Rejuvenating old fluorophores with new chemistry

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Abstract

The field of organic chemistry began with 19th century scientists identifying and then expanding upon synthetic dye molecules for textiles. In the 20th century, dye chemistry continued with the aim of developing photographic sensitizers and laser dyes. Now, in the 21st century, the rapid evolution of biological imaging techniques provides a new driving force for dye chemistry. Of the extant collection of synthetic fluorescent dyes for biological imaging, two classes reign supreme: rhodamines and cyanines. Here, we provide an overview of recent examples where modern chemistry is used to build these old-but-venerable classes of optically responsive molecules. These new synthetic methods access new fluorophores, which then enable sophisticated imaging experiments leading to new biological insights.

Keywords

fluorescence; organic chemistry; rhodamine; cyanine; microscopy; imaging

Introduction

In 1856, 18-year-old William Perkin accidentally synthesized the first synthetic dye, Mauveine (**1**) by condensing a mixture of toluidines (Figure 1A). This preparation of a synthetic colorant was the spark that transformed the field of organic chemistry from a curiosity to a viable industry [1]. That same year the first cyanine dye was synthesized through base-promoted condensation of a mixture of *N*-alkylated quinoline and 4-methyl

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quinoline to yield **2** (Figure 1B) [2]. In addition to early cyanines, triarylmethane dyes were discovered during this decade via condensation of aniline and its derivatives. Reaction of aniline (**3**) with CCl_4 at elevated temperature produced pararosaniline (**4**), a component of the magenta dye Fuchsine, in 1859 (Figure 1C) [3]. Dye discovery continued, with the synthesis of fluorescein in 1871 by Bayer [4] and rhodamine dyes at *Badische Anilin-und Sodafabrik* (BASF) in 1887 [5]. An key example of rhodamine dye synthesis is the acid-catalyzed condensation of 3-*N,N*-dimethylaminophenol (**5**) and phthalic anhydride (**6**) to yield tetramethylrhodamine (TMR; **7**; Figure 1D). These 19th-century advances provided the chemical foundation that enabled further development of these highly absorbing and sometimes fluorescent molecular scaffolds.

Dyes dominated the field of organic chemistry for decades and played an important role in the development of pharmacological agents. The synthesis of Benzoazurine G, an important blue textile dye that competed with natural indigo, resulted in the production of large quantities of *p*-nitrophenol as a byproduct (Figure 1E. **8**); this material was eventually repurposed by Bayer to create the analgesic acetaminophen (*i.e.*, paracetamol; **9**) [6]. The first antibiotic, Prontosil (**10**) was discovered by screening a collection of synthetic dyes for antibacterial activity [7, 8]. Methylene Blue (**11**), another BASF product, found use as a treatment for several maladies including malaria [9]. In the mid 20th century, synthetic dyes gained prominence due to the development of color photography and laser dyes [10]. This was further bolstered by the rise of modern fluorescence imaging techniques, giving rise to the excellent collections of fluorescent labels (*e.g.*, CyDye, Alexa Fluor, ATTO) that consist primarily of rhodamine and cyanine dyes [11].

Despite the 150-year history of synthetic dyes, the chemistry underpinning synthetic colorants remained stubbornly rooted in 19th-centrury chemical methods [12]. These typically involved harsh reaction conditions such as acid catalyzed condensations, which severely limited the scope of dye synthesis. Now, in the 21st century, fluorescent dyes are enjoying a renaissance that is driven by the application of modern synthetic techniques to these classic dye scaffolds. As synthetic organic chemists trained in total synthesis, we advocate that probe discovery be given the same treatment as natural products, replete with retrosynthetic analysis along with adaptation and refinement of modern synthetic methodology for these important targets. Here, we showcase examples from our laboratories and others where new chemistry yields new dyes that enable new, sophisticated biological imaging experiments.

