biological systems, combinatorial libraries targeting biologically relevant molecules have captured rising attention in recent years. The ubiquitous nature of ATP and its crucial role in many metabolic pathways, as well as the strong resemblance to guanosine triphosphate (GTP), make it an important and challenging target in probe development.

Anslyn and co-workers assembled a 4 913-member split-and-mix library in which an aromatic biguanidinium scaffold with electrostatic affinity for anionic triphosphates was derivatized with tripeptides to improve its selectivity toward ATP.⁸⁷ Positive binders were coupled to fluorescein and coumarin to obtain FRET-based ATP probes with a marginal binding to adenosine monophosphate (AMP) and GTP.

Morii and co-workers designed libraries of potential ATP probes on the basis of ribonucleopeptides (RNPs), which are complexes formed by RNA aptamers and peptides (Figure 5).⁸⁸ Like other nucleic acid-based sensors, the authors used SELEX to identify RNA sequences with good affinity to ATP. However, instead of derivatizing the aptamers, the authors labeled their complementary peptides with a pyrene fluorophore, thus keeping the binding characteristics of the RNA sequences unaffected. These RNPs are exceptionally versatile in fluorescence emission wavelengths because different fluorophores can be conjugated to the peptide units without any loss of molecular recognition on the receptor elements.

Small peptides play a crucial role in most cellular systems.⁸⁹ Like nucleotides, their molecular similarity has activated combinatorial chemistry as the main approach to discover probes for these molecules. Hioki et al. made use of the supramolecular properties of the calix[4]arene scaffold, which organizes into a pocket capable of recognizing small molecules, to prepare a library of peptide calix[4]arenes hybrids.⁹⁰ Two identical tripeptide arms capped with pyrene motifs were appended to the calix[4]arene core to prepare a 3 375-member library, and its evaluation led to a fluorescent sensor for the tetrapeptide Tyr-Gly-Gly-Phe. Iorio et al. synthesized an analogous library with a dual tripeptide arm scaffold and prepared fluorescent probes for Ac-Gly-Lys(Boc)-D-Gln(Trt) and Ac-D-Arg(Pmc)-D-Leu-Asn(Trt).⁹¹

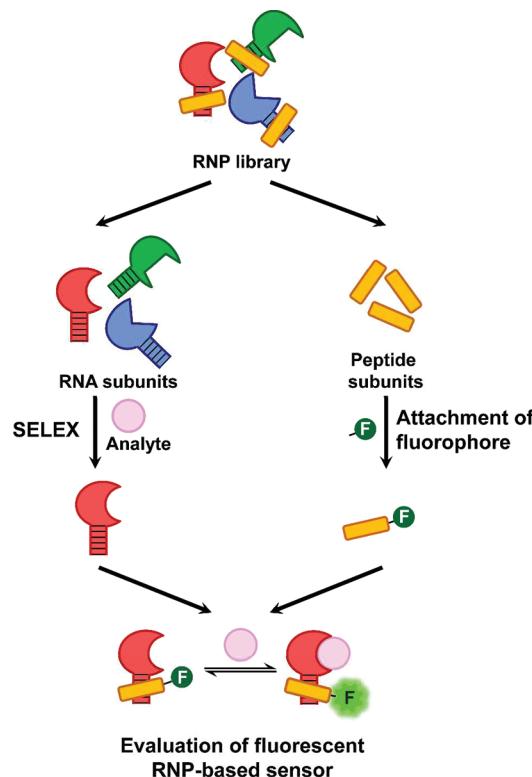


Figure 5. Combinatorial libraries of RNPs. Schematic representation of the preparation of RNP probes, which combine target-selective RNA subunits obtained by SELEX with their complementary fluorescently labeled peptide subunits. Adapted with permission from ref 88. Copyright 2006 American Chemical Society.

2.4. Fluorescent Libraries for Volatile Analytes

Whereas nucleic acids and peptides have garnered immense attention to develop probes for biological molecules, unnatural synthetic polymers are preferred for analytes that might compromise the chemical stability of the probes.⁹² The group of Lippard reported a small library of conjugated polymers to obtain fluorescent sensors for nitric oxide (NO).⁹³ The library of conjugated polymers was composed of phenylene vinylene, phenylene ethynylene, fluorene, and thiophene polymers in π -conjugation with bipyridyl or terpyridyl substituents. The pyridyl substituents were complexed and quenched by Cu²⁺ ions so that a fluorescence increase could be detected in the presence of NO due to the reduction of Cu²⁺ to Cu⁺.

Walt and co-workers also worked on combinatorial libraries of polymer sensors for volatile analytes.^{94,95} The authors employed a polymerization initiator (i.e., benzoin ethyl ether) and different proportions of methyl methacrylate and PS802 [(80–85%) dimethyl-(15–20%) (acryloxypropyl)-methylsiloxane copolymer] to generate a set of polymer matrices entrapping the fluorescent dye Nile Red. The copolymerization was performed at the fiber tip surfaces to monitor the fluorescent response against different organic vapors (e.g., butyl alcohol, butyl acetate, toluene, benzene, and pentyl acetate) using fiber optics.

Wolfbeis and co-workers pursued the combinatorial development of fluorescent sensing materials for gaseous oxygen (Figure 6).⁹⁶ Because of the large number of permutations derived from the preparation of the polymers, the authors used robotic instrumentation to divide the variability into smaller sublibraries. Beginning with 16 different polymer matrices and

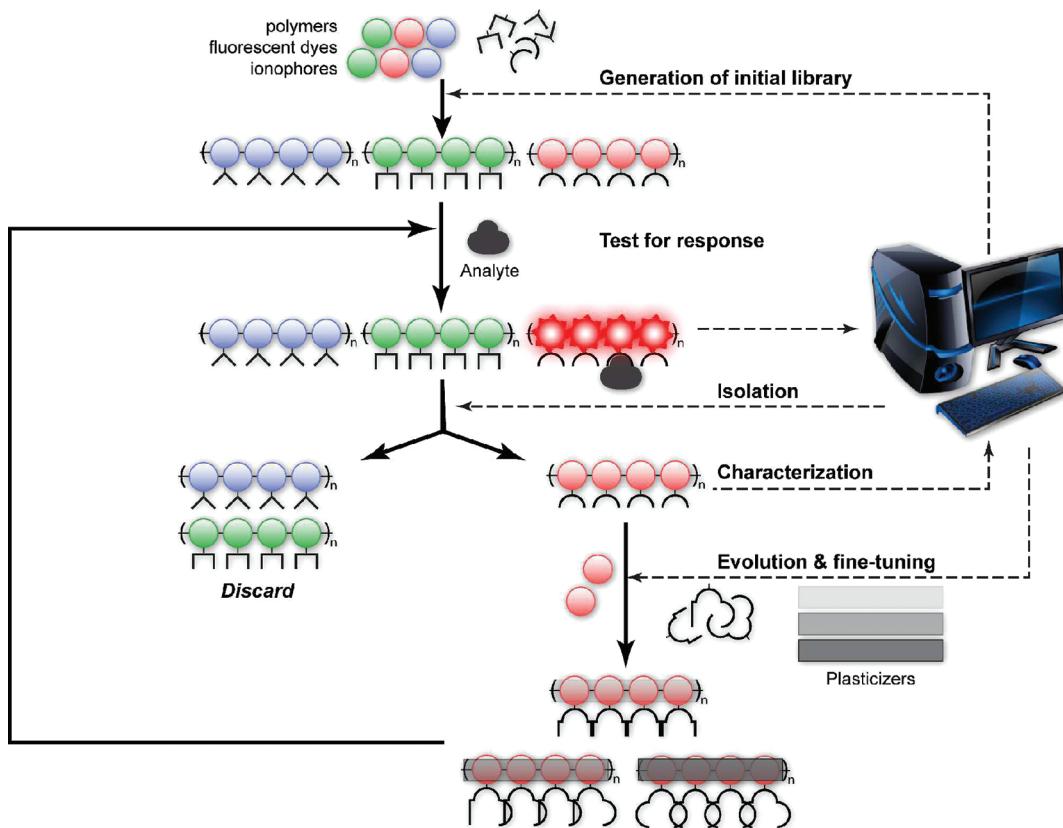


Figure 6. Evolution-based strategies in target-oriented fluorescent polymer libraries. Robotic instrumentation can assist the preparation of polymer libraries by subdividing the large number of potential permutations into smaller sublibraries. An iterative process including the synthesis of small collections of polymers including only a limited number of variables (polymers and fluorescent dyes) is followed by their screening against the target analyte to fine-tune and evolve them into fluorescent polymer probes.

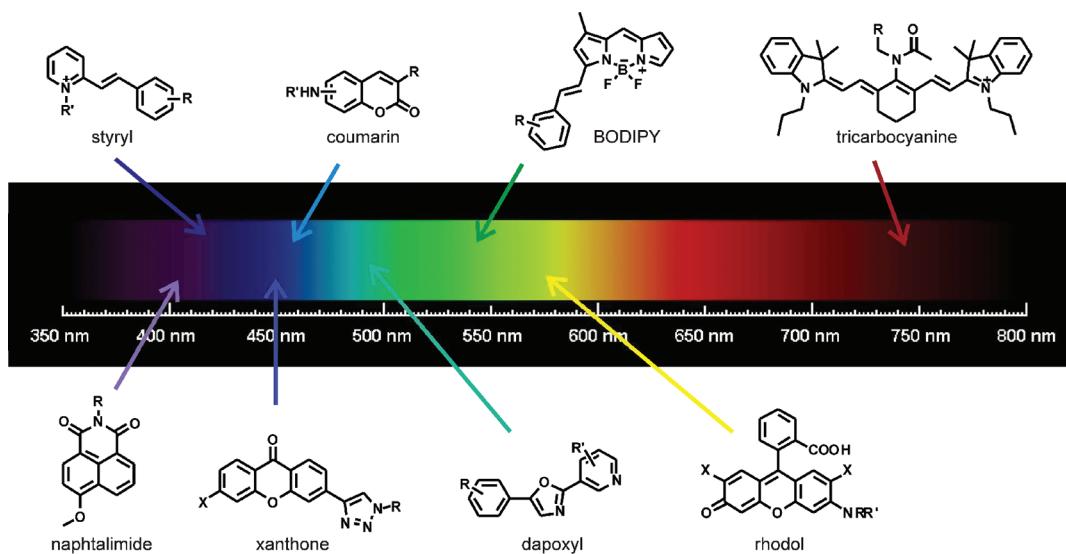


Figure 7. General structures of conventional fluorescent scaffolds for diversity-oriented libraries. Arrows point at the approximate fluorescence emission wavelengths of the fluorophores. Adapted with permission from ref 100. Copyright 2011 Wiley-VCH.

4 oxygen-sensitive fluorescent indicators, 64 sensor materials were prepared and screened to identify six matrices and two indicator dyes as hits. A second library was then constructed, this time including four plasticizers at different concentrations, to generate 192 combinations that were evaluated in a similar manner. The iterative process of library screening, fine-tuning, and evolution was repeated to improve the characteristics of the

probes and exemplifies the power of evolution-based strategies in polymer-based libraries when suitable instrumentation is available.

3. DIVERSITY-ORIENTED FLUORESCENT LIBRARIES

The success of target-oriented libraries in probe development depends on the incorporation of features enhancing the affinity

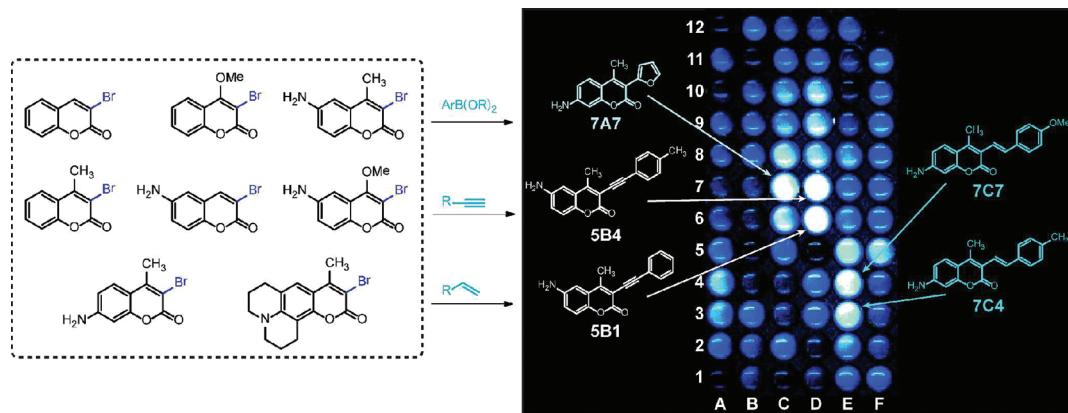


Figure 8. Diversity-oriented coumarin library. Coumarin derivatives prepared by palladium-catalyzed couplings were one of the first examples of diversity-oriented fluorescent libraries. A number of compounds with relatively high fluorescence quantum yields were identified after screening the library in a microtiter plate. Partly reproduced with permission from ref 101. Copyright 2001 Wiley-VCH.

of the probe toward its target (e.g., receptor elements and molecular interactions) and the adaptation of suitable mechanisms to transduce the recognition event into a fluorescent signal. In an attempt to accelerate fluorescent probe discovery, diversity-oriented libraries make use of combinatorial chemistry to generate libraries of fluorescent molecules that play both roles (i.e., receptor and reporter). The libraries are screened for multipurpose sensing applications against targets of very diverse chemical nature (e.g., metabolites, proteins, and cells).⁹⁷ Diversity-oriented libraries offer some advantages: (i) they have a broad scope of applications and can be employed when no prior molecular knowledge of the target is available, and (ii) the newly discovered receptors do not need an additional reporter. On the other hand, the unbiased character of diversity-oriented libraries requires the exploration of an extensive chemical variability in order to successfully identify new probes. To this end, combinatorial chemistry using fluorescent scaffolds has experienced an outstanding growth in the past few years.

