CHAPTER FIFTEEN

Quinoline-based fluorescent small molecules for live cell imaging

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Abstract

Small molecule probes are essential tools for biomedical applications, with utility as cellular stains, labels for biomolecules, environmental indicators, and biosensors. However, a fluorophore's characteristics are difficult to predict solely through calculations or rational design, making the development of a core scaffold that is amenable to late stage functionalization particularly desirable. In this chapter, we describe the synthesis and application of a tunable quinoline scaffold that can be readily functionalized and

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optimized for a variety of imaging applications. We present a facile synthesis that results in three functional domains that influence the compound's photophysical properties, structural diversity, and polarization. We demonstrate a method with which to study the scaffold's tunable photophysical properties as a result of its structure and environment, and finally exhibit its utility in pH sensitive, live-cell imaging.

Abbreviations

DCM	dichloromethane
DMAQ	dimethylamino quinoline
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
EtOAc	ethyl acetate
EtOH	ethanol
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectroscopy
K_2CO_3	potassium carbonate
LCMS	liquid chromatography-mass spectroscopy
MeOH	methanol
NMR	nuclear magnetic resonance spectroscopy
$POCl_3$	phosphorus oxychloride
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
UP-LCMS	ultra-performance liquid chromatography-mass spectrometry

1. Introduction

1.1 Small molecule fluorophores

Fluorescent tools are indispensable in the imaging, study, and understanding of biological systems. Compared to fluorescent proteins, synthetic fluorophores are often characterized by their smaller size, greater brightness, and higher photostability, allowing for the elucidation of biological information without the need for genetic engineering (Terai & Nagano, 2008). These attributes make synthetic small molecule fluorophores essential tools that can be employed as cellular stains, biomolecular labels, environmental indicators, and biosensors. However, development of new fluorescent dyes and probes presents a challenge due to the often-unpredictable relationship

between molecular structure and photophysical properties. Furthermore, there is the challenge of developing facile syntheses of core scaffolds that are amenable to late-stage functionalization and easy derivatization (Finney, 2006). As a result, most fluorophore development has focused on a limited number of core scaffolds such as BODIPY, coumarin, fluorescein, rhodamine, and cyanine (Lavis & Raines, 2008). The perfect fluorophore has yet to be developed, as many existing probes suffer from cell toxicity, impermeability, low aqueous solubility, and poor sensitivity. Therefore, a demand exists for simple scaffolds that can allow researchers to combine rational design and a high-throughput approach (Vendrell, Zhai, Er, & Chang, 2012).

1.2 Environmentally sensitive dyes

Many biologists aim to study processes in which relatively small changes to the environment, such as fluctuations in pH, interactions between biomolecules, or the presence of ions and other analytes, can have major downstream effects. Therefore, dyes that are sensitive to changes in the microenvironment are particularly useful. Dyes that can respond to local changes through variations in either fluorescence or intensity are called chromogenic or fluorogenic, respectively.

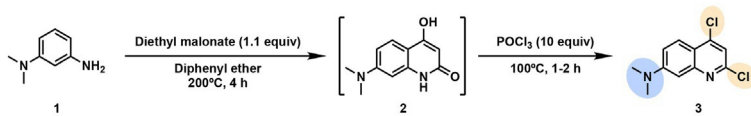
One subcategory of chromogenic or fluorogenic dyes undergoes a change in fluorescence as the dye's microenvironment's polarity or viscosity changes. This phenomenon is known as solvatochromism. Solvatochromism can be caused by a number of factors, including intramolecular charge transfer, intramolecular proton transfer, conformational changes, aggregation, or combinations thereof. Solvatochromic dyes have been applied to the study of lipid rafts, apoptosis, and interactions with proteins or nucleic acids (Klymchenko, 2017).

A second subcategory is pH sensors, which can be excited or emit at different wavelengths depending on the presence or absence of a proton (Han & Burgess, 2010; Shi, Li, & Ma, 2014). Changes in cellular pH are associated with a number of normal and abnormal processes, including ion transport, phagocytosis, inappropriate cell growth, function, or division, and endocytosis. Furthermore, pH is not necessarily consistent across the cell, with some compartments, such as the lysosome, being more acidic than others. pH sensing dyes can be used to develop ratiometric spectroscopic

methods, in which the dye can be observed under two wavelengths and the ratio of the signal can be calibrated and used to determine the exact pH across different cellular compartments.