Rhodamines

The first rhodamine dyes absorbed relatively short wavelength light giving rise to a red color —their name stems from the Greek *rhodon* (rose). The high brightness and photostability of rhodamines made them prominent laser dyes and many of these dye scaffolds were repurposed in the 1990s for use as antibody or oligonucleotide labels—most Alexa Fluor and ATTO dyes are simply sulfonated or amidated laser dyes, respectively [11, 13]. Still, as mentioned above, the use of harsh 19th century chemistry—acid-catalyzed condensation at elevated temperature—is incompatible with all but the simplest functional groups, which severely limited the scope of rhodamine synthesis. This is reflected in the structures of

many rhodamines, which incorporate *N*-methyl or *N*-ethyl anilines along with quinoline and julolidine moieties. This limited chemistry produced suboptimal dyes such as TMR that exhibits a relatively low quantum yield ($\Phi_F = 0.41$) due to nonradiative decay involving twisted internal charge transfer (TICT) [14].

The first step in solving this problem is the development of new chemistry that would allow the exploration of a more diverse set of auxochromes beyond *N*,*N*-dimethylamine. The Lavis lab developed a simple strategy starting from dibromofluoran [15] or fluorescein ditriflate [16, 17], using Pd-catalyzed cross-coupling reactions to generate rhodamines (Figure 2A). This idea was supported by the tremendous effort by the Buchwald lab and others developing ligands to facilitate general Pd-mediated reactions [18]. It is important to note that ditriflates of electron-deficient fluoresceins bearing fluorines or chlorines are prone to aminolysis when using primary or secondary amine reactants. This can be remedied by using *t*-Boc-protected amines or switching from ditriflates to dibromides or diiodides, which can be accessed through direct synthesis [15, 19] or via metal-catalyzed reactions [20].

This cross-coupling chemistry allows almost any amino auxochrome to be installed into a rhodamine, making many new derivatives possible. Inspired by photochemistry principles and computational chemistry, the Lavis lab hypothesized that switching the *N*,*N*-dimethylamino group to the diminutive four-membered azetidine could improve fluorophore properties [14]. Azetidine exhibits a higher oxidation potential than dimethylamine and ground-state calculations of azetidinylrhodamines suggested a planar structure necessary for a fluorescent species; together these data suggested a fluorophore with improved properties. This hypothesis was confirmed with the azetidine-containing dye **12** (*i.e.*, Janelia Fluor 549, JF$_{549}$; Figure 2A) showing substantially higher fluorescence quantum yield ($\Phi_F = 0.88$) and better photostability. This general strategy was applied to many different rhodamines, resulting in a palette of dyes spanning a substantial section of the visible spectrum [21, 22]. To further improve brightness and photostability, deuteration of the *N*-alkyl groups was proposed since the oxidation of amines shows a surprisingly high secondary isotope effect [23]. Deuterated azetidine and deuterated pyrrolidine could be incorporated into rhodamines via Pd-catalyzed cross-coupling, yielding bright and photostable dyes such as JFX$_{554}$ (**13**; Figure 2A). Combined with the self-labeling HaloTag system [24], the JF and JFX dyes have enabled sophisticated single-molecule experiments in live cells [25-27]. This cross-coupling approach has been adopted to prepare other useful derivatives such as photoactivatable ("caged") fluorophores [16, 28].

The Pd-catalyzed cross-coupling approach is generalizable but has two limitations. First, it does not allow the construction of rhodamines with fused rings. This problem was addressed by scientists at Promega Biosciences, who showed that condensation of 3-aminophenols and lactols could produce rhodamine dyes in the presence of O_2 (Figure 2B) [29]. This allowed the synthesis of rhodamine **14**, which is an excellent acceptor dye for bioluminescence resonance transfer (BRET). Second, the cross-coupling approach requires synthesis of fluorescein starting materials. This is straightforward for classic, oxygen-containing dyes but not for other variants where the xanthene oxygen is replaced with another moiety such as carbon or silicon—such fluorescein starting materials can be difficult to synthesize [17]. To complement the cross-coupling approach, the Lavis lab developed an alternative approach

where diarylbromides can be metalated and added to either esters or anhydrides (Figure 2C) [30]. Suitable dibromide starting materials can be synthesized in a few steps and this method allows direct access to many rhodamines and their analogs; this strategy can also be used to prepare fluorescein analogs. This so-called "dibromide" synthetic approach has been expanded upon by the Lavis lab and several other groups to prepare numerous rhodamine dyes [22, 31, 32]. In addition to allowing direct access to standard rhodamine derivatives, the approach allows introduction of alternative substituents at the 9-position of the xanthene system, replacing the classic o-carboxy moiety. An example is dye **15**, which contains a carboxy-thiophene moiety. This dye shows high visible absorption in aqueous solution and can be used as a photocatalyst to oxidize dihydrotetrazines, allowing light directed polymerization *in vivo* [33].