Small molecule combinatorial chemistry, originally developed in the context of drug discovery,^{98,99} approaches the synthesis of diversity-oriented fluorescent libraries by two different strategies. One strategy considers the derivatization of known fluorescent scaffolds with commercially available building blocks using conventional synthetic procedures. This approach typically results in large chemical libraries, whose size is generally defined by the availability of the building blocks. Some of these libraries can be designed to exploit a physical or biological feature of a fluorescent scaffold, such as tricarbocyanines as near-infrared fluorescent dyes or rhodamines as mitochondria-targeting agents (Figure 7).¹⁰⁰ The second strategy, less common and synthetically more challenging, assembles building blocks using multicomponent, one-pot, or tandem reactions to obtain de novo fluorescent structures. Although the resulting libraries are generally smaller in size than in the previous approach, they often cover a broad chemical diversity and have unpredictable sensing capabilities, which can facilitate the discovery of probes for biomolecules that are difficult to target using conventional fluorescent structures. In this section we will summarize the preparation of diversity-oriented fluorescent libraries, mainly in the context of small molecules but also using larger structures such as peptides and nucleic acids.

3.1. Combinatorial Derivatization of Known Fluorescent Scaffolds

3.1.1. Palladium-Catalyzed Couplings. Diversity-oriented libraries were initially prepared on the basis of known fluorescent cores. One of the first reported libraries was prepared by derivatization of the coumarin scaffold (i.e., 3-oxo-3H-benzopyran) using palladium-catalyzed coupling reactions. These reactions are useful for combinatorial purposes because they are compatible with different building blocks (e.g., boronic acids, alkenes, and alkynes). Bäuerle and co-workers synthesized a library of 151 dyes by modifying eight different 3-bromocoumarin structures using Suzuki, Sonogashira, and Heck cross-coupling reactions (Figure 8).¹⁰¹ The authors proved that fluorescent skeletons could be derivatized using combinatorial chemistry and observed that the library members exhibited different photophysical properties (i.e., fluorescence quantum yield, absorbance, and emission wavelengths). Notably, a number of structural patterns leading to relatively high fluorescence quantum yields were identified. In subsequent coumarin libraries, chemical diversity was introduced at other positions of the core (e.g., 6-arylcoumarins).¹⁰² Similar approaches have been used for the preparation of 4-iodoisocoumarins¹⁰³ and 1,2-disubstituted (hetero)aryl fused imidazoles.¹⁰⁴

Palladium-catalyzed couplings have been applied later to fluorophores with better inherent photophysical properties. The rhodol structure (i.e., a hybrid of fluorescein and rhodamine cores) shows good photostability, a high extinction coefficient, and low pH-dependence. Peng and Yang prepared 22 rhodol compounds via palladium-phosphine catalyzed Buchwald–Hartwig amination reactions.¹⁰⁵ The authors designed a monoprotected fluorescein scaffold, which was activated as a triflate and derivatized with different amines. In addition to exhibiting longer absorption and emission wavelengths than coumarin (absorption wavelengths, 470–530 nm; emission wavelengths, 510–560 nm), rhodol derivatives have tunable fluorescence quantum yields (from <0.0001 to 0.98), which makes them a versatile structure for the development of both “turn-on” and quenching fluorescent probes.

3.1.2. Huisgen 1,3-Dipolar Cycloaddition. The simplicity, high efficiency, and mild reaction conditions of Huisgen cycloaddition (one of the prototypes of “click chemistry”) favored its application in diversity-oriented fluorescent libraries. Wang and co-workers employed a set of azide-containing

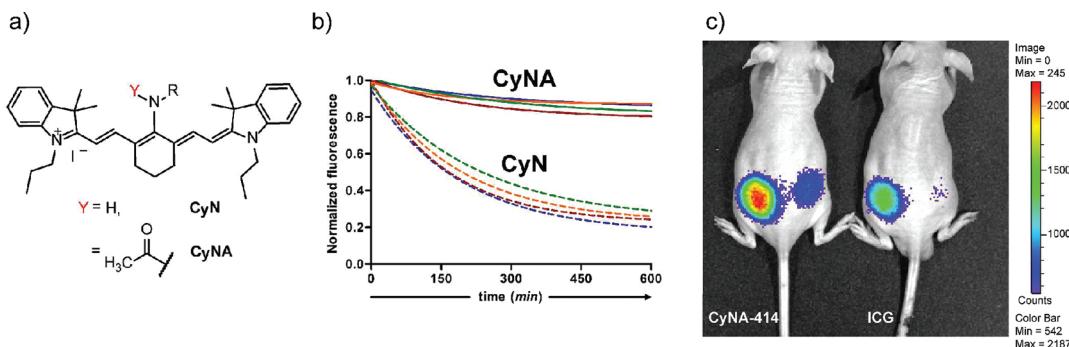


Figure 9. Photostable NIR tricarbocyanine dyes. (a) Chemical structures of acetylated (CyNA) and nonacetylated (CyN) tricarbocyanine dyes; (b) a photostability analysis showing that the acetyl group of CyNA compounds minimize their photodegradation; (c) translation into a photostable NIR protein labeling dye (CyNA-414) for *in vivo* imaging and comparison to the NIR standard Indocyanine Green (ICG). (a, b) Reproduced with permission from ref 119. Copyright 2010 The Royal Society of Chemistry. (c) Reproduced with permission from ref 121. Copyright 2011 Wiley-VCH.

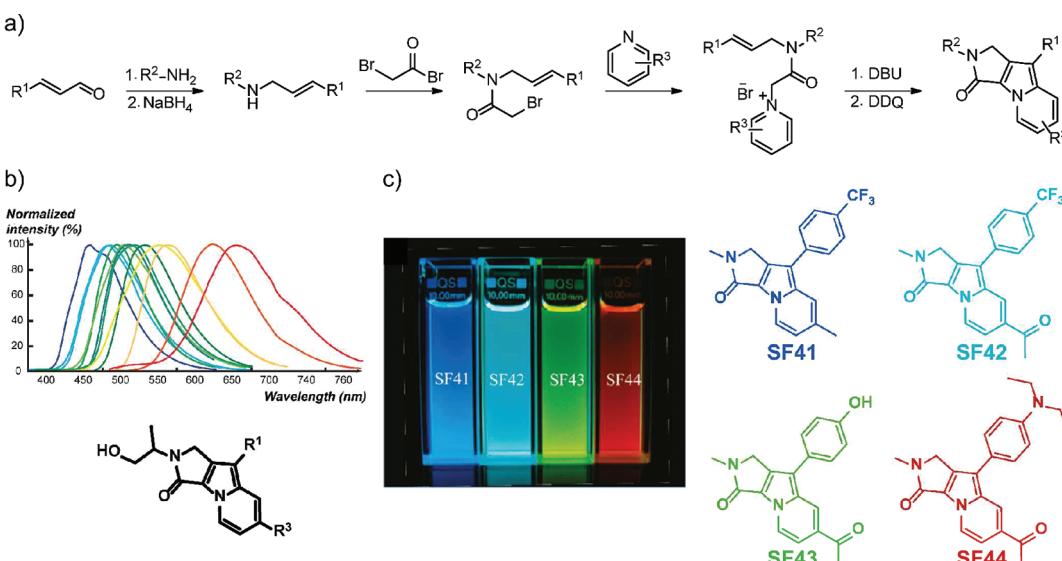


Figure 10. De novo construction of fluorescent scaffolds. (a) Synthetic scheme for the preparation of 1,2-dihydropyrrolo[3,4-*b*]indolizin-3-ones; (b, c) representative chemical structures and tunable fluorescence emission properties of the library. Partly reproduced with permission from refs 122 and 123. Copyright 2008 and 2011 American Chemical Society.

coumarins to “click” with a number of alkynes.¹⁰⁶ The resulting purification-free triazolocoumarins displayed a broader range of spectral properties than previous coumarin libraries (absorption wavelengths, 298–455 nm; emission wavelengths, 388–521 nm).

The lab of Yao employed Huisgen cycloaddition to derivatize both xanthene and xanthone scaffolds and discover molecules undergoing a fluorescence increase upon formation of the triazole structure.¹⁰⁷ After examining a library of 102 compounds, the authors selected two molecules showing a 10-fold brightness increase upon conjugation. This significant “turn-on” effect and the compatibility of Huisgen cycloaddition with physiological conditions provide these molecules with good potential as fluorescent probes for live cell imaging.

3.1.3. Knoevenagel-like Condensation, Amide Formation, and Nucleophilic Substitution. Knoevenagel condensations are particularly important in combinatorial fluorescent chemistry because the coupling of aromatic aldehydes typically results in an extended π -conjugation that red-shifts the emission wavelengths of the fluorescent skeletons. One of the first examples was reported by Chang and co-

workers, who prepared a 276-member library of styryl dyes by combining 14 pyridinium salts with 41 commercially available aldehydes.¹⁰⁸ Styryl dyes demonstrated excellent features as live cell imaging probes, with emission wavelengths covering the whole visible range, good cell permeability, and variable localization in different cell organelles. In subsequent libraries the authors extended the chemical diversity of the styryl scaffold to discover new fluorescent probes for DNA and RNA imaging.^{109,110}

Knoevenagel condensations have been successfully applied to the BODIPY (boron dipyrromethene) core. The group of Chang expanded the limited molecular diversity previously explored in the scaffold^{111–113} by preparing the first library of BODIPY dyes.¹¹⁴ The reaction between 1,3-dimethyl BODIPY and a wide range of aldehydes under microwave conditions rendered a collection of 160 BODIPY molecules with red-shifted emission wavelengths. The good fluorescent properties and neutral character of these compounds contributed to their development as probes for cell imaging¹¹⁵ and *in vitro* applications.¹¹⁶

Diversity-oriented libraries can be designed to optimize particular features of fluorescent structures. Heagy and co-workers prepared a library of *N*-aryl-1,8-naphthalimides by coupling three 1,8-naphthalenedicarboxylic anhydrides to 14 different anilines and identified four compounds with two-color fluorescence emission.¹¹⁷ Dapoxyl libraries have been also constructed by conventional amide coupling. Yao and co-workers prepared 140 dapoxyl dyes by condensing nicotinic acid and 2-aminoacetophenone analogues.¹¹⁸ The authors studied the solvent-dependency properties of the library and identified the compounds with the highest environmental sensitivity.

Chang and co-workers reported a diversity-oriented fluorescent library for the development of photostable near-infrared (NIR) dyes (Figure 9).¹¹⁹ The authors derivatized a tricarbocyanine structure by nucleophilic substitution with 80 amines and subsequent acetylation. Interestingly, the electron-withdrawing effect of the acetyl group minimized the photodegradation of the tricarbocyanine dyes, and one compound (CyNA-414) with better photostability than the NIR standard Indocyanine Green (ICG) was identified. Further studies validated the application of CyNA-414 as a NIR labeling dye for proteins and metabolites for *in vivo* imaging.^{120,121}

3.2. De Novo Construction of Fluorescent Scaffolds

In view of the limited number of fluorescent scaffolds with synthetic flexibility, several research groups have pursued the construction of novel fluorescent skeletons in a diversity-oriented manner. The group of Park reported a library based on the 1,2-dihydropyrrolo[3,4-*b*]indolizin-3-one structure and demonstrated its tunable fluorescent properties (absorption wavelengths, 298–440 nm; emission wavelengths, 420–613 nm) (Figure 10).¹²² Five α,β -unsaturated aldehydes and five pyridine derivatives were used as starting materials to obtain azomethine ylides, which were then subjected to 1,3-dipolar cycloaddition and further oxidized in a one-pot fashion. The authors evaluated the spectral properties of the 24 novel fluorophores and selected two compounds with high quantum yields and large Stokes shifts (i.e., over 100 nm). After incorporating a maleimide group for bioconjugation to thiol moieties, the two new derivatives were used as labeling dyes for immunofluorescence cell imaging. In a subsequent report, the authors expanded the modifications on the 1,2-dihydropyrrolo-[3,4-*b*]indolizin-3-one core with 44 new derivatives.¹²³ Remarkably, the increase in chemical diversity improved the theoretical calculations correlating Hammett constants and energy gaps, which led to a more accurate prediction of the emission wavelengths for the synthesized compounds.

Multicomponent reactions (MCRs) have been also reported for the preparation of novel fluorescent cores. A 2,6-dicyanoaniline library was synthesized via a three-component, one-pot microwave reaction, in which the *in situ* generation of α,β -unsaturated ketones from different aldehydes and ketones was captured by propanedinitrile to form the 2,6-dicyanoaniline core.¹²⁴ The authors prepared 21 new derivatives and screened their optical properties to eventually discover two dicyanoanilines with high quantum yields. Dicyanoanilines constitute an acceptor–donor–acceptor system with longer-lived charge separation than single donors and acceptors and have the potential to be used as intramolecular PeT probes.

In a recent example, Balakirev and co-workers combinatorially explored the three-component Ugi reaction to discover novel fluorophores with drug-like properties.¹²⁵ Taking

advantage of the easy parallelization of MCRs, droplet arrays with 1600 unique combinations of imidazo[1,2-*a*]pyridine-related heterocycles were prepared from 8 heterocyclic amidines, 40 aldehydes, and 5 isocyanides. The array platform facilitated the fluorescence screening of the library, which eventually yielded a number of fluorescent probes for benzodiazepine receptors. With this example, the authors demonstrated that the discovery of novel fluorescent skeletons based on drug-like structures might accelerate the development of live cell-imaging probes.

The synthesis of new fluorescent platforms can be also designed on the basis of known motifs. Chang and co-workers constructed a benzylideneimidazolinone (BDI) library to prepare new probes based on the chromophore of the green fluorescent protein (GFP).¹²⁶ The authors considered previous reports indicating that the BDI scaffold exhibits a notable fluorescent increase upon freezing its rotation^{127,128} to synthesize a collection of 41 potential “turn-on” probes by condensating an imidate structure to diverse aromatic Schiff bases. A high-throughput *in vitro* screening resulted in the identification of three selective “turn-on” probes for pH, human serum albumin (HSA), and RNA.

3.3. Solid-Phase Diversity-Oriented Fluorescent Libraries

The use of solid phase in the construction of diversity-oriented fluorescent libraries complements the chemistry developed in solution-phase approaches. Solid-phase protocols have been well-established for the synthesis of peptides and nucleic acids, and on-bead screenings are the most direct and practical way to evaluate these libraries. Regarding the synthesis of on-bead small molecule sensors, the potential interference of the solid support in the recognition event requires a time-consuming optimization of the different components of the sensors (i.e., resin, spacer, and receptor).⁴⁸ Instead, most libraries of small molecules prepared in the solid phase are oriented toward increasing the chemical diversity of the probes and facilitating their purification process.