With this in mind, quinoline-based dyes, which contain a nitrogen heteroatom (a site for analyte recognition) and can be functionalized with push-pull donor/acceptor moieties (for intracellular charge transfer), have immense potential as biological sensors (Fahrni & O'Halloran, 1999; Hanaoka, Kikuchi, Kojima, Urano, & Nagano, 2004; Klymchenko, 2017; Li, Zhu, Xue, & Jiang, 2013; Meeusen, Tomasiewicz, Nowakowski, & Petering, 2011; Nowakowski & Petering, 2011). Though quinine, the first fluorescent molecule to be studied, was reported in 1845, quinoline-based fluorophores are understudied due to a myriad of synthetic challenges (Herschel, 1845; Kaufman & Rúveda, 2005; Lavis & Raines, 2008). Previously reported synthetic routes require harsh conditions, complex or inefficient purification, and specialty glassware. Furthermore, they result in moderate yields that may be difficult to reproduce (Eisenhart, Howland, & Dempsey, 2016; Nasr, Drach, Smith, Shipman, & Burckhalter, 1988).

We report a facile, high-yielding, one-pot synthesis of a key quinoline precursor that can be performed on a gram-scale (Scheme 1). This critical scaffold contains an electron-donating motif that acts as a polarization domain and two tunable domains for late-stage chemical modification *via* either a regioselective or sequential Suzuki-Miyaura coupling (Jun, Petersson, & Chenoweth, 2018). We refer to the resulting scaffold as dimethylamino quinoline (DMAQ). We also present optimized, selective syntheses for both mono- and bisarylated quinolines to create a library of dyes with varied photophysical properties (Schemes 2 and 3). This method also describes a procedure for high-throughput analysis of this library and for performing live cell imaging of a hit compound in HeLa cells (Fig. 1).



Scheme 1 Synthesis of core scaffold **3**, containing a polarization domain (highlighted in blue) and two tunable domains (highlighted in orange).

2. Chemical synthesis

2.1 Reagents, equipment, and general procedures

Chemical reagents are listed below. Solvents were used directly from a JC Meyer Solvent System (Laguna Beach, CA), and Mili-Q filtered (18 MΩ) water was used for all solutions unless otherwise stated (Millipore; Billerica, MA, USA). Liquid chromatography-mass spectrometry (LCMS) was performed using a Waters Acquity Ultra Performance LC connected to a single quadrupole detector (SQD) mass spectrometer (Milford, MA, USA). High resolution mass spectrometry (HRMS) was obtained with a Waters LCT Premier XE, time-of-flight LCMS with electrospray ionization (ESI). Nuclear magnetic resonance (NMR) spectra were acquired using Bruker UNI-500 or AVII-500B and processed with either MestReNova or TopSpin software. Analytical HPLC was performed using a JASCO-FC-2088-30 HPLC fitted with a Phenomenex Luna Omega 5 μm PS C18(2) 100A; 250 × 4.60 mm column.

2.1.1 Equipment and instrumentation

Fume Hood
Rotary evaporator system
Stirring Hot Plate (IKA RCT basic; No: 003810001)
Glovebox
Pipettes (100, 1000 μL)
Pipette tips
Nitrogen or argon gas (Airgas NI R200SS or AR 300)
NMR, HRMS, and analytical HPLC for analysis
Column chromatography for purification
Automated Flash chromatography (Teledyne ISCO Combiflash Rf+)
Silica gel (Silicycle silica gel 55–65A° pore diameter)
Sodium sulfate (Na_2SO_4)
Scintillation vials (4, 10, and 40 mL)
Green Open Top SURE-LinkTM Polypropylene Cap (Chemglass Life Sciences CG-4909-05)
Stirbars
Teflon tape (Fisherbrand™ Pure Low Density PTFE Thread Seal Tape, 15-078-260)
TLC silica gel plates (60G F254, Millipore Sigma)
TLC chamber

Handheld UV lamp with longwave (365 nm) and shortwave (254 nm)
Potassium permanganate ($KMnO_4$) staining solution
1.5 g $KMnO_4$ + 10 g K_2CO_3 + 1.25 mL 10% NaOH + 200 mL water
Syringe
Needle
Balloon
pH testing strips (Hydrion (93) S/R Inst-Chek Disp 0.0-13.0)
Separatory funnel