Another useful structural modification is fluorination of the pendant phenyl ring. Dyes with this motif exhibit a bathochromic shift in absorption and emission maxima and fluorination also adjusts chemical properties. The electron-deficient phenyl ring is also a good substrate for nucleophilic aromatic substitution reactions (S_NAr), which allow facile installation of a chemical handle for bioconjugation. This has typically been realized by reaction with thiols [34, 35], but the Lavis lab discovered that masked acyl cyanide (MAC) reagents [36] such as compound **16** add in a regioselective manner to the 6-postion of the pendant ring, deprotection of the MOM group yields a putative acyl cyanide that can be transformed to an acid, ester, or anhydride (Figure 2D) [22]. This approach allows the facile synthesis of functional derivatives of dyes such as JFX_{673}-HaloTag ligand (**17**) which combines deuteration, fluorination, and this *umpoloung* chemistry to yield a bright, photostable, and bioavailable dye ligand suitable for pulse–chase experiments *in vivo* [37].

Cyanines

Since the introduction of the "CyDye' series in the early 1990s, polymethine indocyanines have become the molecules of choice as covalent labels to visualize proteins and nucleic acids. More recently, polymethine cyanine dyes are emerging as the molecular core of responsive fluorescent sensors and photocages [38-46]. Despite finding use across the spectrum of preclinical to clinical biomedical applications, the chemistries used to assemble and diversify these probes had changed relatively little until recent years. Here we highlight recent synthetic advances, which are enabling the creation of promising probes for challenging imaging applications.

The quantum yield of a typical pentamethine cyanine ($\Phi_F \approx 0.1$ to 0.2 in aqueous solvent) can present a limitation, particularly for demanding single molecule applications. A major non-emissive pathway for excited-state deactivation involves polymethine isomerization [43, 47]. Using the trimethine scaffold, this pathway was blocked by installing 6-membered rings along the polyene chain to form Cy3B, leading to a dramatic improvement in quantum yield $\Phi_F = 0.09$ for Cy3 to $\Phi_F = 0.85$ for Cy3B [48]. Applying this approach to penta- and hepta-methine congeners required the development of new synthetic strategies to assemble the more complex polycyclic ring systems. This challenge was recently addressed by the Schnermann lab using the synthetic strategy shown in Figure 3A. In the key sequence, a protected aldehyde precursor undergoes a ruthenium-mediated cross-metathesis reaction

followed by an intramolecular Michael addition and dihydropyran ring-forming cascade to form the desired tetracyclic ring system. These compounds exhibit dramatically improved Φ_F relative to a typical Cy5-type fluorophore ($\Phi_F = 0.69$ *vs.* $\Phi_F = 0.15$) and extended fluorescence lifetimes [49]. While mono-sulfonated variants were only suitable for small molecule labeling, second-generation di- and tri-sulfonated Cy5B probes such as **18** enabled the preparation of labeled mAb and nucleic acid conjugates. These probes have been applied for single molecule FRET and single molecule localization microscopy where they improve photon output relative to non-constrained cyanine variants [50, 51]. The Schnermann lab also applied the cross-metathesis/cyclization chemistry on heptamethine cyanine scaffold —an approach that entailed a pentacyclization reaction [52]. Surprisingly, the resulting conformationally restrained heptamethine cyanine did not exhibit significantly improved quantum yields or lifetimes at room temperature in protic solvents. This finding reveals an important insight about the excited state-quenching of heptamethine cyanines—the role of polymethine isomerization is modest relative to the dominant role of water/solvent-mediated excited-state deactivation [53]. Critically, this observation suggests future studies should not focus on the issue of photoisomerization in the development of cyanine probes that absorb at longer wavelengths (>700 nm) [54].