3.3.1. Small Molecule Libraries. With the aim of expanding the diversity achieved by solution-phase chemistry, the group of Lam developed a solid-phase approach to incorporate multiple derivatization points in the coumarin scaffold.^{129,130} The synthesis started with the loading of 7-fluoro-4-methyl-6-nitro-2-oxo-2*H*-1-benzopyran-3-carboxylic acid onto Rink amide resin (i.e., an acid-labile polystyrene resin widely used for the preparation of amide derivatives). The aryl fluoride group was substituted by different nucleophiles, and the reduction of the nitro group yielded an additional heterocyclic ring that introduced further diversity and allowed the preparation of imidazocoumarins, lactam coumarins, and thioimidazocoumarins. Park and co-workers recently reported an extension of the solid-phase chemistry of benzopyrans with a 434-member library obtained by different chemical reactions (i.e., Suzuki coupling, Stille coupling and subsequent Diels–Alder, asymmetric hydrogenation, aromatization, and click chemistry).¹³¹

Solid-phase synthesis can be useful to surmount challenging purification steps and accelerate the preparation of diversity-oriented libraries. Many fluorescent scaffolds contain both hydrophobic regions and polar groups, which often make their purification difficult. The Knoevenagel derivatization has been adapted to positively charged structures to prepare solid-phase diversity-oriented libraries of styryl,¹³² benzimidazole,¹³³ and quinaldine dyes (Figure 11a, 11d, and 11f, respectively).¹³⁴ All

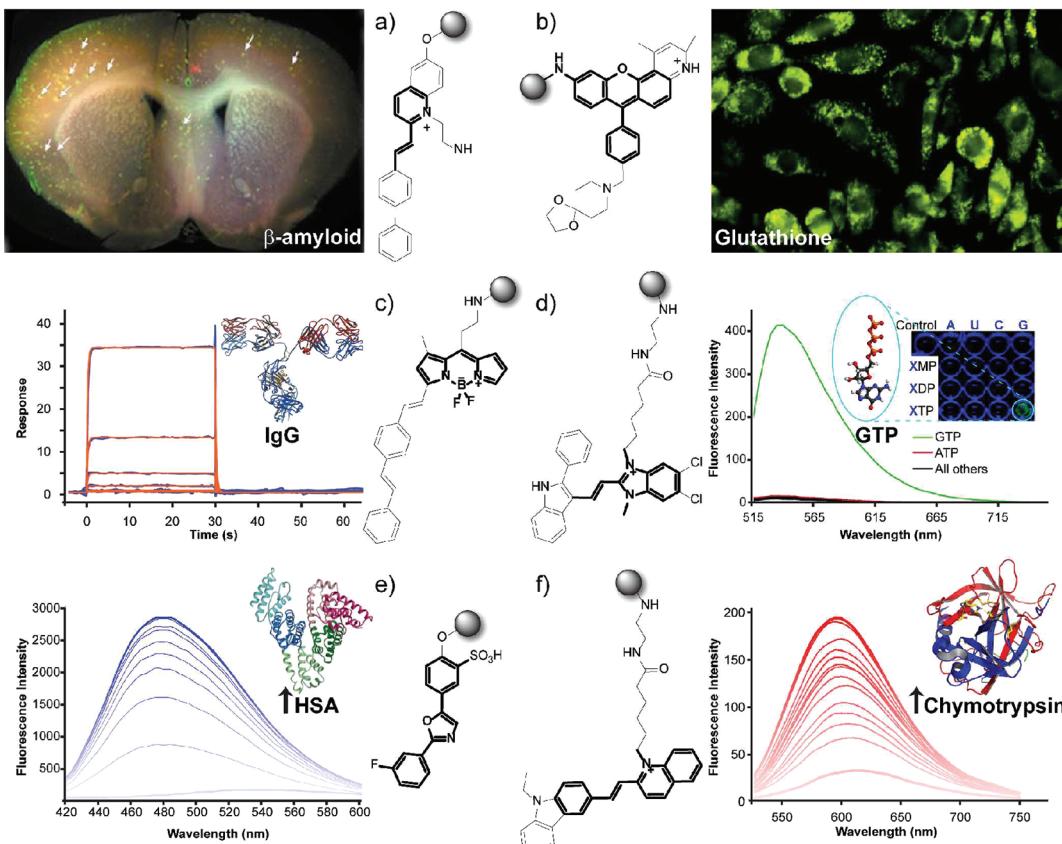


Figure 11. Diversity-oriented libraries of small molecules prepared by solid-phase synthesis. (a) Styryl dyes and labeling of amyloid deposits (white arrows) in mouse brain tissue; (b) rosamine compounds and glutathione staining in 3T3 cells upon activation with α -lipoic acid; (c) BODIPY dyes and binding sensorgrams of Ig Orange upon injection across an immobilized immunoglobulin chip; (d) benzimidazolium compounds and fluorescence spectra of GTP Green upon incubation with GTP and other nucleotides; (e) dapoxyl dyes and fluorescence increase upon incubation with HSA; (f) quinaldine dyes and fluorescence increase upon incubation with chymotrypsin. Reprinted with permission from refs 132, 139, 135, 133, 138, and 134, respectively. (a) Copyright 2004 Wiley-VCH. (b, d, e, f) Copyright 2006 and 2008 American Chemical Society. (c) Copyright 2011 The Royal Society of Chemistry.

these syntheses began with the incorporation of short linkers (e.g., ethylenediamines and amino alcohols) that connected the fluorescent skeletons to the resins, which was followed by the combinatorial diversification and the release of the fluorescent compounds by cleavage of the resins. The simplicity of this approach allowed the rapid construction of collections of 320 styryl, 96 benzimidazolium, and 96 quinaldinium dyes, and their application to probe development has been demonstrated with the discovery of fluorescent sensors for β -amyloid plaques, GTP, and chymotrypsin.

In a similar approach, the lab of Chang reported the first solid-phase library of BODIPY dyes (Figure 11c).¹³⁵ Although the adaptability of the BODIPY core to solid-phase chemistry had been controversial because of its lability under both basic and acidic conditions,¹³⁶ the authors derivatized an aminoethyl BODIPY scaffold on chlorotriptyl chloride resin and minimized the purification steps by optimizing very mild cleavage conditions. Notably, the aminoethyl linker introduced into the BODIPY core favored the interaction of the compounds with different proteins, and a fluorescent probe for immunoglobulin was discovered.

The versatility of this strategy allows its adaptation to numerous fluorescent scaffolds undergoing different chemical reactions. Xanthone derivatives have been prepared on chlorotriptyl chloride resin employing click chemistry,¹³⁷ and the harsh cyclodehydration conditions for the synthesis of the

dapoxyl core have been adapted to solid phase to prepare environmentally sensitive dyes.¹³⁸ Chang and co-workers prepared the first diversity-oriented library based on the rosamine scaffold (Figure 11b).¹³⁹ The authors envisioned that the removal of the 2'-carboxylic acid of the highly fluorescent rhodamine core would enhance the flexibility of rosamine dyes and moderate their quantum yields while retaining their high extinction coefficients and photostability. Twelve unsymmetrical xanthones and 33 Grignard reagents were combined to prepare 240 rosamine derivatives. Subsequent studies have proven the outstanding discriminatory power of these compounds, with fluorescent probes for glutathione, mitochondrial proteins,¹⁴⁰ or stem cells.¹⁴¹

Traceless solid supports, which release the loaded compounds without leaving any chemical group due to their linkage to solid phase,¹⁴² are alternative tools to assist the purification of small molecule fluorescent libraries. Soon after Isacsson and Westman reported the first solid-phase synthesis of asymmetric cyanine dyes with DNA recognition properties,¹⁴³ Balasubramanian and co-workers developed a catch-and-release strategy to prepare unsymmetrical trimethine cyanine dyes.¹⁴⁴ Hemicyanine intermediates were loaded on sulfonyl chloride resin and attacked by a heterocyclic carbon nucleophile to release the final cyanine dyes, hence avoiding the removal of contaminating symmetrical dyes or hemicyanine intermediates. Similar combinatorial approaches were applied later to other cyanine

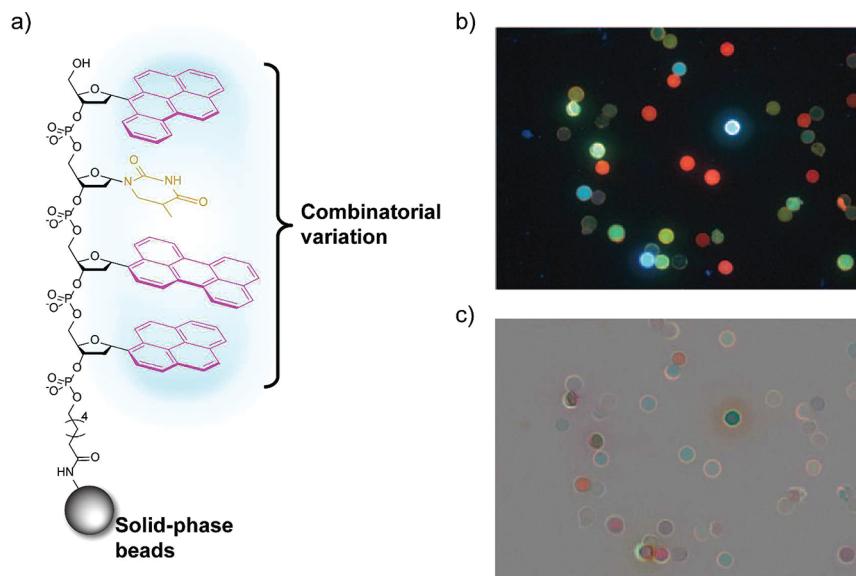


Figure 12. DNA-polyfluorophore on-bead libraries. (a) Chemical components forming one representative library member; (b) image of the on-bead library; (c) the fluorescent changes in response to the dimethylaniline are observed in the subtracted image after exposing the beads to dimethylaniline. Adapted and partly reproduced with permission from ref 168. Copyright 2011 Wiley-VCH.

structures (i.e., pentamethine and water-soluble,¹⁴⁵ heptamethine cyanines,¹⁴⁶ and phthalocyanine dyes¹⁴⁷).

Traceless resins have been also used in combinatorial libraries of thiazole orange derivatives,¹⁴⁸ thiazolo[4,5-*b*]-pyridines,¹⁴⁹ and unsymmetrical *trans*-stilbenes.¹⁵⁰ Regarding the preparation of *trans*-stilbenes, Park and co-workers synthesized a sulfonate-based solid support by loading 4-bromobenzenesulfonyl chloride on a hydroxyethylmethyl resin that was further modified with a benzaldehyde group. The authors designed a two-dimensional library, first with the reaction between the solid support and different benzylphosphonates to obtain polymer-bound stilbenesulfonates and second with a nickel(0)-catalyzed cleavage and cross-coupling reaction using Grignard reagents to release the final collection of unsymmetrical stilbenes.

Although many solid-phase libraries of small molecules are cleaved in their final step to afford combinatorial collections of potential fluorescent probes, on-bead fluorophores might overcome some shortcomings of fluorescent small molecules (e.g., aggregation and self-quenching). O’Shea and co-workers immobilized the highly hydrophobic aza-BODIPY compounds on alcohol-functionalized polystyrene (PS) beads with an oxygen–fluorine displacement reaction between the resin and the fluorine atoms of the aza-BODIPY scaffold.¹⁵¹ The resulting constructs exhibited good photostability and might be used as acid-sensitive NIR fluorescent probes.

3.3.2. Peptide and Nucleic Acid Libraries. The conventional methodologies for the solid-phase synthesis of peptide and nucleic acid libraries have favored their screening as solid-supported platforms. Notably, the interference of the resin in the recognition process of peptides and nucleic acid probes is usually marginal because the probes are generally large macromolecules. Unlike fluorophores or synthetic conjugated polymers,¹⁵² peptides and nucleic acids are inherently non-fluorescent, and several approaches have been described to facilitate their application to fluorescence screenings. One strategy involves the screening of on-bead libraries against fluorescent targets so the resulting fluorescent beads can be sorted and decoded after washing the excess of substrate. These

approaches are adaptable to numerous targets (e.g., small molecules,¹⁵³ antibodies,¹⁵⁴ proteins,^{155,158} and cells¹⁵⁷) provided that the fluorescent tag does not impede the recognition event (e.g., nitrobenzoxadiazole (NBD) is preferentially used to label small biomolecules because of its small size^{158,159}) nor bind nonspecifically to the solid support.¹⁶⁰

Alternatively, on-bead peptide libraries can be labeled with fluorophores. A few methods to incorporate fluorescent tags in peptide libraries have been described. Rademann and co-workers optimized the preparation of fluorescein-labeled peptides,¹⁶¹ whereas Ellman’s and Katritzky’s laboratories worked on coumarin derivatives.^{162,163} Unnatural amino acids containing fluorogenic motifs¹⁶⁴ or FRET pairs^{165,166} are useful tools but are rather used to study protein–protein interactions than to develop fluorescent probes. Split-and-mix libraries of fluorescent peptides have been also reported as optical encoding tools.¹⁶⁷

Although it was synthetically more challenging, Kool and co-workers constructed solid-phase libraries of short DNA-like oligomers in which nucleobases were replaced by fluorophores (Figure 12).^{168–170} These libraries were prepared in a DNA synthesizer using a standard phosphoramidite synthetic procedures and split-and-mix to ensure an extensive molecular diversity. In a representative application, polyethyleneglycol (PEG)–PS beads were screened against different volatile organic compounds (e.g., acrolein, acrylonitrile, mesitylene, 2,6-lutidine, and dimethylaniline, among others). The fluorescent beads exhibited variable responses (e.g., quenching, lighting up, and wavelength changes) that enabled the discrimination of closely related analytes. The authors concluded that the composition and the sequence and order of the monomers were critical in the recognition process. The broad chemical diversity and wide-ranging spectral properties of these DNA-polyfluorophores have enabled the discovery of a number of fluorescent probes for different applications.^{171,172}