2.1.2 Chemical reagents

N,N-dimethyl-1,3-phenylenediamine dihydrochloride (CAS: 536-46-9; Acros Organics AC204350250)
Potassium carbonate (CAS: 584-08-7; ACROS 424081000)
Diethyl malonate (CAS: 105-53-3; Alfa Aesar A1546836)
Phosphorus oxychloride [*Highly Corrosive*] ($POCl_3$) (CAS 10025-87-3; Acros Organics 105-53-3)
Brine (saturated aqueous sodium chloride)
Sodium sulfate (Na_2SO_4) (CAS 7757-82-6; Aldrich 822286)
Tetrahydrofuran, anhydrous, degassed (from the solvent system)
Dioxane, anhydrous, degassed (from the solvent system)
Degassed water
Ethyl acetate (EtOAc)
Hexanes, reagent grade
Diphenyl ether (CAS: 101-84-8; Aldrich 240834)
Dichlorobis(triphenylphosphine)palladium(II) dichloride ($PdCl_2(PPh_3)_2$) (CAS: 13965-03-2; Aldrich 208671)
(Hetero)aryl boronic acids

2.2 Synthesis of 2,4-dichloroquinoline core

The DMAQ scaffold can be readily synthesized through a one-pot condensation of 3-(dimethylamino) aniline and diethyl malonate to form **2** (Scheme 1). Though previously published syntheses involve running the cyclo-condensation reaction neat, this results in a heterogeneous reaction, which leads to poor mixing and low yields. We address this challenge by performing the reaction in diphenyl ether, a nonpolar, high boiling (258 °C) solvent that is found to be inert to both the cyclo-condensation and chlorination reaction. Compound **3** is then prepared by addition of $POCl_3$ prior to purification.

(a) *N,N*-dimethyl-1,3-phenylenediamine dihydrochloride (5.70 g, 270 mmol, 1.0 equiv.) is placed in a dry 500 mL Erlenmeyer flask and dissolved in 100 mL of deionized water.

(b) Neat K_2CO_3 (~15 g) is added until pH approaches 10–11.

(c) The substance is then extracted with EtOAc (3 × 100 mL) in a 500 mL separatory funnel.

(d) The organic layers are then combined and washed once with 200 mL brine.

(e) After drying over Na_2SO_4 for 15 min, the liquid is concentrated using a rotary evaporator, giving the **1** as a brown oil (3.63 g). (See Synthetic note 1.)

(f) In a 40 mL scintillation vial, free-based *N,N*-dimethyl-1, 3-phenylenediamine (1.20 g, 8.81 mmol, 1.0 equiv.) is dissolved in 2.0 mL diphenyl ether (See Synthetic note 2).

(g) Diethyl malonate (1.55 g, 9.69 mmol, 1.1 equiv.) is added, the threads of the cap are wrapped tightly with Teflon tape for a tight seal, and the reaction is capped with a Green Open Top SURE-Link™ Polypropylene Cap.

(h) The reaction vial is then heated and stirred at 200 °C for 4 h (See Synthetic note 3) or until TLC shows complete formation of **2** ($R_f = 0.34$ in 10% MeOH in DCM or 0.0 in 1:1 EtOAc/hexanes).

(i) The reaction vial is cooled to room temperature.

(j) Without isolation or purification of **2**, a syringe is used to add $POCl_3$ (13.5 g, 88.1 mmol, 10.0 equiv.) to the reaction vial.

(k) The reaction mixture is stirred at 100 °C for 1–3 h. Upon conversion of starting material to product ($R_f = 0.47$, 1:5 EtOAc/hexanes) the vial is removed from heat and cooled over ice for 30 min.

(l) Following cooling, ice water is added slowly without removing the vial from the ice bath (See Synthetic note 4). As the ice melts, the solution will begin to bubble and smoke violently.

(m) After the exotherm settles and the vial is cool enough to handle, the reaction mixture is carefully poured into a 500 mL flask containing additional ice and slowly neutralized with K_2CO_3.