The most broadly used approach to modify the heptamethine cyanine chromophore entails sequential derivatization of cyclohexanone derived precursors [55-58]. Stacko, Klan and coworkers recently reported a new approach to rapidly prepare a range of substituted polymethine probes [59]. This chemistry converts 2,4-dinitroaryl pyridine (Zincke) salts to the corresponding ring-opened dianiline intermediate, which can be carried on to various heptamethine cyanines (Figure 3B). This new assembly strategy has been adopted for a variety of applications, including the development of novel cyanine-based photocages as well as aggregation-resistant fluorophores [40, 45]. Representative of these efforts, s775z (**19**), developed by Smith and colleagues, is a promising compound for *in vivo* imaging applications [60]. This new chemistry has the strategic benefit of starting from broadly available pyridine precursors to enable chemical diversification of the polymethine chromophore unit.

Over the last decade, a variety of studies have shown that wavelengths between 1000 and 2000 nm can enable high-resolution *in vivo* multicolor imaging at depths not possible with conventional optical wavelengths [61]. While significant early efforts in this area were enabled by a range of nanoparticles quantum dots, and carbon nanotubes, there are significant benefits to using small molecule organic probes [62-64]. Recently it has been found that heptamethine cyanines emit readily observed short wavelength infrared (SWIR) signal, which has been broadly applied including in clinical efforts with ICG [65, 66]. To enable multicolor imaging in this wavelength regime, bright, readily accessible, and easily modified probes with absorbance and emission maxima beyond the Cy7 range are needed. However, the design and synthesis of such molecules represents a significant, but now approachable, chemical challenge.

Towards the goal of shifting cyanine absorption wavelengths into the SWIR, recent studies by Sletten and colleagues replaced the conventional indoline ring with modified flavylium heterocycles to provide tunable agents with emission beyond 1000 nm [67, 68]. The

critical synthetic advance used cyanine precursors to assemble the array of tri-, penta-, and hepta-methine probes, which could be applied for a range of mutlicolor imaging efforts (Figure 3C) [68]. Further tuning of the heterocycle has led to compounds such as JuloFlav7 (**20**), which is ideally matched for excitation with the broadly available 1064 nm laser [69]. There remains a significant need for the type of bioconjugatable probes that have proven invaluable for multiplexed imaging in the visible and NIR range. The Schnermann lab hypothesized that nonamethine cyanines, which had been only sparingly reported, might be suitably modified to enable targeted imaging efforts. A rational design process led to the insight that direct aryl fusion onto the nonamethine scaffold might lead to a dramatic bathochromic shift. This supposition was validated through the preparation of the persulfonated indocyanine dyes, FNIR-866 (**21**) and FNIR-1072 (**22**) through catechol- and aryl-ring fusion, respectively, onto the nonamethine scaffold (Figure 3D). These probes were applied in a range of imaging applications, including three-color imaging in a model surgical setting [69].

Conclusion and Future Outlook

The century old rhodamine and cyanine dyes continue to dominate biological imaging. First-generation rhodamines held the blue-orange excitation window and were primary used as antibody labels and live-cell stains. Cyanine dyes filled the far-red and NIR region of the spectrum making them useful for in vivo imaging, while also finding broad use as oligonucleotide and protein labels. Although the inherent advantages of the rhodamine and cyanine scaffolds will ensure different utility, the traditional divisions of these two classes are beginning to blur. This is due largely to new chemistry. The improved methods to make rhodamine dyes are pushing excitation wavelengths longer by incorporating silane, phosphine oxide, and ketone functionalities; these also allow fine-tuning of chemical properties [22]. Likewise, chemistry is providing strategies to broadly tune the cyanine chromophore, enabling a broader set of applications. Looking forward, we hypothesize that the union of advances in the synthetic chemistry and imaging probe design will provide versatile molecules to interrogate complex biological systems with increasing precision.

