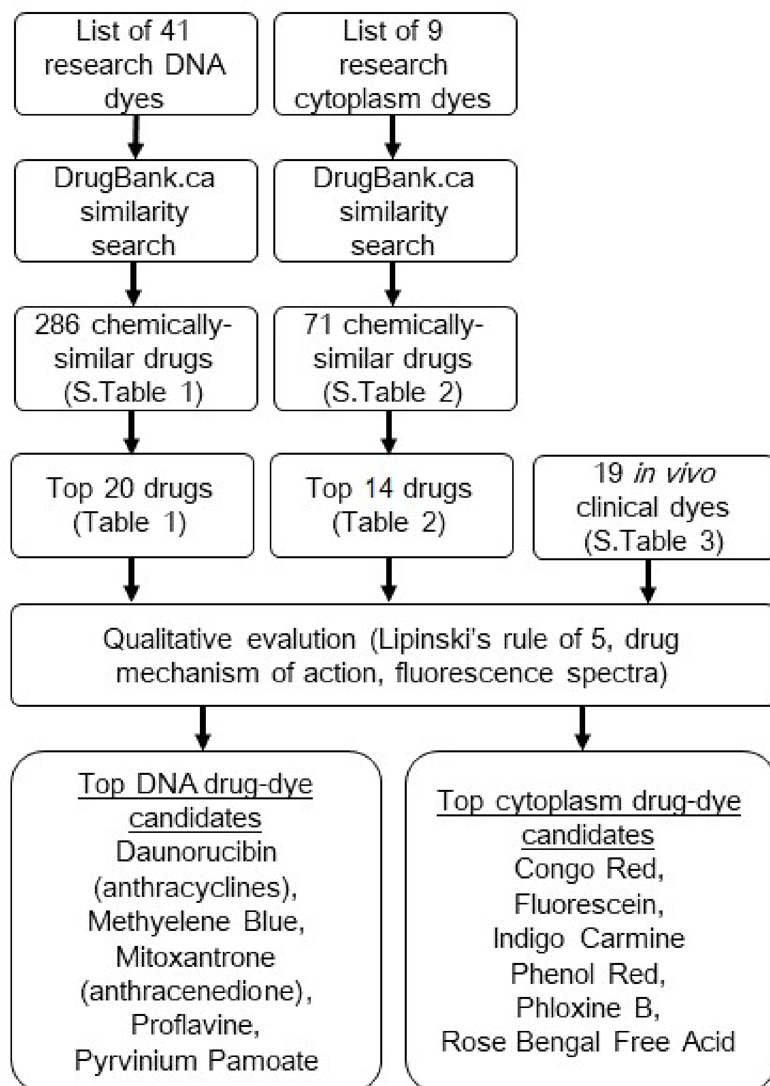
Supplemental Methods and Figures



Supplemental Diagram 1: *In silico* workflow developing drug-dye alternatives to H&E.

In general, combination drugs, protein-based drugs, peptides, or other pepitdomimetics were excluded based on simplicity and/or large molecular weight and poor molecular diffusion. Fluorophores were excluded if their peak emission was in or near either the UV or infrared region or had a peak width at half max exceeding 200nm.

Nuclear/DNA dye candidates evaluated in silico.

Research fluorophores C61, EVOblue™ 30, PO-PRO-1, Resazurin, Thiazole Orange, and TO-PRO-1 did not have any approved drugs above the arbitrary 0.45 similarity score threshold.

Of the top 20 drugs that matched research fluorescent nuclear dyes, Dactinomycin (also called Actinomycin D, AMD) had the greatest similarity to a research dye, 7-AAD, but AMD is relatively diffusion-restricted given its molecular weight of 1255.42 g/mol[62]. Plicamycin was similar to Chromomycin A3, but has been discontinued/not in clinical use, and also has a relatively large molecular weight, 1085.15 Daltons; no further evaluation of these as a nuclear dye was undertaken.

Mitoxantrone, an antineoplastic agent that intercalates and crosslinks nuclear, is very similar to DRAQ5™ (similarity score of 0.92). Mitoxantrone fluorescence excitation peaks are reported around 610nm and 670nm, and emission peak near 685-700nm[63,64]. While it has a relatively large polar

surface area (163 Angstroms^2) all other properties qualitatively made it a candidate for further evaluation.