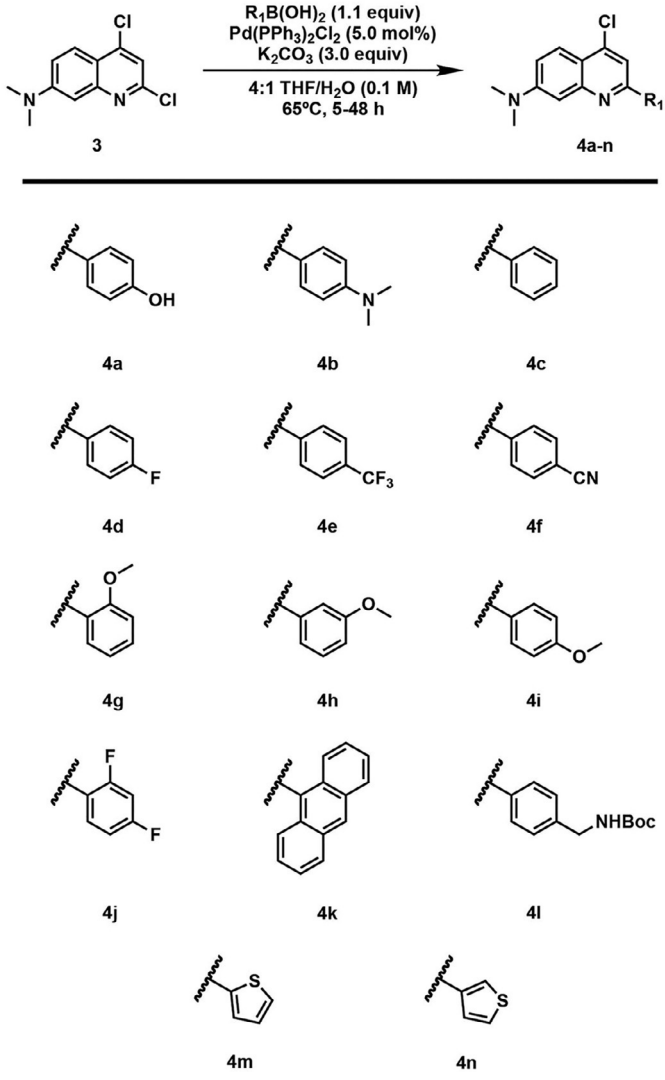
(n) The crude mixture is then extracted with EtOAc (3 × 100 mL).

(o) The organic layers are combined and washed once with brine, then dried over sodium sulfate for 15 min and concentrated *in vacuo*.

(p) The product is purified by flash column chromatography (0%–30% ethyl acetate in hexanes). Diphenyl ether elutes at 100% hexanes, and **3** is obtained as a yellow, needle-like, crystalline solid (2.03 g, 95.6%).

2.3 Synthesis of monoarylated quinolines, 4a-n

A regioselective Suzuki coupling was optimized for selectivity of addition at the 2-position over the 4-position of **3**. The optimized conditions were extended to couple 14 boronic acids of varying electronic and steric properties. This reaction tolerates phenyl boronic acids containing a range of electron-donating, -withdrawing, and -neutral functional groups, such as methoxy, dimethylamino, fluoro, cyano, and a Boc-protected primary amine (Scheme 2). Further detail about this reaction's optimization and



Scheme 2 Substrate scope for monoarylated DMAQ, **4**.

substrate scope, as well as characterization and isolation information for **4a–n,** can be found in the supplementary section of Jun et al. (2018).

(a) Under inert atmosphere, such as inside of a glovebox (see Synthetic note 5), 696 mg **3** (2.88 mmol) is dissolved in 1.6 mL of dry, degassed THF to make a stock solution.

(b) 100 μL of stock solution is then added to each 4.0 mL scintillation vial (43.5 mg, 0.18 mmol, 1.0 equiv.) that has been fitted with a small stir bar and wrapped with Teflon tape.

(c) To the stock solution is added $PdCl_2(PPh_3)_2$ (6.3 mg, 0.009 mmol, 0.05 equiv.), boronic acid (0.198 mmol, 1.1 equiv.), and 1.4 mL THF.

(d) K_2CO_3 is then dissolved in 361 μL degassed water and added to the THF solution, giving a final concentration of 0.1 M.

(e) The reaction mixture is sealed under nitrogen and removed from the glovebox to stir at 65 °C.

(f) As the reaction proceeds, the vial can be cooled and returned to the glovebox. An aliquot is removed for monitoring by TLC and LCMS (avoiding the introduction of oxygen during the course of the reaction) and the reaction vessel is again removed from the glovebox to be heated and stirred.