4. IN VITRO FLUORESCENCE SCREENINGS

Combinatorial strategies in fluorescent probe development consider not only the design and synthesis of fluorescent

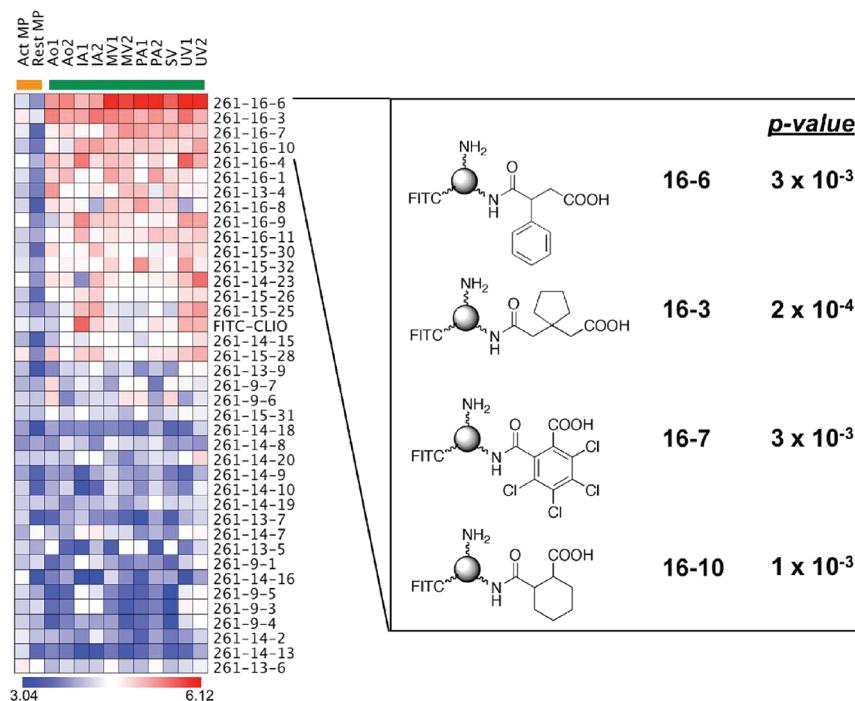


Figure 13. Heat-map analysis of the cellular uptake of fluorescent nanoparticles. The map illustrates the binding of small-molecule-modified nanoparticles to macrophages (columns demarcated by the orange bar) and endothelial cells (columns demarcated by the green bar). The \log^{10} of bound nanoparticle concentrations (in pM) are plotted in each cell of the heat map. On the right are structures of four highly ranked nanoparticles, along with their asymptotic permutation *p*-values. Reprinted with permission from ref 177. Copyright 2009 The Royal Society of Chemistry.

libraries but also the evaluation and validation of the probes in a high-throughput manner. The most conventional screening platforms assess the spectral properties (e.g., fluorescence intensity and emission wavelength) of target- or diversity-oriented libraries in the presence of different analytes. Evolution-based screens, arrays, and differential sensing can accelerate this evaluation process and enhance the discriminatory power of fluorescent probes.^{173,174} Image-based screenings have been recently adapted to fluorescent probe development. With the automatization of high-throughput acquisition and the analysis of fluorescent images of cells and whole organisms, the unbiased screening of diversity-oriented libraries has boosted the discovery of selective fluorescent probes for organelles, phenotypes, cell types, and large macrostructures.

4.1. Fluorescence Spectra Profiling

Target-oriented libraries are conventionally screened by monitoring the changes in the fluorescence spectra of the potential probes after they interact with the target analyte. These screenings are simple and inexpensive but they are limited in throughput, so the selectivity is often only evaluated by measuring the response of probes against some analytes that are structurally related to the target. However, the straightforward readout of these screens enables the easy identification of probes with good sensitivity. Numerous examples have been reported in the development of sensors for metal ions,^{47,51} sugars,⁷⁴ and nucleotides.^{87,133}

The simplicity of these assays has also supported their application as primary screens for targets that are not readily available. Chang and co-workers screened 320 styryl compounds against insulin fibrils with amyloidogenic properties to discover β -amyloid imaging probes.¹³² With this inexpensive primary screening, the authors narrowed down the number of

molecules to be tested in mouse brain tissue and successfully discovered *in vivo* β -amyloid probes.¹⁷⁵ The group of Weissleder reported a screening to profile the cellular uptake of fluorescent nanoparticles derivatized with small molecules (Figure 13). The authors observed that the modifications on the surface of fluorescent nanoparticles modulated their uptake in various cell types and employed nanoparticles with the desired cell targeting properties as *in vivo* imaging probes for pancreatic cancer¹⁷⁶ and endothelial cells.¹⁷⁷

Spectra profiling in diversity-oriented fluorescent libraries is conceptually different from the one in target-oriented libraries because there is no prior definition of a target of interest. Diversity-oriented libraries can be screened against molecules that complement the structural characteristics of the library members and induce a change in their fluorescence spectra (e.g., positively charged benzimidazolium dyes were reported as probes for the negatively charged heparin).¹⁷⁸ The screening of diversity-oriented fluorescent libraries can be assisted by cell-imaging data, which may direct the evaluation of the libraries against analytes localized in particular cellular organelles. For instance, Chang and co-workers exploited the nuclear localization of styryl dyes to discover fluorescent styryl probes for DNA and RNA.^{110,179}

Unbiased fluorescence screenings, which can be applied to any collection of probes and targets, are powerful approaches to profile the response of diversity-oriented fluorescent libraries. The success rate of unbiased screenings is enhanced when they include targets covering a broad range of biological events and the library members have moderate-to-low quantum yields, which facilitates the detection of changes in their fluorescence emission. Unbiased screenings using libraries of GFP chromophores¹²⁶ and BODIPY dyes have been recently reported (Figure 14)¹¹⁵ and proved that the combination of diversity-oriented fluorescent libraries and multianalyte screen-

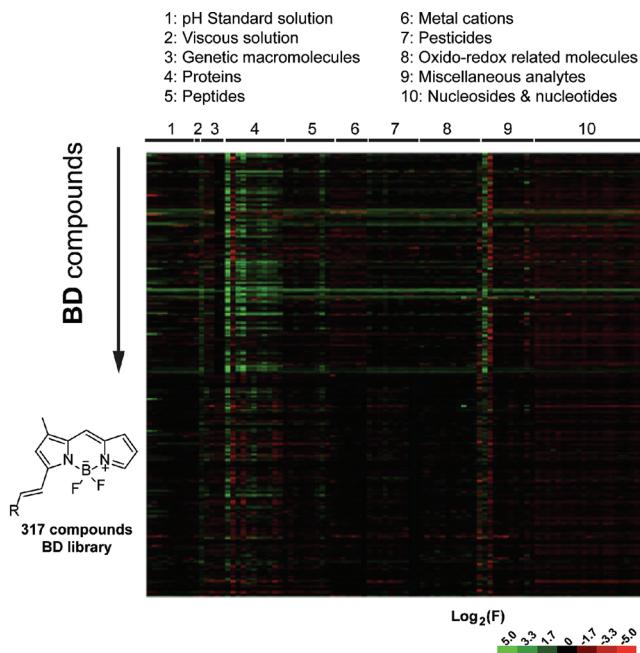


Figure 14. Heat-map plot of the fluorescent pattern of BODIPY dyes. BODIPY dyes (317) were screened *in vitro* against 94 different biomolecules. Green and red colors, respectively, show the resulting increases and decreases in fluorescence at the maximum emission wavelengths of every compound (F represents the ratio between the fluorescence intensity with analyte and the fluorescence intensity without analyte). Reprinted with permission from ref 115. Copyright 2011 The Royal Society of Chemistry.

ings can significantly accelerate the discovery of probes for proteins and metabolites. The spectroscopic profiling of fluorescent libraries also can be oriented toward optimizing the screening design¹⁸⁰ or tailoring the fluorescent properties of small molecule fluorophores.¹⁸¹

4.2. Evolution-Based Strategies

Evolution-based approaches are seldom applied to collections of small molecules or synthetic polymers. On the other hand, they are common in nucleic acid and peptide libraries, where the immense possible combinations of the building blocks give rise to collections of an unpractical size for screening by other methodologies (e.g., spectra profiling). Evolution strategies rely on SELEX for DNA- and RNA-based libraries and phage and yeast display technologies (generally in combination with cell-sorting tools) for peptide libraries. The main advantage of these strategies is the improvement of the characteristics of the library members through an iterative process of screening-selection-derivatization until fluorescent probes with the desired selectivity and sensitivity are obtained.

4.2.1. Libraries Using Systematic Evolution of Ligands by Exponential Enrichment (SELEX). First developed by the groups of Szostak and Gold,^{182,183} SELEX makes use of the PCR to evaluate collections of nucleic acid libraries against specific biomolecules and amplify those sequences with desirable recognition properties. Inspired by the outstanding fluorescence enhancement of malachite green upon aptamer binding,¹⁸⁴ SELEX-based screenings have appeared as a powerful complement of on-bead split-and-mix RNA libraries to develop probes for RNA imaging.¹⁸⁵ Sparano and Koide reported an interesting platform for the discovery of fluorescent small-molecule-based RNA sensors (Figure 15). The authors

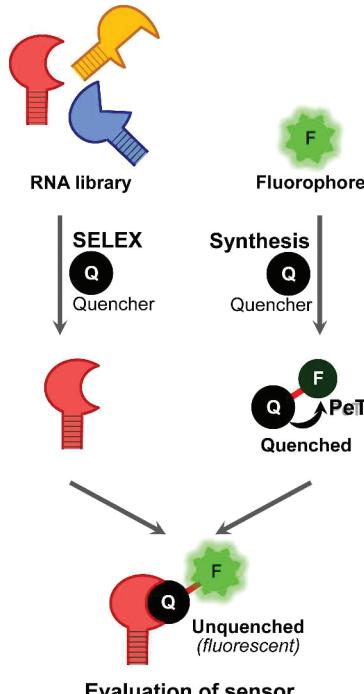


Figure 15. Schematic development of fluorescent RNA probes by SELEX. The organic synthesis to incorporate quenchers into a fluorophore and the *in vitro* evaluation of a RNA library to isolate aptamers against the quencher resulted in the development of RNA-selective fluorescent probes. F, fluorophore; Q, quencher. Adapted with permission from ref 186. Copyright 2007 American Chemical Society.

derivatized 2',7'-dichlorofluorescein (DCF) with aniline quencher units and raised RNA aptamers against the same quencher moieties. The authors proved that the selected RNA aptamers “turned on” the emission of DCF–aniline conjugates and could be employed as fluorescence-inducing RNA tags.¹⁸⁶

Similar approaches were described later to study the interaction of RNA with other fluorescent structures. Rao and co-workers used SELEX in *E. coli* to discover RNA aptamers that were capable to “activate” rationally modified sulforhodamine derivatives. The chemical optimization of the fluorophores improved their binding affinity by 33-fold, and the authors eventually developed a pair fluorophore: RNA with an overall 88-fold increase in fluorescence emission.¹⁸⁷

SELEX strategies have been successfully used to prepare fluorescent DNA probes, such as FRET-based DNAzymes or aptamers for metal ions.^{23,28} To expand SELEX to DNA libraries incorporating “artificial” chemical groups on the nucleobases, the group of Wang recently described the enzymatic incorporation of a fluorescent naphthalimide B-TTP into the side-chain of a functionalized DNA.¹⁸⁸ Notably, the fluorophore maintained both emission and sensing properties after its integration into the DNA structure. The combination of this approach with SELEX strategies will certainly broaden the applications of fluorescent DNA libraries in probe development.

4.2.2. Display Libraries and Fluorescence Activated Cell Sorting (FACS). Phage and yeast display technologies enable the expression of exogenous peptides on the surface of yeast cells or phage particles (the filamentous phage M13 is the most frequent vector).¹⁸⁹ Peptide or protein display libraries are much larger than those prepared by protein engineering

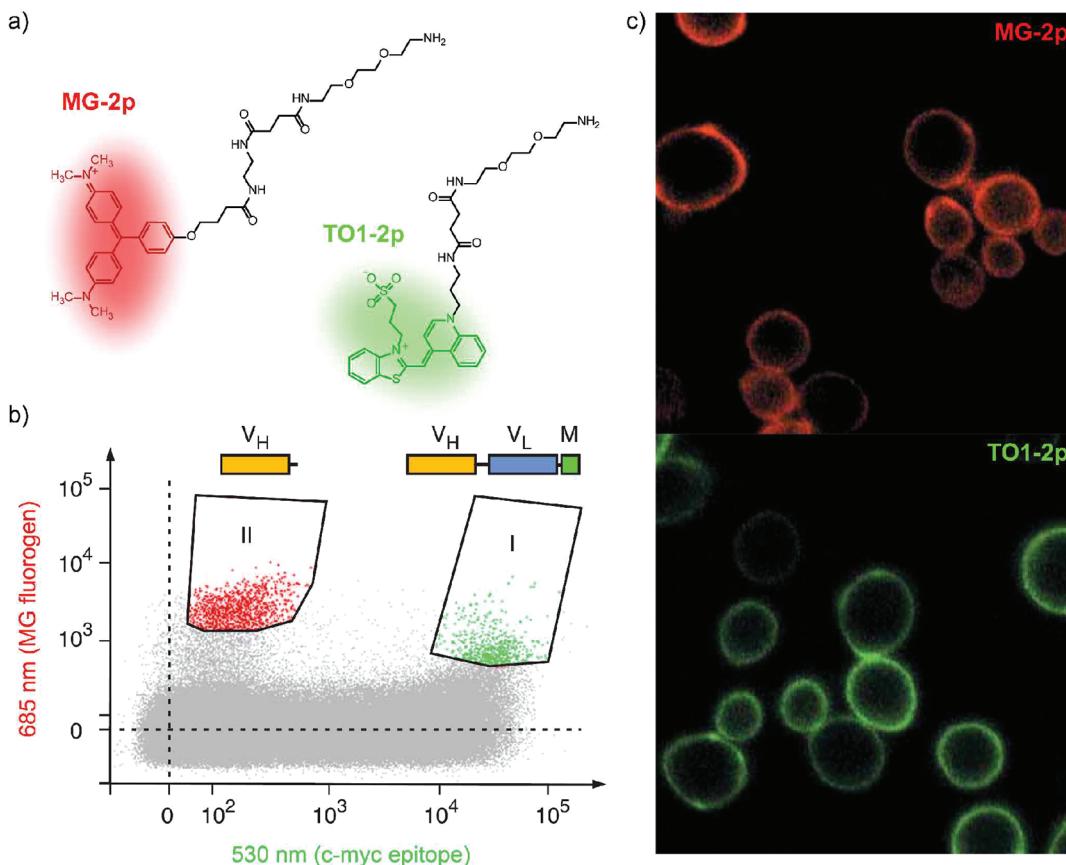


Figure 16. FAPs from a yeast display library of scFvs. (a) Chemical structures of the fluorogenic dyes (thiazole orange as TO1-2p and malachite green as MG-2p); (b) FACS screening separates yeast cells bearing MG-activating scFvs from bulk yeast (horizontal axis, distribution of cells by fluorescein isothiocyanate (FITC)-labeled c-myc epitope; vertical axis, distribution of the cells by the fluorescence generated upon binding to MG [window I collects cells enriched for FAPs composed of heavy chain (V_H), light chain (V_L), and c-myc epitope (M) and window II collects cells enriched for FAPs composed only of V_H]); (c) two-color confocal fluorescence microscopy of yeast display FAPs using a single laser excitation. Reprinted with permission from ref 192. Copyright 2008 Nature Publishing Group.