Proflavine, a topical antiseptic and wound irrigant, is similar to Acridine Orange (AO, similarity score of 0.81). It has peak excitation and emission near that of AO, 450 nm and 510 nm, respectively[65], and conforms to Lipinski's rules of 5's, and was thus a candidate for further evaluation.

Tamoxifen, one of numerous selective estrogen receptor modulators (SERM), has been shown to be excited at 275 nm with emission peaks at approximately 370 and 390 nm[66]. This and other SERMs (Ospemifene, Toremifene, and Clomifene) showed similarity to Z-TPE3 (similarity score>0.60). Tamoxifen's high partition coefficient (log P of 7.1) and the SERM mechanism of acting on estrogen receptors suggested against further evaluation of this class as fluorescent nuclear dyes in this study, though this may be useful as a breast-specific optical biopsy dye.

Dequalinium, another topical antiseptic, is somewhat similar to a few fluorescent nuclear/DNA dyes including LDS-751, Acridine Orange, Hexidium, Ethidium and Propidium. Excitation of Dequalinium has been reported at 335 nm, and emission at 360 nm[67]. The drugs proposed mechanism of action by increasing cell permeability (and therefore likely localization to the cell membrane) and log P of 7.3 suggests against use of this as a nuclear dye.

Niclosamide demonstrated similarity to SYBR Gold, and is an old antihelminth drug thought to decouple oxidative phosphorylation (suggesting at least some degree of localization to mitochondria). Niclosamide is excited in the UV range[68]. The mechanism of action location and unfavorable excitation excluded Niclosamide from further evaluation as a DNA dye.

Hesperidin is a glycoside found in citrus fruits and oral formulations have been used to help venous diseases (chronic venous insufficiency/varices). It is excited in the deeper UV range and emits a shoulder at approximately 325 nm, still in the UV range[69]. This, combined with its large molecular weight (610.565 Daltons) and very high polar surface area (234 Angstroms^2) excluded it from further analysis as a nucleic acid dye.

Daunorubicin, Doxorubicin, Epirubicin, Idarubicin, and Valrubicin are all anthracyclines known to intercalate DNA, and many are also known fluorophores[70], with excitation in blue wavelengths and emission near green-yellow. These demonstrated similarity to Chromomycin A3 (similarity score >0.6). Given their mechanism of DNA interaction and known fluorescence, anthracyclines as a class represent lead nuclear drug-dyes. Being alphabetically first, Daunorubicin served as the prototypical anthracycline studied.

Etoposide and Teniposide, as topoisomerase inhibitors, were excluded from further analysis based on the combination of their large molecular weight (588.14 and 656.655 Daltons), polar surface area (161 A^2 and 189A^2) and mechanism of action inhibiting topoisomerase during certain parts of the DNA synthesis cycle.

Pyrivinium, with 0.623 similarity score to LDS-751, is an antihelmenthic and antineoplastic with multiple reported mechanisms of actions as well as transient photosensitization[71] and fluorescence in the green[72] or yellow wavelength[58] when bound to DNA. Further evaluation of the approved formulation Pyrvinium Pamoate as a drug-dye candidate was undertaken.

Of the known clinical dyes and photosensitizers localizing to the nucleus, a few of such dyes are listed as example possibilities. Toluidine blue, used in chromoendoscopy, can be excited with red light[73] and does stain DNA-protein complexes[74], but also stains polysaccharides and other acidic cellular components, which makes it non-ideal as a nuclear stain. Methylene Blue did not have a similarity score to any of the research fluorophores, but has been shown to intercalate and electrostatically bind DNA, which alters/quenches its fluorescence[75]; this may or may not be clinically useful and so Methylene Blue was evaluated *in vitro*. Non-steroidal anti-inflammatory drugs have photosensitizing properties, with Carprofen having some similarity to DAPI (similarity score >0.5). Carprofen has fluorescence excitation and emission in the UV range, with a peak near 360-370 nm[76]; its relative low similarity and non-ideal wavelength excluded it from further analysis. Psoralens are photosensitizing compounds known to bind DNA, with Trimethylpsoralen[77] and Methoxsalen[78] as two FDA-approved agents; however their photoactivation and resultant DNA cross-linking[79] may hinder downstream (genetic) testing should a physical biopsy be taken.