(g) The reaction will proceed to completion in a period of 5–48 h, dependent on the boronic acid being coupled (see Synthetic note 6). Upon reaction completion, the reaction vial is cooled and transferred to a 100 mL separatory funnel, to which 30 mL each EtOAc and deionized water are added.

(h) The organic layer is extracted with EtOAc and rinsed once with brine solution.

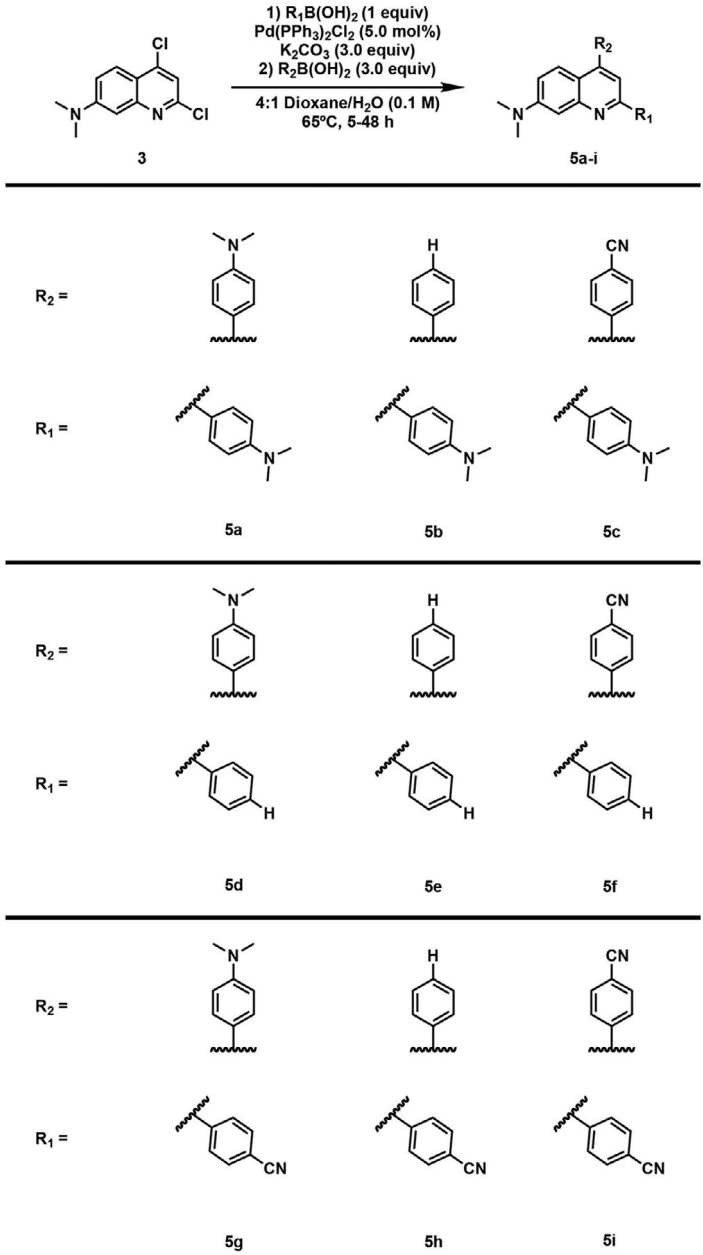
(i) The organic layer is then dried over Na_2SO_4 for 15 min before being concentrated *in vacuo* and purified by column chromatography.

2.4 Synthesis of bisarylated quinolines, 5a-i

Further detail about this reaction's substrate scope, as well as characterization and isolation information for **5a–i**, can be found in the supplementary section of Jun et al. (2018).

(a) Under inert atmosphere, such as inside of a glovebox under nitrogen, 200 mg **3** (0.83 mmol) is dissolved in 1.0 mL dry, degassed dioxane to create a stock solution.

(b) 100 μL of stock solution is then added to each 4.0 mL scintillation vial (20 mg, 0.083 mmol, 1.0 equiv.) that has been fitted with a small stir bar and sealed with Teflon tape.



Scheme 3 Synthesis of bisarylated DMAQ via a one-pot, two step Suzuki cross-coupling.

(c) To the stock solution is added $PdCl_2(PPh_3)_2$ (2.9 mg, 0.004 mmol, 0.05 equiv.), first boronic acid (0.087 mmol, 1.05 equiv.), and 664 μL of dioxane.