methods,¹⁹⁰ and they are screened against particular analytes to identify those sequences with high binding affinity and selectivity for the target. As a result, peptide display libraries render fluorescent peptide probes by conjugating fluorescent dyes to the hit peptides obtained after several rounds of screening.¹⁹¹

Protein tags for imaging purposes can be developed when fluorophores or fluorogens (i.e., molecules that undergo a process in which fluorescence is generated) are used as the screened analytes. Analogous to the fluorescence-inducing RNA tags, Waggoner and co-workers designed a platform to identify fluorogen-activating peptides (FAPs) from yeast display libraries of single-chain variable-fragment (scFv) antibodies (Figure 16).¹⁹² After performing a FACS screening of 10^9 human scFv antibodies against two fluorogenic unrelated structures (i.e., thiazole orange and malachite green), the authors identified FAPs enhancing the fluorescence emission thousands-fold and reaching intensity levels comparable to conventional fluorescent proteins. FAPs are excellent probes for live cell imaging when incorporated into fusion proteins because they can provide spatiotemporal resolution and have a significantly lower background than chemically labeled proteins.

Subsequent efforts have been directed to screening peptide libraries against other fluorescent structures. Berget, Armitage, and co-workers screened a yeast display scFv library to identify

one peptide with nanomolar affinity for a range of cyanine dyes.¹⁹³ Weissleder and co-workers obtained peptide tags for in vivo imaging after screening a phage-display 7-mer library against NIR cyanine chromophores. The authors discovered that the peptide IQSPHFF (named as IQ-tag) bound to NIR dyes with subnanomolar affinity and proved its compatibility with different in vivo assays, including cell-labeling experiments.¹⁹⁴

Peptide libraries attached to fluorescent reporters are also compatible with FACS screenings. Daugherty and co-workers engineered a method to display libraries of 10^9 peptides on the surface of *E. coli* that coexpressed an intracellular GFP. The FACS screening of the bacterial fluorescent cells against breast cancer tumor cells was used to identify peptides with an 80-fold increased binding to tumor cells (ZR-75-1) relative to cells from healthy tissues (HMEC, MCF-10A).¹⁹⁵ Cell-specific peptides can be conjugated later to quantum dots so that the fluorescent peptides are directed to the cells of interest.¹⁹⁶

The detection of two different fluorescent signals can improve the efficiency of FACS screenings.¹⁹⁷ Dual-wavelength FACS have been reported to screen a library of surface-functionalized fluorescent nanoparticles and evaluate their capacity to discriminate normal and apoptotic cells.¹⁹⁸ Untreated cells and apoptotic cells (i.e., after treatment with camptothecin) were incubated with Cy5.5-annexin and fluorescein-labeled nanoparticles and analyzed by FACS to

determine the ratios of single- and double-stained cell populations. The authors observed that guanidinium-functionalized nanoparticles showed selectivity for apoptotic cells. This example demonstrates the potential of FACS screenings in the development of probes for functional cell states without prior knowledge of the molecular target.

4.3. Arrays and Pattern Sensing

Arrays are practical tools to organize collections of potential sensors and screen them in a high-throughput format. Libraries of peptides, DNAs, and polymers can be easily immobilized on different surfaces (e.g., cellulose,¹⁹⁹ microarrays,²⁰⁰ and micro-titer plates²⁰¹) to facilitate their screening against analytes of interest, typically fluorogens or fluorophores. Alternatively, arrays can be prepared using fluorescent macromolecule probes. Bradbury, Waldo, and co-workers constructed an array of fluorobodies by inserting diverse antibody binding loops into four of the exposed loops of GFP.²⁰² Fluorobodies combined the intrinsic fluorescence of GFP with the recognition characteristics of antibodies, and their emission intensity correlated well with their binding properties. The authors discovered fluorobodies with high affinity for proteins (e.g., myoglobin, tubulin, and ubiquitin, among others) and successfully applied them to enzyme-linked immunosorbent assays (ELISAs), flow cytometry, immunofluorescence, and gel-shift assayss.

Unlike other screening platforms, arrays can take advantage of the combined response of their components to create pattern responses or fingerprints. Similar to biological sensory processes like smell and taste, pattern sensing (also known as differential sensing) employs collections of not highly selective receptors and their nonspecific response patterns for analyte recognition.²⁰³ The groups of Walt and Suslick pioneered the use of fluorescent arrays as cross-reactive probes mimicking the olfactory system. Whereas the group of Walt worked with polymer matrices immobilizing the Nile Red dye,²⁰⁴ Suslick described an array of metalloporphyrins to detect different organic vapors (e.g., alcohols, amines, ethers, and phosphines, among others).²⁰⁵

Afterward, the lab of Anslyn created a tripeptide array to obtain differential receptors for ATP and GTP by using an indicator displacement assay.²⁰⁶ The array consisted of a cationic tripeptide library that was first incubated with a fluorescent anionic indicator (i.e., fluorescein) and later exposed to solutions of different anionic nucleotides. The nucleotides displaced fluorescein from the array components at different rates, and the resulting pattern was used to discriminate structurally similar molecules. Mihara and co-workers employed off-bead fluorescent peptide libraries with diverse secondary structures (i.e., α -helices and β -strands) to construct dry peptide arrays for proteins. The pattern responses of the array after protein binding successfully clustered different families of proteins.^{207,208} The detection of proteins with fluorescent peptide microarrays has been also achieved with arrays relying on enzymatic activities rather than binding affinities.²⁰⁹ Nucleic acid structures have been also utilized in differential sensing. Dye-labeled, immobilized single-stranded DNAs were described as sensors for volatile chemicals in the vapor phase.²¹⁰

Several strategies have been developed to maximize the power of arrays while using the smallest number of components. For example, small molecule fluorophores can be combined with metal complexes^{211,212} or supramolecular

structures.^{213,214} In the first examples, Severin and co-workers successfully discriminated closely related peptides with an array of six fluorescent dyes and three metal-transition complexes. As for the latter, Anslyn et al. reported a micelle–fluorophore array to detect nitrated explosives. The authors prepared micelle-solubilized pyrene and anthracene derivatives in polysorbate surfactant Tween 80, and envisioned that the explosives, according to their hydrophobicity, would distribute differently between the micellar interior (where most of the fluorophore dissolved) and the aqueous medium. As a result, the different partition of the explosives resulted in unique fluorescent quenching fingerprints.

Other pattern sensing approaches have incorporated binding recognition motifs into fluorescent reporters. Anzenbacher and co-workers prepared an array for metal cations by conjugating a common receptor (i.e., 8-hydroxyquinoline) to various chromophores.²¹⁵ The coordination of the cations to the quinoline scaffold resulted in metalloquinolinolate complexes that exhibited in their fluorescent properties. The combination of spectral modifications including fluorescence enhancement, energy transfer, and metal quenching generated a distinct pattern response for every cation (e.g., Ca^{2+} , Mg^{2+} , Cd^{2+} , Hg^{2+} , and Co^{2+} , among others).

Molecules with large hydrophobic recognition surfaces are potentially good cross-reactive sensors. The group of Hamilton explored the binding capabilities of tetraphenylporphyrins (TPPs), which are inherently fluorescent structures, to create differential arrays for proteins (Figure 17).^{216,217} The authors prepared a set of TPPs with diverse binding properties after modifying their periphery (*m*-, *p*-phenyl or β -pyrrole positions) with amino acid derivatives. These modifications included electrostatic charges and hydrophobic groups that could match complementary domains of the proteins. In addition to their detection capabilities, TPPs may be used to gain knowledge of the surface characteristics of proteins.

Rotello, Bunz, and co-workers reported the detection of proteins with fluorescent conjugated polymer arrays (Figure 18).²¹⁸ The authors synthesized water-soluble PPE polymers with potential to bind protein surfaces through multivalent interactions. PPE polymers are highly sensitive to the environment and contain various structural features (e.g., charges and molecular scales) that can lead to a large binding diversity when they interact with proteins. An array of six PPEs generated distinct fluorescent patterns to discriminate a total of 17 proteins covering a broad range of structures, molecular weights, and isoelectric points. Subsequent applications of these arrays include their adaptation to nanoparticle-based materials²¹⁹ and to cell sensing (e.g., discrimination between cancerous and metastatic cells).²²⁰

Proteins with inherent low binding specificity (i.e., serum albumins) have been recently adapted to the preparation of fluorescent sensor arrays. The combination of different serum albumins with a fluorescent indicator (6-propionyl-2-dimethylaminonaphthalene, PRODAN) has been described as an effective source of cross-reactive arrays for terpenes²²¹ and fatty acids.²²²

5. IMAGE-BASED FLUORESCENCE SCREENINGS

5.1. Recent Advances in High-Throughput Microscopy

High-throughput fluorescence screenings are versatile assays to study the function of specific proteins as well as signaling pathways and cellular phenotypes.²²³ In particular, fluorescent

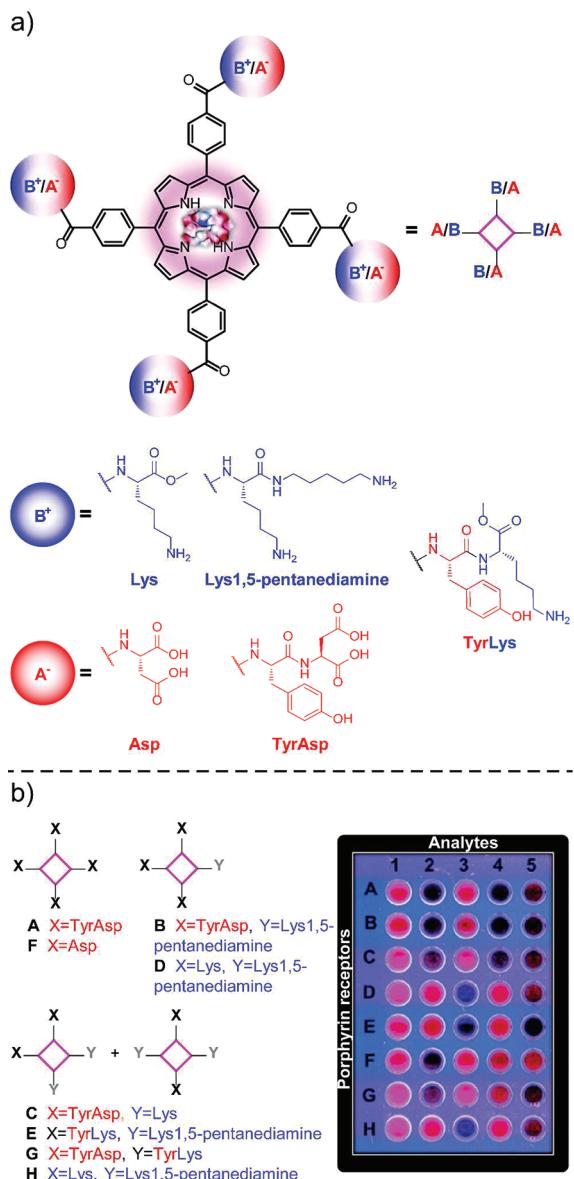


Figure 17. Array of TPPs for pattern sensing of proteins. (a) Chemical structures of TPP receptors; (b) fluorescent image upon UV irradiation of a 96-well plate with eight TPP receptors and four proteins (columns: cytochrome *c* (2), ferredoxin (3), cytochrome *c551* (4), and myoglobin (5)). Adapted with permission from ref 216. Copyright 2004 American Chemical Society.

image-based screenings have undergone a notable progress in the past few years with major advances in the development of high-throughput imaging and analysis software tools (Figure 19).²²⁴ This improvement has been reflected in both the quantity and quality of the readouts, with larger libraries of chemicals and the expansion of the data content from population averaged to individual cellular information. Although image-based screenings are relatively recent in fluorescent probe discovery,²²⁵ its progress has been directly linked to the advances in instrumentation and data analysis of high-throughput fluorescence microscopy.

With the development of automated high-throughput imaging systems, large fluorescent libraries can be now routinely screened in numerous cell lines (with layouts ranging from 96 to 1 536 well plates) and generate reproducible cell-

imaging data.²²⁶ Current efforts in the field are directed to the full adaptation of the instrumentation to live cell imaging and the improvement of the spatial resolution beyond the diffraction limit using printing technologies or microfluidics.²²⁷

After the acquisition of a large volume of images, image analysis software tools are required to process the data in a quantitative and unbiased manner. Although commercial software is generally well-suited for commonly studied cell features (e.g., cell counting, cytotoxicity, translocation, and neurite outgrowth), it is limited to a number of phenotypes and cannot be used to learn and define new features. Sabatini, Carpenter, and co-workers developed CellProfiler (<http://www.cellprofiler.com>) as an open-source imaging-analysis software that enables the measurement of a large number of cell features.^{228,229} In addition to a remarkable reduction of the time needed for image analysis, this software opened the possibility of processing of complex phenotypes that were inaccessible with other analysis tools.