Acknowledgements

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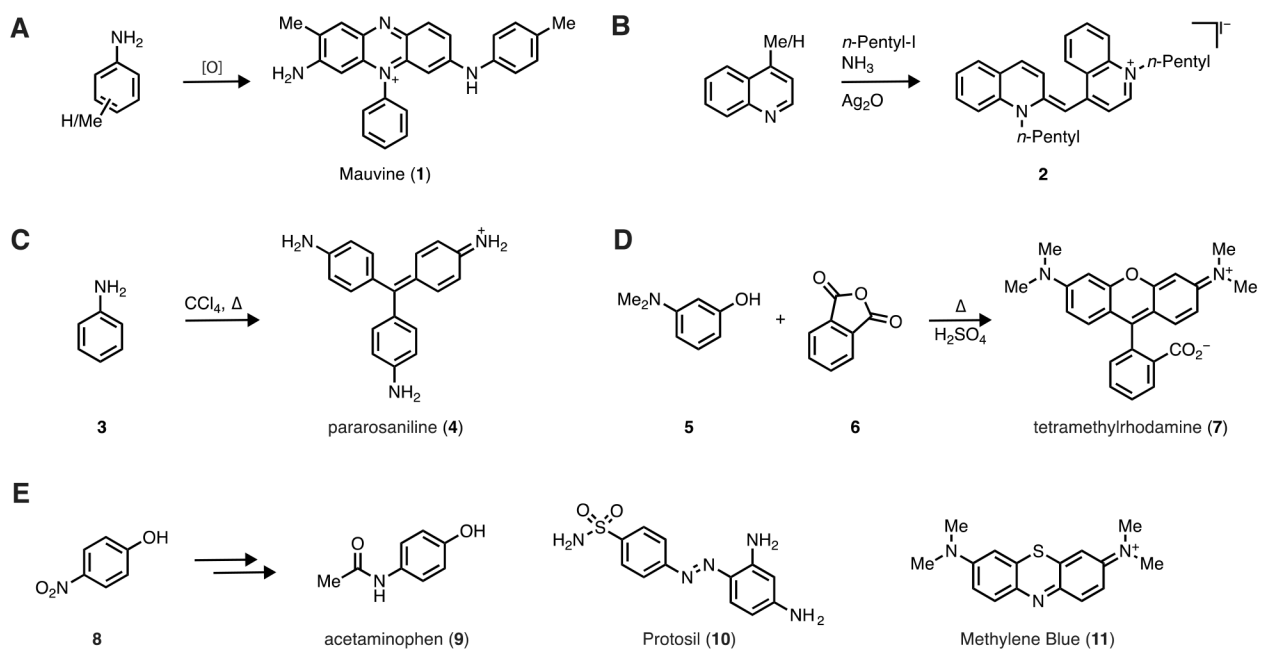
Figure 1. Early developments in dye chemistry.
(**A**) Synthesis of mauveine (**1**). (**B**) Synthesis of cyanine dye **2**. (**C**) Synthesis of pararosaniline (**4**). (**D**) Synthesis of tetramethylrhodamine (**7**). (**E**) Examples of dye chemistry inspiring medicinal chemistry.
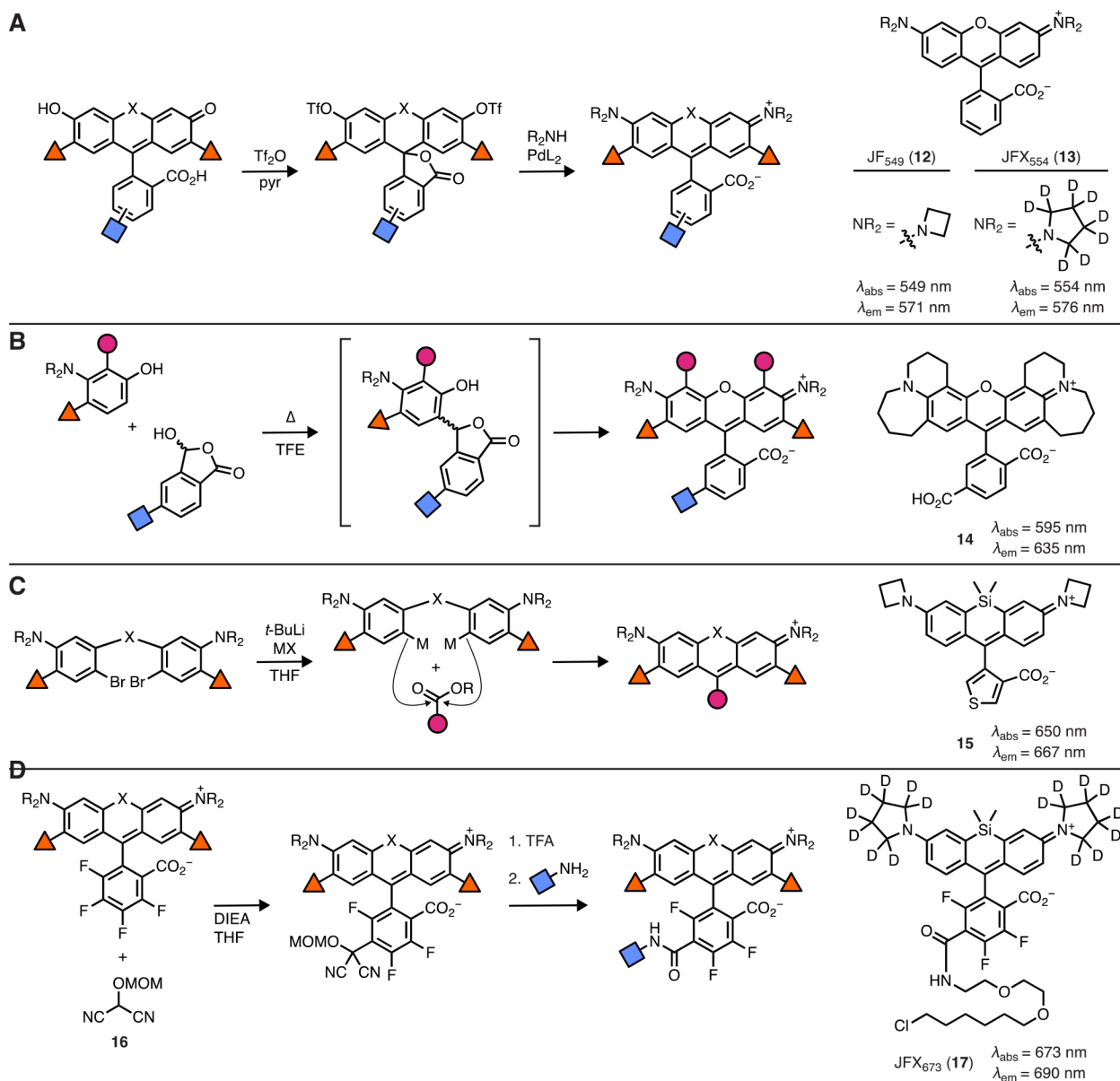
Figure 2. Modern chemical approaches for rhodamine dye synthesis.