(d) K_2CO_3 is then dissolved in 165.9 μL degassed water and added to the dioxane solution, giving a final concentration of 0.1 M.

(e) The reaction vial is sealed under nitrogen and removed from the glovebox to stir at 65 °C for 24 h.

(f) After the first coupling has reached completion, the solution is cooled to room temperature before being returned to an inert atmosphere for the addition of a second boronic acid (0.249 mmol, 3.0 equiv.). The vial must be resealed with fresh Teflon tape.

(g) The temperature is then increased to 85 °C and the vial is stirred under inert atmosphere for an additional 24 h, with monitoring by TLC and LCMS.

(h) Upon completion of the second coupling, the reaction vial is cooled to room temperature and the contents are transferred to a 100 mL separatory funnel.

(i) 30 mL EtOAc and water are added, and the organic layer is extracted before being dried over Na_2SO_4.

(j) Solvent is then removed *in vacuo* and the compound is purified by column chromatography (Scheme 3).

2.5 Synthetic notes

(1) **1** may react with acetone, so it is important to ensure that all glassware is dry prior to free-basing and to avoid using acetone to transfer compound.

(2) Due to diphenyl ether's high melting point (~27 °C), care must be taken when dispensing liquid diphenyl ether from a syringe, as it may solidify at room temperature, causing a buildup of pressure that can cause the needle to separate from the syringe.

(3) We found that we could push the equilibrium of the cyclocondensation reaction forward by removing EtOH without the use of special glassware. To do this, the plunger of a 2 mL syringe is removed, and a balloon is stretched across the top and sealed with parafilm and a rubber band. The syringe is fitted with a long needle and the needle is pushed through the cap, making sure it does not touch

the reaction solution. Liquid EtOH will condense in the syringe as the reaction proceeds.

(4) Care must be taken to slowly add ice water to the reaction vessel and to ensure that the vessel is no more than half-full to minimize the danger of exothermic quenching of corrosive $POCl_3$. We find that adding a minimal amount of ice and allowing several minutes before adding more is necessary, as quenching can take several minutes to begin. Adding excessive water or ice too quickly can cause the vial to bubble over or splash.

(5) If not using a glove box, the catalyst is bench stable and can be handled in air prior to dissolving in solution, at which point care must be taken to ensure maintenance of an air-free reaction vessel.

(6) For more detailed information regarding the reaction times and yields for each boronic acid, see Jun et al. (2018).

3. Plate reader analysis of DMAQ derivatives

Having developed a straightforward synthesis of a library of DMAQ derivatives, we desired a method by which to quantitatively evaluate the probes in a high-throughput manner. The photophysical properties of these probes were anticipated to be highly impacted by their environment, due both to the quinoline nitrogen as a site for analyte recognition and to the push-pull scaffold. The protocol below describes a multi-well assay we employed to screen our library of compounds and assess their response to changing solvent polarity and pH in a high-throughput manner. We chose to excite our compounds at 405 nm, as this is the wavelength of a commonly used laser line in confocal microscopy. However, the wavelength of excitation can be optimized based on the compound of interest. This method can be extended to monitoring the fluorescent response to a variety of other elements, such as metals, peptides, DNA, and other biological macromolecules prior to application in biological systems.

3.1 Reagents and equipment

Plate reader:

Tecan M1000 plate reader (Mannedorf, Switzerland)
Greiner 96 Flat Bottom Black Polypropylene plate
Greiner 96 Flat Bottom Black Polystyrene plate
Thermo Scientific™ Nunc™ Sealing Tapes (Thermo Scientific™ 232698)

Solvents:

DMSO
Water
Toluene
DMF
DMSO
DCM
EtOH (200 proof)
Trifluoroacetic acid
Aluminum oxide, activated, basic, Brockmann I

Buffers:

Hydrochloric acid (10 mM, pH 2)
Citric acid/phosphate (10 mM, pH 4)
Citric acid/phosphate (10 mM, pH 6.9)
Phosphate (10 mM, pH 7.3)
Carbonate (10 mM, pH 10)
Sodium hydroxide (10 mM, pH 11)

Miscellaneous

Pipettes (10, 100, 1000 μL)
Pipette tips
Eppendorf tubes

3.2 Procedure

(a) Solvents are prepared. For each plate, a minimum of 2.0 mL of each solvent is required, but it is advisable to prepare an excess of stock to accommodate evaporation or loss during transfer. For neutral and acidic DCM: 6–10 mL of DCM are dried over activated alumina for several minutes, to neutralize. To 3.0 mL of DCM is added 3 μL TFA (0.1% by volume) to acidify.