Image analysis software also aims at more specific applications. Stochaj and co-workers reported an analysis method to quantify fluorescent signals from different subcellular compartments,²³⁰ and Shedd and Rosania analyzed the behavior of a fluorescent styryl dye library to extract structure–localization relationships.²³¹ The authors succeeded in correlating the contribution of different synthetic building blocks to the staining patterns of the fluorescent molecules and concluded that charge migration and partial charge distribution played a major role in determining the localization of the library compounds.

Other imaging informatics projects have targeted the standardization of high-throughput imaging data. The group of Swedlow made a remarkable contribution to this field with the open microscopy environment (OME).²³² OME was designed as an informatics tool to standardize the storage, process, and analysis of microscope imaging data. The development of OME has been essential in quantitative analysis of biological imaging because it enables automatic analysis, modeling, and mining of large image sets while it is compatible with all types of optical microscopes and different data models.²³³

5.2. Cell-Based High-Throughput Screenings

The improvement in high-throughput fluorescence microscopy has significantly changed the scope of fluorescence cell imaging in combinatorial probe development. The initial goal of cell imaging in probe development was limited to evaluating the response of the fluorescent probes (generally identified *in vitro*) in living cells.¹³⁹ Occasionally, validated probes were used in fluorescence image-based high-throughput screens to identify chemicals with a particular biological activity.^{234,235} In the past few years, cell imaging-based screenings have significantly expanded their scope and have been used to identify fluorophores with low cellular retention²³⁶ or profile different cancer cell lines in a systematic way.²³⁷ As a result, unbiased fluorescent libraries, which had been little explored in cell-imaging screens, are now regularly employed in screening laboratories to discover organelle, phenotype, or cell-selective fluorescent probes. Their expansion has been possible because of the progress in high-throughput fluorescence imaging and the rise of combinatorial chemistry using fluorescent scaffolds. As previously mentioned, extensive efforts have been aimed at preparing large collections of small molecule diversity-oriented fluorescent libraries. These libraries constitute an excellent

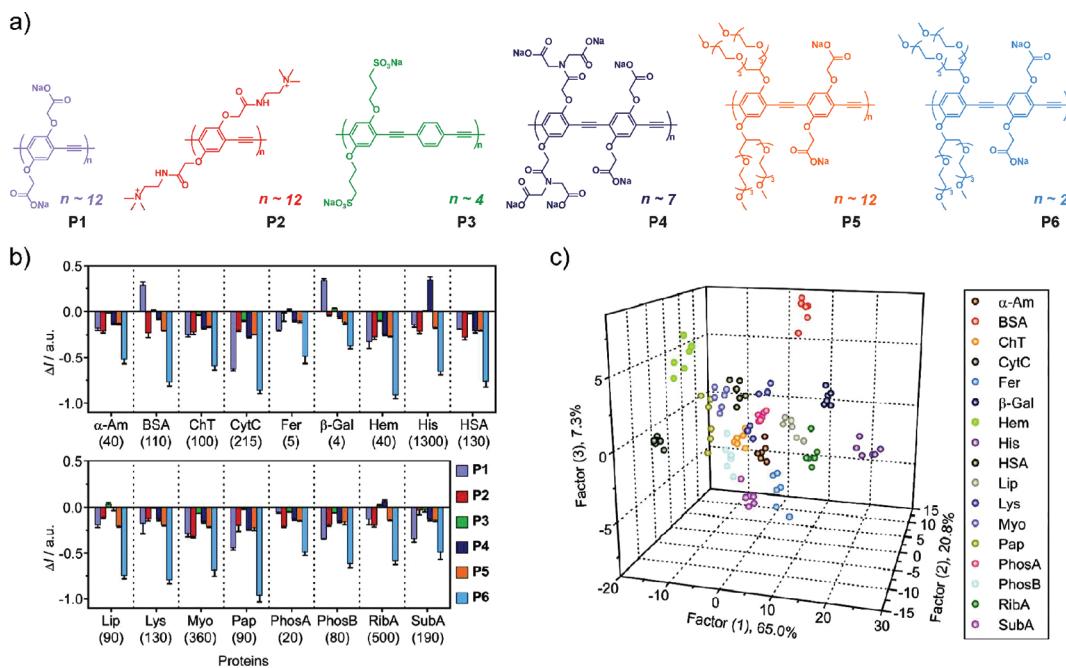


Figure 18. PPE polymers for protein detection. (a) Chemical structures of six functionalized PPE polymers (P1–P6); (b) fluorescent response patterns of the array (P1–P6) against different proteins; (c) canonical score plot for the first three factors of simplified fluorescent response patterns obtained with the array against 17 different proteins. Adapted with permission from ref 218. Copyright 2007 American Chemical Society.

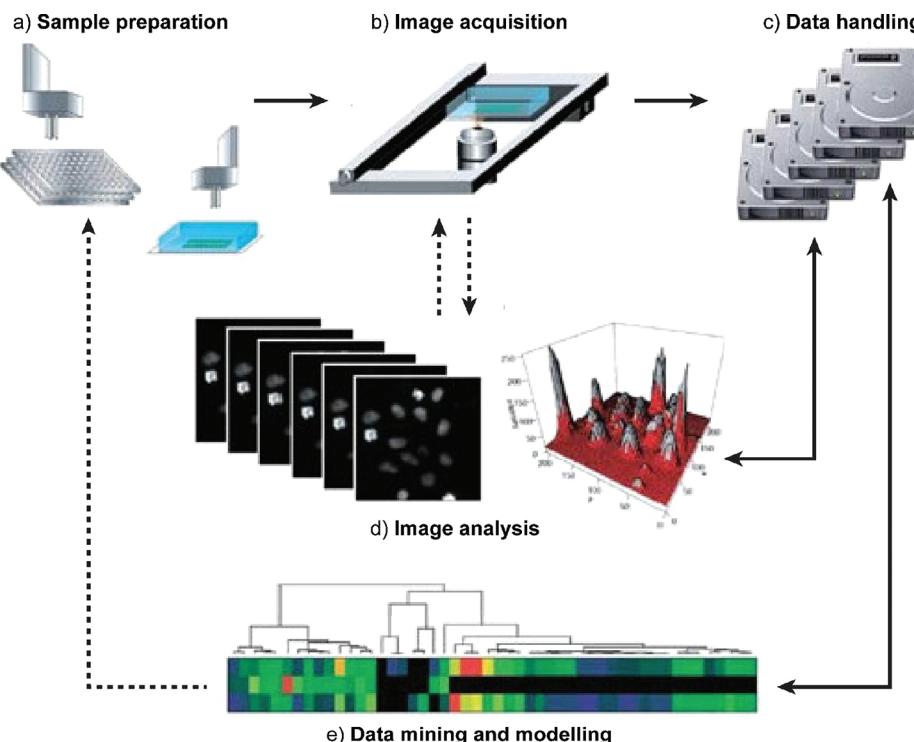


Figure 19. Different steps in a high-throughput fluorescence microscopy experiment. Reproduced with permission from ref 226. Copyright 2006 Nature Publishing Group.

toolbox for unbiased cell-based screenings^{238–240} and enclose important advantages over peptide and nucleic acids (e.g., cell permeability and chemical stability), which make them the most abundant chemical source of cell-imaging screenings.

One of the first reports of unbiased cell-imaging screenings of fluorescent libraries was described by the group of Chang, who analyzed the subcellular localization of 276 styryl dyes in

human melanoma cells (Figure 20).¹⁰⁸ From this library, 119 compounds showed specific localization in a defined cellular compartment (e.g., mitochondria, vesicles, nucleus, cytoplasm, granules, and endoplasmic reticulum), indicating that different chemical modifications on the styryl core could guide the dyes toward different organelles.

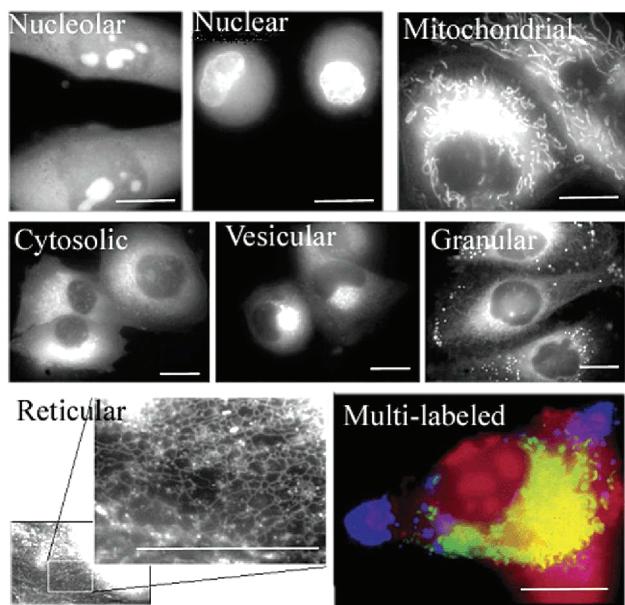


Figure 20. Cell-imaging screening of a fluorescent styryl library. The analysis of the subcellular localization of a styryl library in UACC-62 human melanoma cells proved that different chemical modifications on the styryl core directed the dyes toward different cellular organelles. Scale bar: 10 μm . Reprinted with permission from ref 108. Copyright 2003 American Chemical Society.

With the demonstration that high-throughput cell-imaging screens could successfully identify organelle-selective fluorescent compounds, subsequent approaches were focused on probes for particular cell states or phenotypes. Wagner, Clemons, and co-workers screened a collection of 1606 optically active compounds in C2C12 myoblasts and differentiated myotubes to obtain molecules that fluoresced selectively in only one of the two cell states (Figure 21a).²⁴¹ The authors discovered a myotube-specific rosamine dye, which was effectively applied to a high-throughput screening of inhibitors for muscle differentiation.

In contrast to in vitro screening platforms that rely on spectral or emission intensity readouts, cell-image screenings

are more comprehensive and can track morphological changes of the cells. This feature was exploited to discover a fluorescent compound that regulates muscle differentiation (Figure 21b).¹⁴⁰ With a phenotype-based screening of rosamine dyes in myoblasts, the authors could monitor the formation of myotubes and identify an inhibitor (B25) of the differentiation process. Further experiments indicated that B25 induced the fission of myotubes into mononucleated fragments through activation of the NF- κ B signaling pathway.

These two phenotype-based screens exemplify the versatility of fluorescence cell-imaging assays and how their high information content can be derived into cell-state-specific compounds or probes able to induce a particular cellular response.

Cell-imaging screenings of fluorescent libraries have been also used to obtain cell-specific probes. These assays involve the parallel screening of two or more cell lines that are functionally related or in close proximity within a tissue. Chang and co-workers reported one of the first examples with a fluorescence image-based screen to discover an α -cell probe (Figure 22a).¹¹⁴ Three cell lines (α TC1 and β TC6 cells, which secrete glucagon and insulin, respectively, and HeLa as a control cell line) were treated with a collection of 160 BODIPY derivatives, and their fluorescent images were taken to identify one compound (BD-105) showing a high fluorescence intensity only in α TC1 cells. Moreover, the authors validated the sensing mechanism with in vitro experiments, confirming that BD-105 underwent a significant fluorescence emission increase upon binding to glucagon, the main peptide hormone present in α TC1 cells.

An important advantage of unbiased screenings is their adaptation to probe development without prior definition of the biological target. The emerging interest in stem cell research and the lack of tools for their isolation and characterization encouraged Chang and co-workers to explore fluorescent small molecules as potential stem cell probes (Figure 22b).¹⁴¹ The authors tested a collection of 280 rosamine dyes in mouse embryonic stem cells (mESCs) and mouse embryonic fibroblasts (MEFs), which are generally used as feeder cells. From this primary screen, 20 mESC-staining compounds were selected and eventually narrowed down to one fluorescent probe (CDy1) with a significantly higher

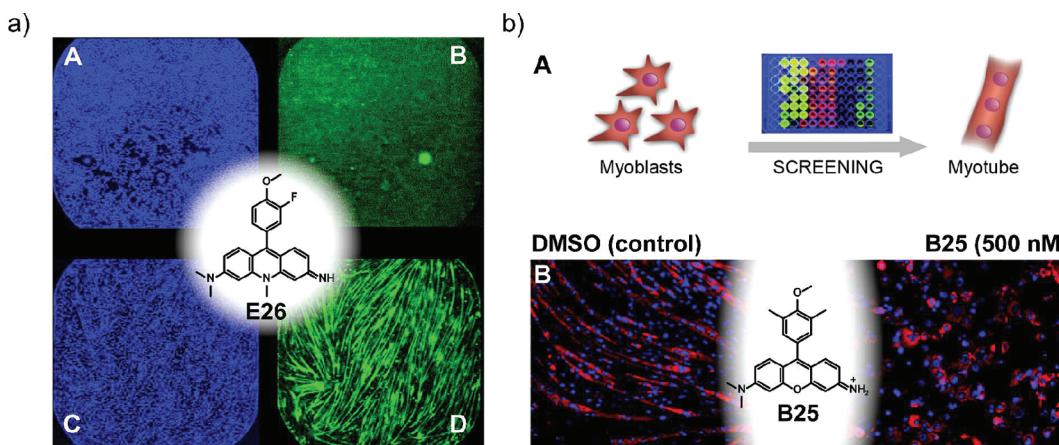


Figure 21. (a) Myotube-specific fluorescent probe. Fluorescent images of myoblasts differentiated from C2C12 cells (A, B) and myotubes (C, D) after treatment with the myotube-specific dye E26 (nuclear counterstaining, Hoechst dye). Scale bar: 150 μm . (b) Screening of a rosamine library in myoblasts to identify compounds controlling muscle differentiation. Schematic diagram (A) and fluorescent images of myoblasts upon differentiation with B25 (B); dimethylsulfoxide (DMSO)-treated control cells were stained with MitoTracker Red (nuclear counterstaining, Hoechst dye). Scale bar: 100 μm . Reprinted with permission from refs 241 and 140. Copyright 2008 and 2010 American Chemical Society.