Photosensitizers and in vivo clinical dyes considered in silico.

English literature and drug databases for FDA-approved fluorophores and photosensitizing agents through early 2019 were also evaluated by searching for "photosensitizer," "photosensitivity," and "phototherapy" drugs. FDA-approved photosensitizing drugs include multiple classes such as porphyrinoids: Photofrin (Porfimer Sodium), Benzoporphyrin derivative monoacid ring A (BPD-MA), Temoporfin (m-THPC), Verteporfin, Aminolevulinic acid (ALA), Methyl aminolevulinate, Hexaminolevulinate; sulfonamides: including sulfasalazine and anti-infective agents sulfisoxazole, sulfamethoxazole, sulfadiazine; other antimicrobials including tetracyclines (tetracycline, doxycycline), anti-helminthics (albendazole, mebendazole, fenbendazole), and anti-malarials quinolines (quinine, chloroquine, primaquine, quinacrine, mefloquine); psoralens such as Trimethylpsoralen and Methoxsalen; non-steroidal anti-inflammatory drugs (in particular Carprofen, Ketoprofen, Benoxaprofen); cardiovascular drugs (Amiodarone, Hydrochlorothiazide, Quinidine, Captopril as

the model ACE inhibitor, Atorvastatin and Fluvastatin); psychotropics (phenothiazines, most notably Chlorpromazine), retinoids (Vitamin A, Tretinoin, Etretinate) and riboflavin to name many, but not all culprits implicated in drug-induced photosensitivity[80–82]. While many of these may provide clinically useful fluorescent tissue- and cellular-level contrast, the overwhelming number of possibilities as drug-dyes based solely on photosensitivity made further evaluation of such compounds beyond the scope of this manuscript.

Protein & cytoplasm dye candidates from literature review and in silico *evaluation.*

Oftasceine, Phloxin B, Fluorescein and Rose Bengal Free Acid all demonstrated similarity of >0.63 to Eosins, Calcein, and CFSE (ViaFluor® CFSE), which are molecules known to fluoresce and localize to the cytosol. Of these top 10 cytosol drug-dye candidates, Calcein/Oftasceine was not initially further evaluated given its calcium-dependent fluorescence, use as a multidrug resistance transporter substrate and semi-impermeable agent to interrogate the blood-retinal barrier[83]. Noscapine has a number of biological effects[84,85] and is excited in the UV range and emits in the near-ultraviolet range (peak emission reportedly at 402nm)[86] making it not ideal, but not excluded as a possibility for future study. Similarly, Tritoqualine, an atypical antihistamine, similarly may be investigated in the future but not included due to UV-range emission[87]. Phenolphthalein has been withdrawn from use as a laxative due to concerns over genotoxicity/carcinogenicity, which suggests against localization to cytosol. Mycophenolate Mofetil is an immunosuppressant anti-transplant rejection prodrug which active metabolite preferentially affects lymphocytes[88] and it emits in the UV range[89]; it was not further studied at this time. Flavone fluorescence has been shown to be lipid-dependent[90] making it a possibility for cell membrane and other lipid-rich subcellular structures, but not cytoplasm per se and was not further investigated as an Eosin alternative at this time. Valrubicin, as the 10th most structurally-similar drug to research protein & cytoplasm dyes, is actually the 17th on the research nuclear/DNA dyes list, and is known to bind DNA as an anthracycline. Thus, drugs below on the protein/cytoplasm list were not further evaluated in this study.