(**A**) Synthesis of rhodamines using Pd-catalyzed cross-coupling and structures of exemplar dyes: JF$_{549}$ (**12**) and JFX$_{554}$ (**13**). (**B**) Condensation–oxidation of 3-aminophenols and lactols yields dyes such as **14**. (**C**) Synthesis of rhodamines using metalation of dibromides and structure of thiophene-containing Si-rhodamine **15**. (**D**) Use of masked acyl cyanide (**16**) to derivatize fluorinated rhodamines via S$_N$Ar and structure of bioavailable JFX$_{673}$-HaloTag ligand (**17**).
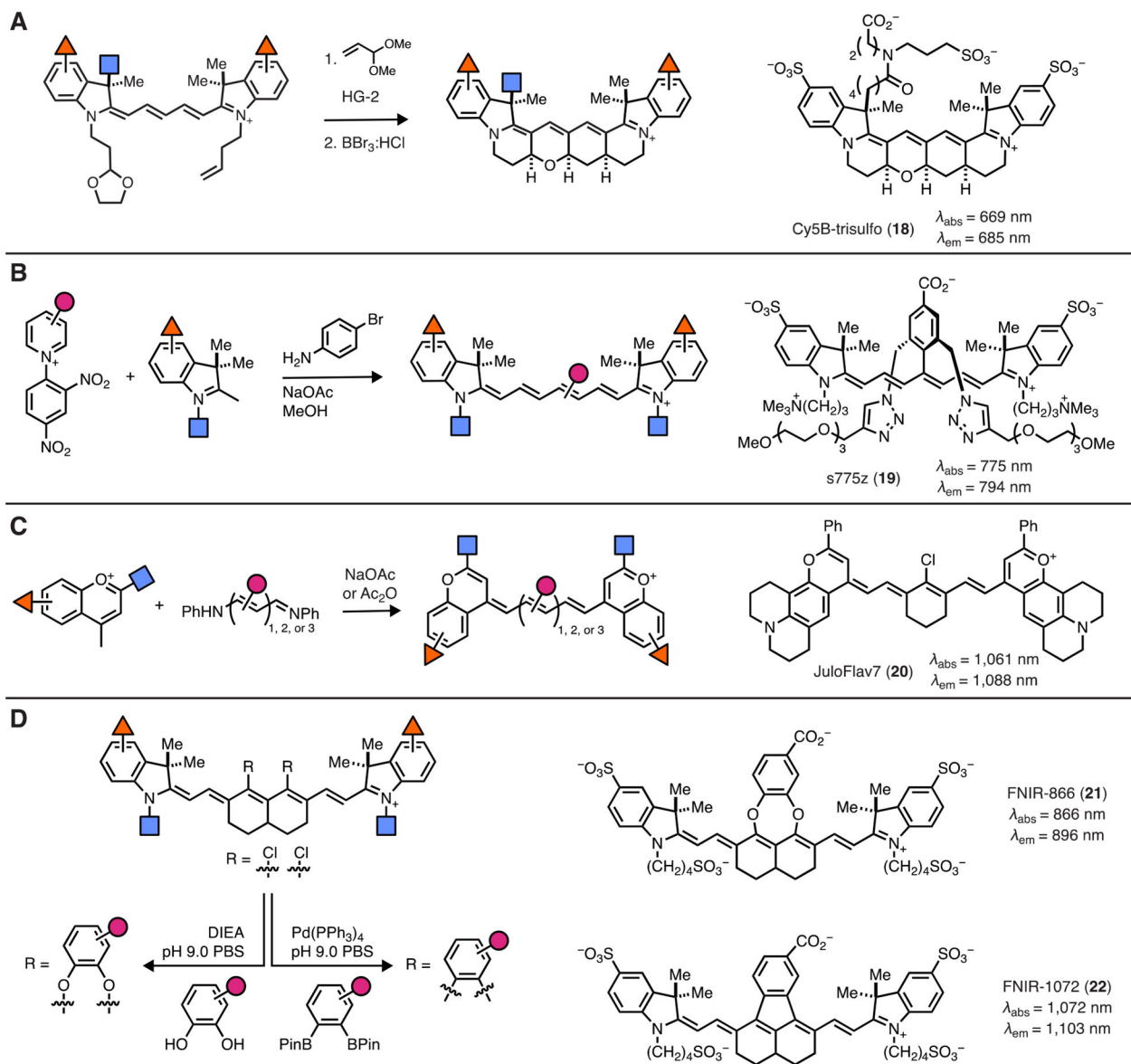
Figure 3. Modern chemical approaches for cyanine dye synthesis.
(**A**) Synthesis of conformationally restricted cyanine dyes and representative compound **18**.
(**B**) Use of Zincke salts in the synthesis of heptamethine dyes such as **19**. (**C**) Replacing the indoline units in cyanine dyes with flavylium moieties yields SWIR-absorbing dyes including **20**. (**D**) Synthesis of nonamethine dyes and representative compounds **21** and **22**.