(b) 1–2 mg of each dye are dissolved in DMSO for a final concentration of 5 mM. The amount of dye stock required will depend on the number of conditions being screened. For each assay of 12 conditions, a minimum of 30 μL of each dye will be required, but it is advisable to prepare an excess of stock to accommodate loss during transfer.

(c) Using more than one plate if necessary, each row is dosed with 2.5 μL of dye stock solution, ensuring the pipette tip is changed each time a new dye is used. (If dye is added before solvent, the same tip can be used across a row.) If using organic solvents or high temperatures, a

polypropylene plate must be used. If using aqueous solutions and buffers and moderate temperatures, polystyrene is sufficient.

(d) Each well is then dosed with 250 µL solvent and mixed well, changing pipette tips each time. Particularly volatile solvents (such as DCM) are added last to avoid evaporation.

(e) At this point, the plate can be visualized under a hand-held UV lamp. The plate is then covered with sealing tape prior to analysis to avoid evaporation and contamination. The sealing tape will be removed for plate reader analysis, though it should be noted that evaporation prior to and during analysis can impact the results. Therefore, intensity values should only be treated as approximate.

(f) Regardless of absorption maxima of the individual dyes, each compound is excited at 405 nm. Emission is scanned from 420 to 780 nm at a constant temperature of 20 °C. Details of additional settings can be found in the supplemental information of Jun et al. (2018).

4. Cell imaging of an environmentally sensitive dye

Following in vitro library analysis, **5a** was found to exhibit interesting pH-sensing properties, with a bathochromic shift in emission of 168 nm

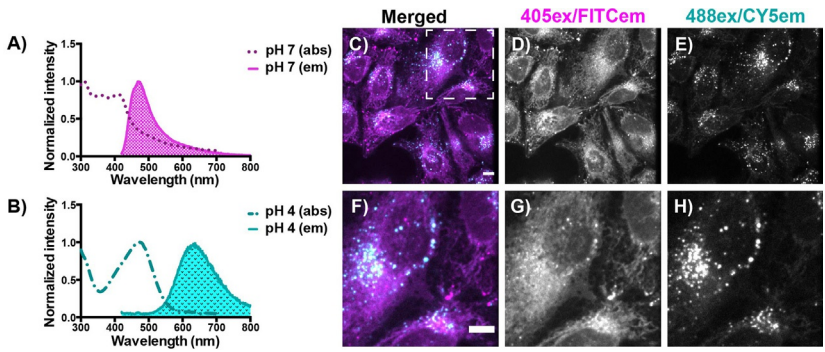


Fig. 1 Fluorescence spectrum and live cell imaging of **5a**. (A) Normalized UV absorption (dotted) and emission (solid) of **5a** at pH 7. (B) Normalized UV absorption and emission of **5a** at pH 4. (C, F) Merged image of magenta (405ex/FITCem) and cyan (488ex/Cy5em) channels following incubation with **5a**. Scale bar represents 10 µm. (White box in (C) represents area shown in greater detail in F–H.) (D, G) Grayscale image of 405ex/FITCem (525/36 nm)[a] channel. (E, H) Grayscale image of 488ex/Cy5em (700/72 nm)[a] channel. [a]Filter center wavelength/full width at half maximum.

from pH 7 to 4 (Fig. 1A-B). We therefore turned our attention to demonstrating this compound's dual emissive properties *in cellulo* (Fig. 1C–G).

4.1 Reagents and equipment

HeLa cells
Biosafety cabinet
Incubator with CO_2
Pipettes (10, 100, 1000 μL)
Pipette tips
Eppendorf tubes
Glass bottom dish, 35 mm, No. 1.5 Coverslip, Poly-D-Lysine Coated (MatTek, P35GC-1.5-14-C)
DMSO, anhydrous, 99.8 +% (CAS: 68-68-5; Afla Aesar 43998)

Growth media

Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, 11965084)
10% Fetal bovine serum (FBS) (Life Technologies, 16000-044)
1% Pen/Strep (Invitrogen, 15140122)
Dulbecco's phosphate-buffered saline (DPBS) (Invitrogen 14040117)

Microscope compatible with live-cell imaging at 405 and 488 nm (Nikon Eclipse Ti2 with Yokogawa CSU-X1 spinning disk confocal).