In vivo clinical dyes are included in Supplemental Table 3. Brilliant Green is effective against Gram-positive bacteria, but its proposed broad mechanisms of action (protein/thiol and DNA binding)[91] suggest against use as a subcellular-compartmentalizing drug-dye. Indocyanine green peak emission is in the infrared[52] and is grossly excluded from intact cells[92], making it useful like Isosulfan Blue in lymphangiography[93], but not likely in live cellular staining. Many other ophthalmic dyes such as Brilliant Blue G[94], Crystal (Gentian) Violet[95], Oftasceine, Patent Blue[96], Prussian Blue, Rose Bengal Free Acid, and Trypan Blue[96], are also primarily excluded from intact epithelial cells and bind non-specifically to proteins, which property would not likely contribute directly to cellular imaging. However, given the high degree of structural similarity to research dyes, Fluorescein, Phloxine B, and Rose Bengal (Table 2) were evaluated. Lugol's Iodine solution as used in Schiller's Test stains glycogen in healthy epithelia but not glycogen-poor (cancerous) tissue and, thus, was not further evaluated. In like manner, porphyrinoid compounds or precursors thereof may be suitable for cell membrane staining given the hydrophobicity[97] of porphyrin but not initially evaluated as a topical protein/cytoplasm dye. Congo Red could also be used as a non-specific protein-binding[98] fluorescent dye[99], and was thus evaluated *in vitro*. Indigo Carmine, while generally thought to be taken up by dysplastic cells, can be taken up into normal tissues at high enough concentrations[100] and was evaluated *in vitro*. Phenol red, used in ophthalmology and chromoendoscopy, can be taken up intracellularly (preferentially into lysosomes)[101] and therefore may help in visualizing (portions of) cytosol; this was also evaluated *in vitro*. Prussian Blue, as a stain for iron, may be useful for that purpose in future fluorescence studies, but structure and *in vivo* mechanism of action as a heavy metal antidote suggest against its utility as a protein & cytoplasm or nuclear & DNA dye.

In vitro *evaluation of candidate drug-dyes.*

Supplemental Figure S1 shows the excitation-emission spectragraphs of leading drug-dye candidates as alternatives to hematoxylin (nuclear dye) from the *in silico* and qualitative evaluation as above. Supplemental Figure S2 shows the excitation-emission spectragraphs of possible eosin-alternative (protein/cytoplasm) drug-dyes. Supplemental Figure S3 presents different factors needed to be considered in terms of concentrations and excitation wavelengths in developing compatible hematoxylin- and eosin-alternative formulations. Supplemental Figures S4 and S5 are examples of fluorescence incompatibility, with the addition of more phloxine B resulting in decreased daunorubicin fluorescence and substantial emission spectral overlap in Figure S4. Supplemental Figure S5 shows how the addition of a nuclear dye resulted in decreased fluorescence signal from otherwise identical concentration of the protein/cytoplasm dye, also with substantial spectral overlap. Supplemental Figures S6-S10 show the individual and combined fluorescence emission spectra of the top candidate nuclear and protein/cytoplasmic dyes. Of note, pyrvinium pamoate was excluded based on substantial overlap of its emission spectra with all the protein/cytoplasm dyes (Supplemental Figure S10).

Supplemental Table S4 shows compatible nuclear and non-specific protein/cytoplasmic dyes, with combination fluorescence emissions spectra indicated.

This *in silico* work resulted in daunorubicin (as the prototypical anthracycline), methylene blue, mitoxantrone (as the prototypical anthracenedione), proflavine and pyrvinium pamoate as the top candidate nuclear drug-dyes; and congo red, fluorescein, indigo carmine, phenol red, phloxine B, and rose bengal free acid as the top candidate protein & cytoplasm dyes to be evaluated *in vitro*.