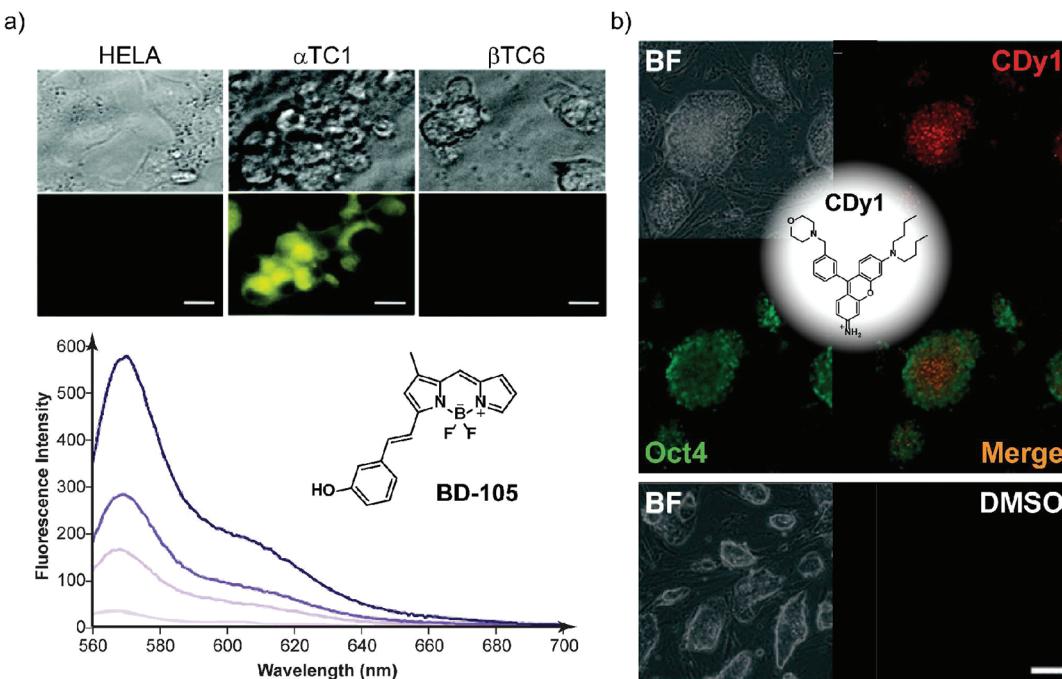


Figure 22. Development of an α -cell selective fluorescent probe. (a) Microscope images of HeLa, α TC1, and β TC6 cells upon treatment with BD-105 (upper row, bright-field images; lower row, tetramethyl rhodamine isothiocyanate (TRITC) fluorescent images; scale bar: 20 μ m) and fluorescence spectra of BD-105 at increasing concentrations of glucagon; (b) development of a fluorescent mESC probe. Fluorescent images of mESC upon staining with CDy1 (upper panel, CDy1-treated mESC were costained with FITC-labeled anti-Oct4 antibody; lower panel, DMSO-treated mESC as a negative control; BF, bright-field). Scale bar: 100 μ m. Reprinted with permission from refs 114 and 141. (a) Copyright 2009 American Chemical Society. (b) Copyright 2010 Wiley-VCH.

intensity in mESC and an earlier fluorescent response than conventional GFP-based genetic markers. Although the mechanism behind the cell selectivity of CDy1 has not been fully unveiled yet, this probe has become an excellent tool for the staining and sorting of live embryonic and induced pluripotent stem cells.²⁴²

The following approaches have pursued a similar objective using different fluorescent libraries. The screening of a xanthone library in mESCs and MEFs resulted in the discovery of a blue-emitting probe for mESCs.¹³⁷ With multispectral cell-imaging approaches showing an enhanced power for discriminating cell populations undistinguishable by conventional methods,²⁴³ we envision that stem cell research may greatly benefit from the combination of multiple mESC-specific small molecule fluorescent probes.

5.3. Tissue and Organism-Based Screenings

Although cell screenings are common in probe development because of their advanced automatization, there is a growing interest in the optimization of tissue-imaging technologies. Cell screens provide imaging data with subcellular resolution but tissue imaging provides a less artificial environment and includes critical factors for the function and activity of the cells.²⁴⁴ However, the complex preparation of histological samples entails a considerable reduction of the throughput, and tissue imaging has been generally reported as a validation method for individual fluorescent probes rather than as a screening technology for fluorescent libraries.^{245–247} One of the few examples of a tissue-based screening was reported in the search of β -amyloid plaque fluorescent probes.¹⁷⁵ A collection of 56 styryl dyes in Alzheimer's disease was screened in human brain sections to find compounds staining β -amyloid plaques

and eventually identified new chemical entities with potential as β -amyloid probes and good blood-brain-barrier penetration.

Current efforts in tissue-imaging technology are focused on multiphoton microscopy, which confines excitation to the focal plane and makes use of long excitation wavelengths to allow deep tissue penetration and minimize photodamage.²⁴⁸ The adaptation of recently described multiphoton structures (e.g., gold nanorods²⁴⁹ and fluorescent small molecules²⁵⁰) to combinatorial chemistry approaches might enhance the development of multiphoton tissue-based imaging screenings in the coming years.

Whole-organism fluorescence imaging has been mainly described on *Danio rerio* (zebrafish), *Caenorhabditis elegans* (nematode worms), and *Arabidopsis thaliana* (thale cress). Fluorescence live imaging in plants is limited to low-throughput studies, usually requiring the expression of GFP-based constructs.^{251–253} On the other hand, zebrafish and *C. elegans* have been increasingly adapted to high-throughput assays.²⁵⁴ In addition to being good models for a number of human diseases, their small size, optical transparency, and short live cycles are key advantages for high-throughput fluorescence imaging screens.^{255,256} An interesting example of this adaptation has been recently reported by Langenau and co-workers, who built a low-cost and easy-to-manipulate light-emitting diode for high-throughput imaging of adult zebrafish. The authors proved the simultaneous imaging of 30 adult animals using five different channels.²⁵⁷

Other recent advances in whole-animal imaging include the lens-free on-chip platform for the ultrawide field-of-view imaging of transgenic *C. elegans*²⁵⁸ or the quantification of the aggregation kinetics of amyloidogenic proteins using time-correlated single-photon counting lifetime imaging.²⁵⁹ The

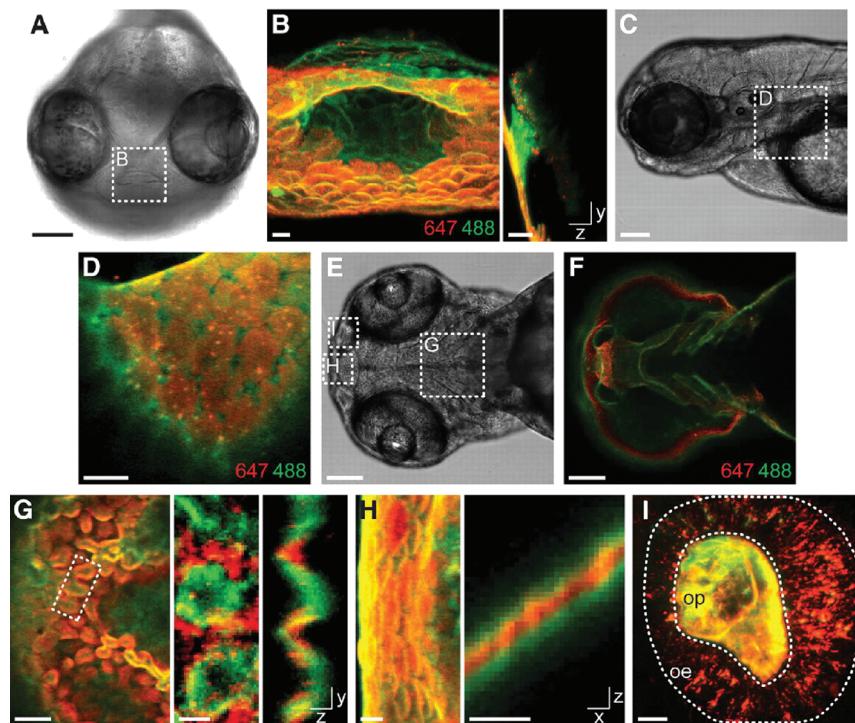


Figure 23. Identification of temporally distinct glycan populations during zebrafish development using two-color labeling. Zebrafish embryos metabolically labeled with Ac₄GalNAz were first reacted with DIFO-647 and then with DIFO-488. Bright-field image of a frontal view (A), z-projection (left panel) and x-projection (right panel) fluorescent images of the mouth region (B), bright-field image of a lateral view (C), single z-plane fluorescent image of the pectoral fin region (D), bright-field image of a ventral view of an embryo (E), single z-plane fluorescent image of E displaying intense DIFO-488 fluorescence but not DIFO-647 fluorescence (F), single z-plane fluorescent image of the jaw region (G, left panel), z-projection (G, middle panel), and x-projection (G, right panel) fluorescent images of the region highlighted in the left panel, z-projection (left panel) and y-projection (right panel) fluorescent images of the mouth (H), z-projection fluorescent image of the olfactory organ (I) highlighting the olfactory epithelium (oe) and olfactory pit (op) regions. Scale bars for A, C, E, and F: 100 μ m; B, D, G, H, and I: 10 μ m; G middle and G right: 5 μ m. Reprinted with permission from ref 261. Copyright 2008 Science.

progress in this field has been accompanied by an expansion of the chemical toolbox to prepare fluorescent animal models. The group of Bertozzi reported an outstanding example of how unnatural sugars and their conjugation to different fluorophores via click chemistry could be used to identify temporally distinct glycan populations in the development of zebrafish embryos (Figure 23).^{260,261} Fluorescent strains of other animal models (e.g., *C. elegans*, zebrafish larvae) have been integrated into high-throughput formats, such as 384-well plates.^{262,263}

Zebrafish larvae can absorb small molecules from the environment through their skin and gills. This trait has been used to image specific compartments *in vivo* (e.g., lipid droplets can be stained by a number of dyes^{264,265}) and to screen collections of fluorescent small molecules in the search for noninvasive visualization probes. Tanaka and co-workers assayed a set of coumarin derivatives and identified probes that preferentially stained retinal cells and enabled imaging of the multiple layers in the retina.²⁶⁶ The group of Chang reported the high-throughput imaging screening of 320 rosamine compounds and discovered two fluorescent molecules with specific localization in the neural bodies and their projections as well as a good colocalization with commercially available neural tracers (Figure 24).²⁶⁷

6. TRENDS AND PROSPECTS

6.1. Challenge of Target Identification

After the successful discovery of fluorescent probes for organelles, phenotypes, or cell states, researchers face the

challenging task of identifying the macromolecules that bind to these probes and are responsible for their selectivity. Target identification remains as one of the most time-consuming and demanding stages of chemical genetics.^{268–270} The challenge also applies to fluorescent probes, with the only advantage that their intrinsic fluorescence can facilitate protein isolation. Although there are numerous tools (e.g., activity-based probes,^{271,272} affinity chromatography,²⁷³ photocross-linking reagents,^{274,275} and bioorthogonal click chemistry,²⁷⁶ among others) that may accelerate this process, chemical modifications that compromise the selectivity of the fluorescent probes are usually required for the identification of a molecular target. Diversity-oriented libraries that consider the incorporation of chemical tools for target identification in their design (as it has been done in medicinal chemistry programs^{277,278}) may offer a faster transition from high-throughput screenings to the elucidation of the binding targets.

Environmental factors also play an essential role in target identification and must be considered as part of the experimental design. An interesting example of how the cellular environment can influence the elucidation of proteins binding to fluorescent probes has been reported recently (Figure 25).²⁷⁹ The authors chemically modified a myotube-staining rosamine dye in order to isolate its target protein(s) by two different methods: (1) affinity pull-down and (2) derivatization with a chloroacetyl thiol reactive group (CDy2) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Interestingly, while affinity pull-down assays