Imaging media

DMEM, no phenol red (Invitrogen, 21063029) **OR** Leibovitz-15 (L15) CO_2-independent media, no phenol red (Life Technologies 11415064)
10% Fetal bovine serum (FBS) (Life Technologies, 16000-044)
1% Pen/Strep (Invitrogen, 15140122)
5 mM dye stock in DMSO
Trypsin 0.25% EDTA (Invitrogen, 25200056)

4.2 Procedure

(a) 1–2 mg **5a** is dissolved in DMSO to give a concentration of 5 mM and aliquots are divided among several Eppendorf tubes. The dye can be stored at 4 °C and warmed to 37 °C when ready for use. Avoid excessive freeze/thaw cycles.

(b) HeLa cells are cultured in glass-bottomed MatTek dishes coated with poly-D-Lysine until they reach 60%–80% confluency.

(c) In a 5 mL Falcon tube, 2 μL of 5 mM **5a** in DMSO is added to 2 mL growth media and mixed well to give a final concentration of 5 μM.
(d) Growth media is removed from cells and exchanged for media containing **5a**.
(e) Cells are incubated under CO_2 for 3 h.
(f) Cells are washed with warm growth media three times.
(g) Growth media is replaced with warm imaging media (DMEM, no phenol red if using an imaging chamber connected to CO_2 **or** Leibovitz-15 if not).
(h) The dish is mounted onto the microscope and a population of multiple cells is located, avoiding overly crowded populations. CO_2 and incubation can be turned on to allow for long-term imaging.
(i) The dye is excited at both 405 nm and 488 nm (10%–20% laser power) and emission is collected at a range of wavelengths. We found that 405ex/FITCem and 488/Cy5em showed optimal dual emission (Fig. 1) but this will be dye and microscope dependent. Care should be taken not to photobleach the dye.

4.3 Analysis

Images can be analyzed using NIS-Elements (Nikon's proprietary image analysis software) or using the open-source software FIJI, provided by the NIH at https://imagej.net/Fiji.

5. Summary and conclusions

In this chapter, we have presented detailed procedures for synthesizing, analyzing, and employing a library of DMAQ derivatives for live cell imaging. Ease of synthesis can be a barrier to biological utility, and we therefore offer a simple, straightforward synthesis of a medically relevant quinoline core with excellent tunability and varied photophysical properties. This core can be modified at the 2-position for structural diversity and the 4-position for further late-stage functionalization, allowing for the facile generation of a library of mono- and bisarylated dyes with emission spectra that collectively span the entire visible light region. These compounds exhibit intriguing pH-sensing and solvatochromic qualities and emphasize the utility of a high-throughput approach for library generation and analysis. We have therefore begun to probe an underexplored scaffold

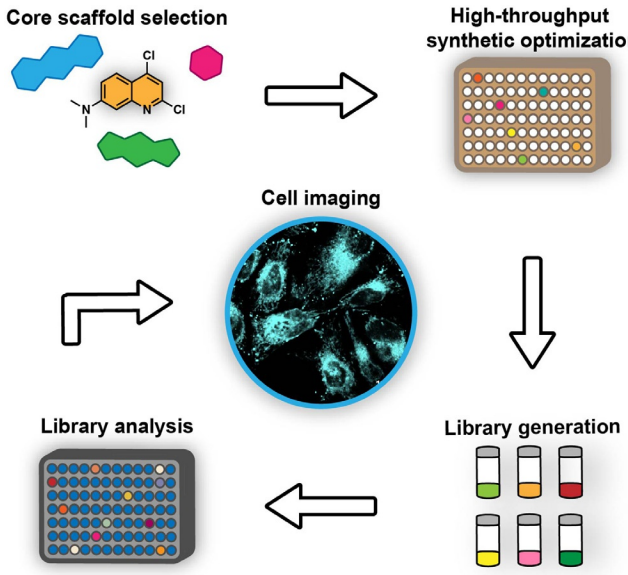


Fig. 2 Workflow for combining rational design and high-throughput approach for fluorophore development.

that has great potential as a biologically relevant dye. Furthermore, we present a strategy for developing and analyzing a library of compounds from underexplored dye families in a high-throughput manner (Fig. 2). Our method, which combines rational design with a combinatorial approach, can easily be extended to other dye scaffolds.

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