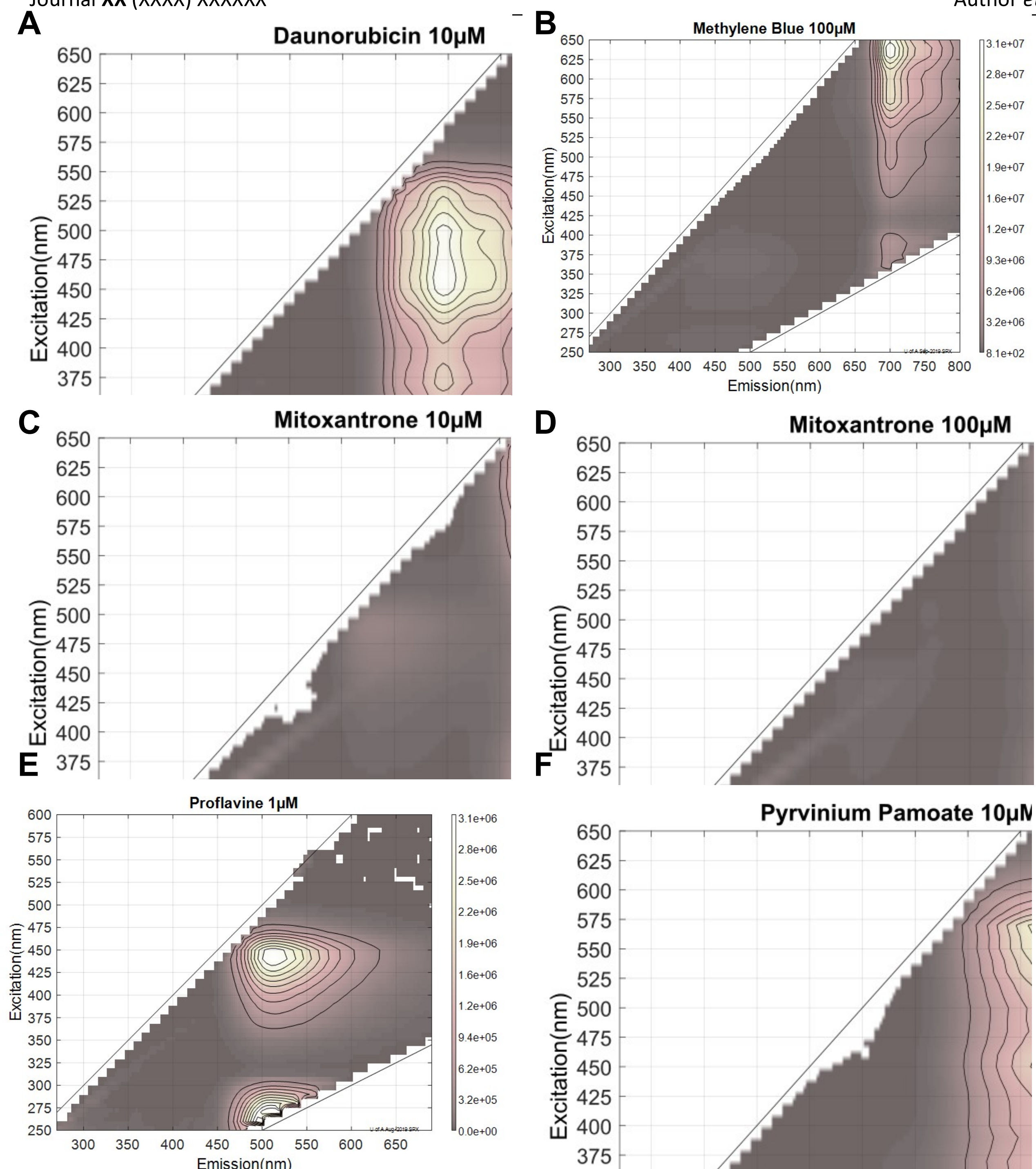
Fluorescence emission and excitation plots of potential drug-dyes are provided for reference, with possible nuclear dyes in Supplemental Figure S1, and protein dyes in Supplemental Figure S2.

Supplemental Table S1: FDA-approved drugs with structural similarity to non-approved research DNA dyes