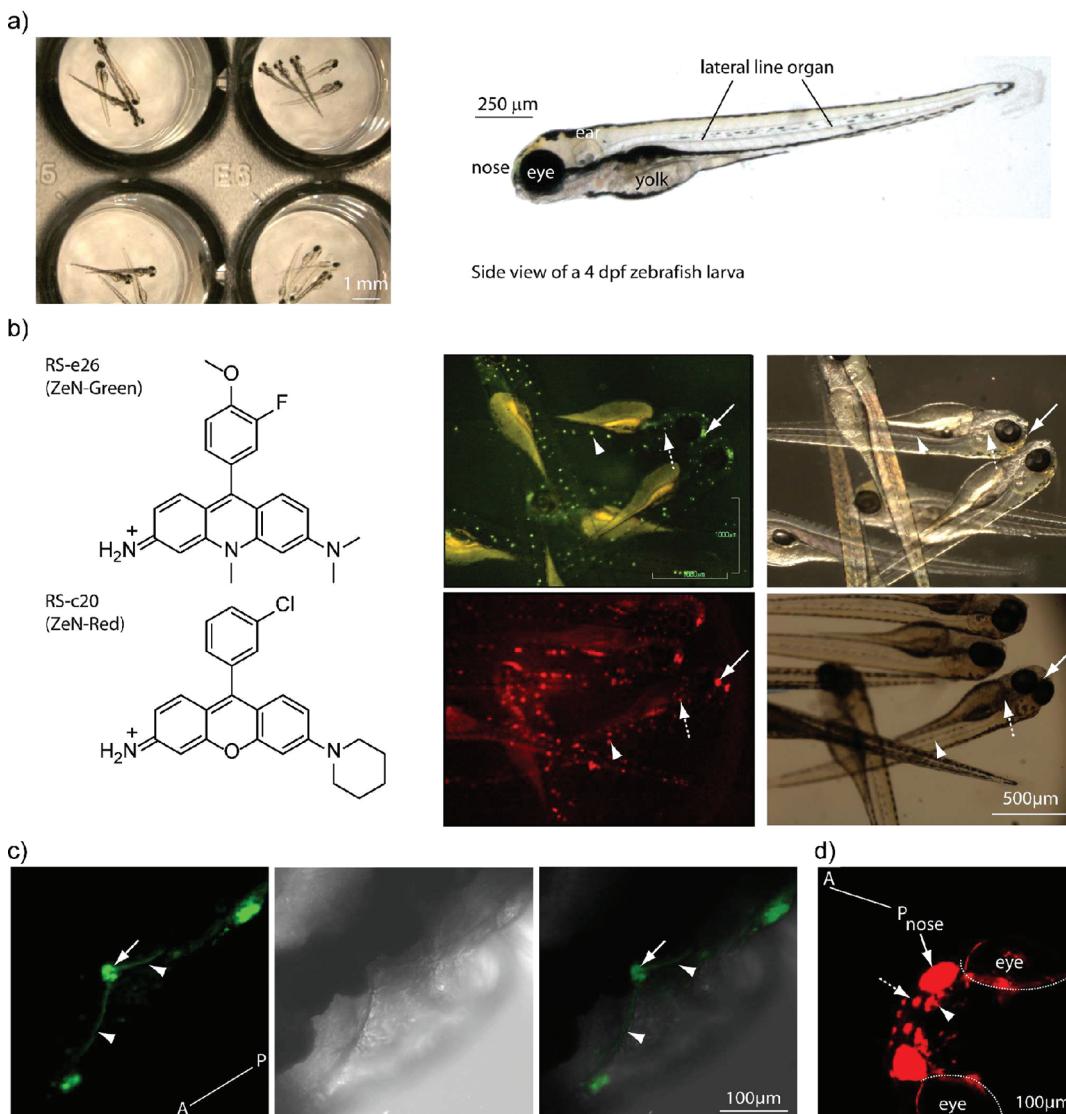


Figure 24. Fluorescence high-throughput screening in zebrafish larvae. (a) 4 dpf larvae in a 96-well plate and a magnified side view; (b) chemical structures of neural tracer dyes RS-e26 (ZeN-Green) and RS-c20 (ZeN-Red) and their fluorescence labeling in larvae; (c) high-resolution confocal reconstruction with ZeN-Green labeling neuron soma in the lateral line organ (arrows); (d) ZeN-Red labeling neuron soma in the nose and their projections as arrowheads (A, anterior; P, posterior). Reprinted with permission from ref 267. Copyright 2010 The Royal Society of Chemistry.

identified tubulin as the main binder, the dye bound to mitochondrial aldehyde dehydrogenase (ALDH2) in living cells, indicating that the cellular environment favored a rapid interaction with the mitochondria before the binding to tubulin could take place. This report proves that complementary methods for target identification may be needed to provide a complete description of the behavior of fluorescent probes in biological systems.

6.2. Design of New Chemical Architectures

6.2.1. Multicolor and Multimodal Probes. The growing interest in developing imaging tools has activated the application of fluorescent probes to visualize complex molecular events *in vivo*.^{280,281} Multicolor toolboxes allow the simultaneous monitoring of multiple (typically up to five) biological processes. Peptide-functionalized quantum dots for multicolor single-cell imaging²⁸² and NIR-labeled dendrimers for *in vivo* lymph node visualization²⁸³ are representative examples. With the development of imaging systems improving the acquisition of multispectral data sets, future combinatorial approaches may

engage in exploiting the multispectral capabilities of fluorescent molecules or even their combination with other multiplexing properties (e.g., Raman scattering²⁸⁴) to render probes with enhanced selectivity and sensitivity.

Another emergent trend for *in vivo* imaging regards the construction of multimodal probes, which combines optical imaging with modalities that provide anatomical information (e.g., magnetic resonance imaging (MRI), positron emission tomography (PET), single-photon emission computed tomography (SPECT), or computed tomography (CT)). Although there are some reports of multimodal probes combining fluorophores, quantum dots, or up-conversion nanoparticles with iron oxide, gadolinium-based materials, or radioisotopes (e.g., ¹⁸F, ⁶⁴Cu, and ¹¹¹In),^{285–288} their adaptation to combinatorial synthesis and high-throughput technologies still requires substantial optimization.²⁸⁹ New materials and imaging systems with higher throughput will be key factors to integrating multimodal probes to combinatorial strategies.

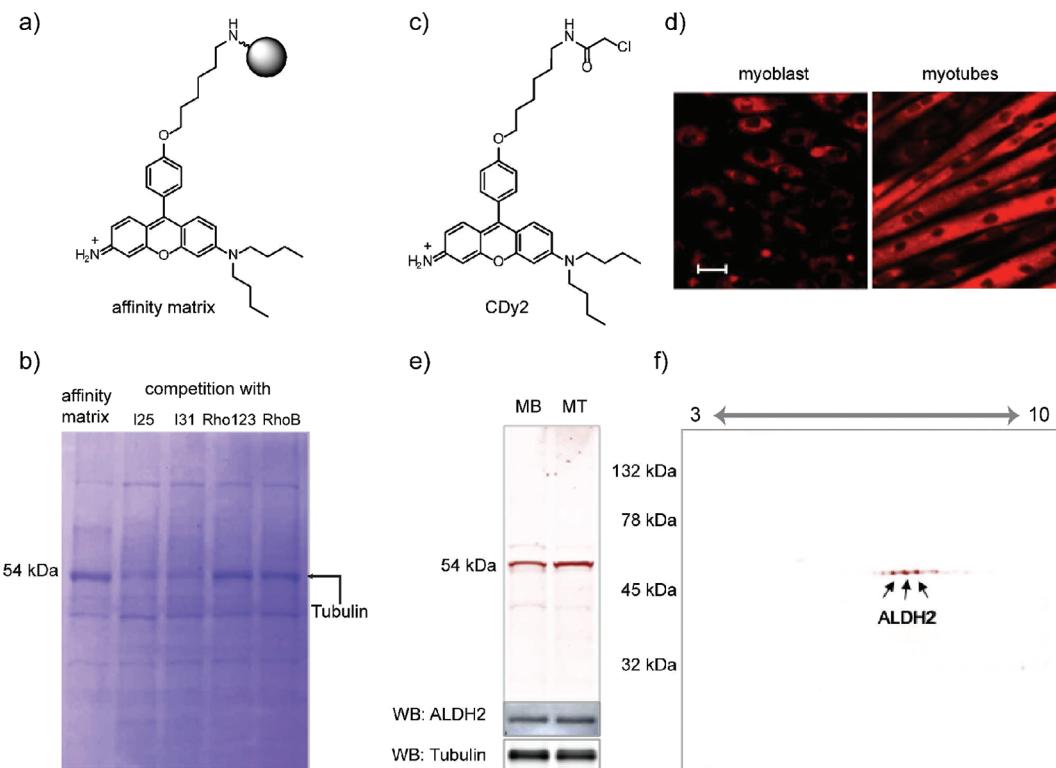


Figure 25. Target identification of a myotube-specific rosamine dye. (a) Chemical structure of the affinity matrix; (b) polyacrylamide gel electrophoresis of bead-bound proteins from C2C12 myotubes; (c) chemical structure of CDy2 for labeling the target protein in living cells; (d) fluorescence microscope images of CDy2-stained myoblasts (MBs) and myotubes (MTs); (e) Western blot analysis; (f) two-dimensional gel analysis for the identification of the labeled protein. Reprinted with permission from ref 279. Copyright 2011 Wiley-VCH.

6.2.2. Dynamic Combinatorial Libraries. The advent of dynamic combinatorial chemistry pioneered by the groups of Lehn and Sanders opened the opportunity to access novel fluorescent architectures that can be rarely achieved by other combinatorial strategies.^{290,291} Dynamic combinatorial libraries (DCLs) are constructed from building blocks that are reversibly connected by imine, hydrazone, acetal, or disulfide linkages. These blocks eventually associate into a composite equilibrium mixture of compounds with concentrations proportional to their thermodynamic stability (Figure 26).²⁹² By exposing the mixture to a target molecule, a new global equilibrium is established to favor those composites with the highest affinity for the analyte of interest.

Lehn and co-workers succeeded in applying fluorescent DCLs as differential sensors for Zn²⁺ by combining three monomers (i.e., 2,7-fluorenedicarboxaldehyde, 2,7-diaminofluorene, and *trans*-1,4-cyclohexanediamine) that were assembled by reversible imine linkages.^{293,294} In the absence of Zn²⁺, the DCLs mainly consisted of a sequence fluorene–cyclohexane including the more nucleophilic diamine. On the other hand, the complexation of Zn²⁺ with *trans*-1,4-diaminocyclohexane shifted the equilibrium toward the formation of polymers with 2,7-diaminofluorene and the emission wavelength shifted from 370 to 493 nm. The isolation and characterization of the components of DCLs remains challenging, but their simple experimental methodology may convert them into a valuable tool to prepare collections of fluorescent probes that are not available by conventional approaches.

7. CONCLUSIONS

The application of combinatorial approaches in fluorescent probe development has experienced a remarkable growth in the last 5–10 years. Several milestones have been achieved: the adaptation of small molecule fluorescent scaffolds to parallel synthesis, the incorporation of fluorescent elements into the automated preparation of nucleic acid and peptide libraries, the integration of profiling and evolution-based strategies to improve the selectivity and sensitivity of the probes, and major technical improvements in high-throughput imaging acquisition and data analysis. With these advances, conventional target-oriented fluorescent libraries have been expanded to diversity-oriented libraries and increased the scope of fluorescent probe discovery. Diversity-oriented libraries are successful in identifying novel structural binding motifs, especially for targets that could not be previously studied (e.g., cell states).

Recent discoveries envisage a promising future for the still-young combinatorial methodologies in fluorescent probe development, yet important limitations must be attended. Libraries of novel and more diverse platforms need to be built. The rising de novo construction of fluorescent skeletons and dynamic combinatorial chemistry are good examples of how complex and new fluorescent probe frameworks can be assembled. Libraries of potential probes must also consider the challenge of target identification from the very first design because the chemical modification of hit probes jeopardizes their selectivity and can slow down the target identification process.

Important efforts are also being driven toward improving instrumentation and data analysis.²⁹⁵ The integration of time-

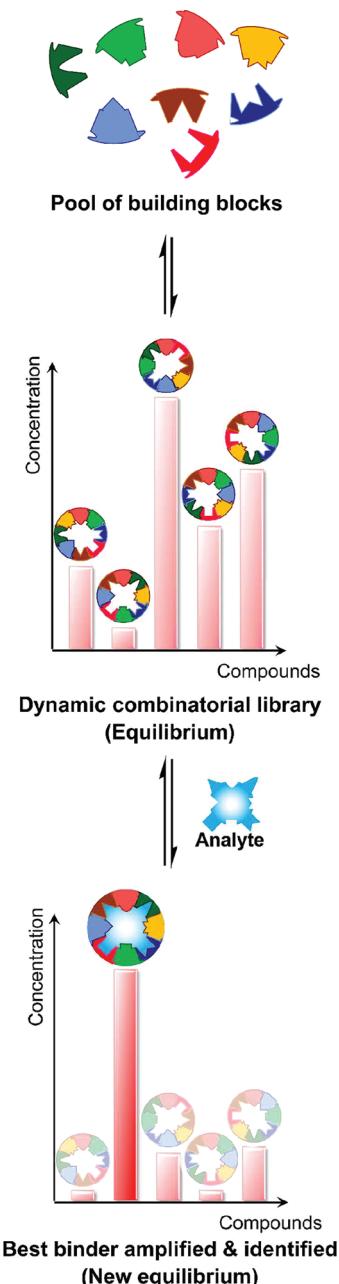


Figure 26. DCLs for the discovery of fluorescent probes with complex chemical architectures. DCLs connect different building blocks by reversible bonds to form an equilibrium mixture of compounds whose concentrations are proportional to their thermodynamic stability. The exposure of the DCL to an analyte shifts the equilibrium toward the probes with the highest affinities for the target. Adapted with permission from ref 292. Copyright 2007 Springer.

course analysis tools to enable the study of the kinetics of cell biology processes, the automation of high-throughput screenings for live cells and whole organisms, and the expansion toward multispectral and multimodal imaging capabilities are important landmarks to ensure the progress of the field. The present of combinatorial strategies in fluorescent probe development is supported by a growing number of successful deliveries. The future of the field will not only depend on how combinatorial chemists tackle the forthcoming technical challenges but also on how they circumvent the problems experienced in past stages of combinatorial chemistry.²⁹⁶

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Notes

The authors declare no competing financial interest.

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Duanting Zhai studied at the University of Soochow (China), obtaining her B.Sc. in Chemistry in 2008. She joined Prof. Chang's research group at the National University of Singapore as a graduate student under the MedChem Programme in 2008. Her research interests are combinatorial synthesis of fluorescent scaffolds,

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Jun Cheng Er was born in Singapore in 1986 and received his undergraduate education at the National University of Singapore (2011, B.Sc. (Hons)) majoring in Chemistry and Life Sciences. He performed research under the guidance of Prof. Yeung Ying Yeung with interludes in the laboratories of Prof. Michael D. Burkart (2010, University of California, San Diego) and Dr. Eric Kantchev (2008, Institute of Bioengineering and Nanotechnology, A*STAR). Jun Cheng was awarded the Outstanding Undergraduate Researcher Prize for his accomplishments in honors research. He is currently pursuing a Ph.D. in the area of Chemical Biology, at the National University of Singapore, School for Integrative Sciences and Engineering, under the supervision of Professor Young-Tae Chang.



Young-Tae Chang studied Chemistry at POSTECH (Korea) and received his B.Sc. in 1991 and Ph.D. in 1997. He did his postdoctoral work with Prof. Peter Schultz at U.C. Berkeley and The Scripps Research Institute. In 2000, he was appointed Assistant Professor at New York University and was promoted to Associate Professor in 2005. In September 2007, he moved to the National University of Singapore (NUS) and the Singapore Bioimaging Consortium (SBIC) at Biopolis. He was promoted to Full Professor in 2012 and is currently running the Medicinal Chemistry Program at NUS and the Laboratory of Bioimaging Probe Development at SBIC. He has published more than 200 scientific papers and 3 books and has filed more than 30 patents so far.

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