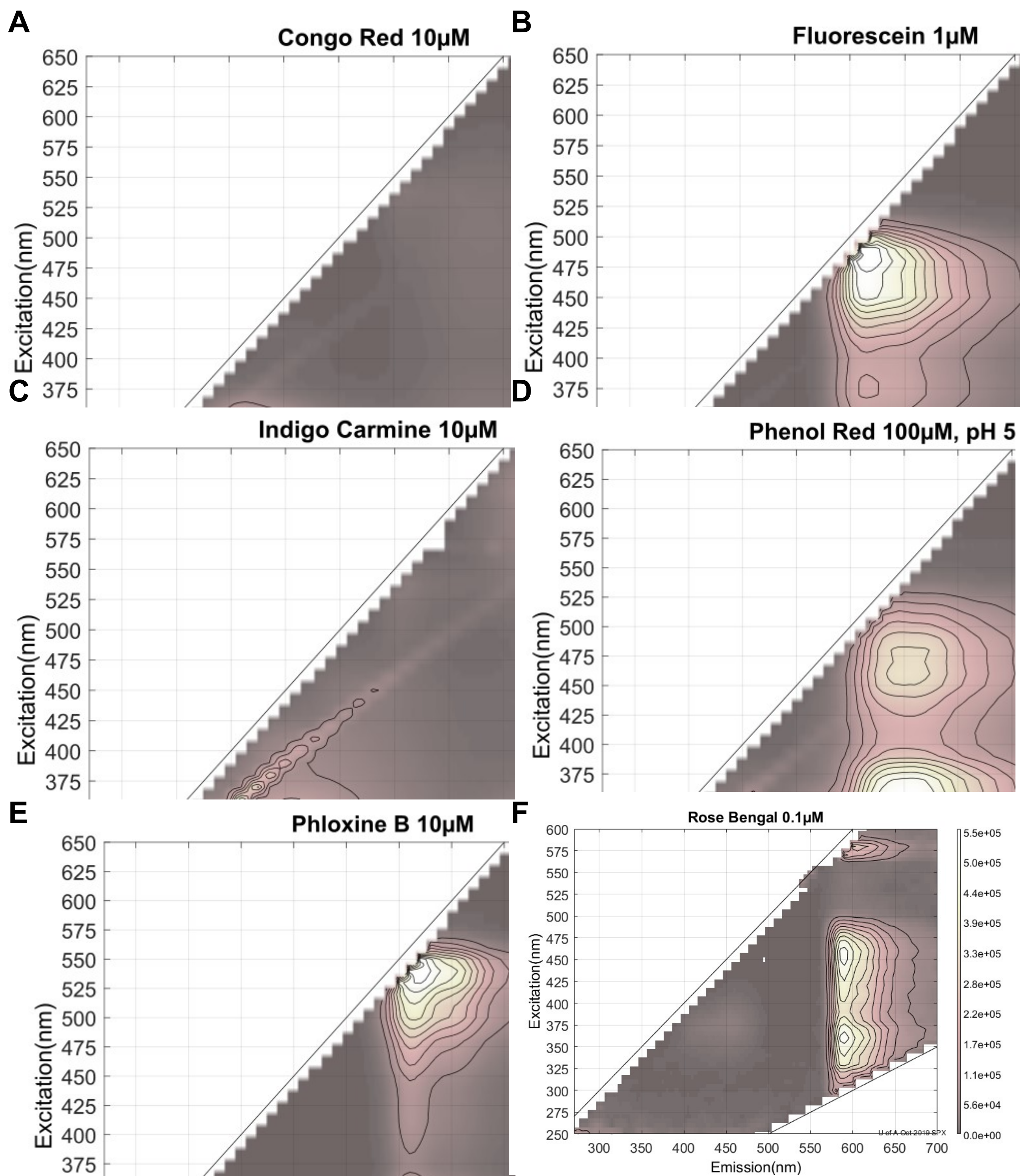
Supplemental Table S2: FDA-approved drugs with structural similarity to non-approved research cytoplasmic dyes

Chromogenic/fluorescent drug	**General *in vivo* Use/Treatment**
Brilliant Blue G (Acid Blue, Coomassie Brilliant Blue)	Ophthalmic contrast
Brilliant Green	Topical antiseptic additive
Congo Red	Chromoendoscopy contrast agent
Crystal/Gentian Violet	Topical antiseptic additive, ophthalmic contrast
Fluorescein	Ophthalmic contrast, fluorescence angiography
Indigo Carmine	Neurosurgical, Gastroinestinal and Urological contrast
Indocyanine Green	Fluorescence angiography, lymphangiography
Isosulfan Blue	Lymphangiography
Lugol's Iodine Solution	Topical antiseptic, Stain for Schiller's Test
Methylene Blue	Antidote for methemoglobinemia, ophthalmic contrast
Oftasceine	Ophthalmic contrast
Patent Blue (Sulfan Blue)	Lymphangiography
Phenol Red	Chromoendoscopy
Phloxine B	Dental disclosing agent
Porphyrinoids/Porphyrin precursors (Hexview/Cysview, 5-ALA)	Neurosurgical and Urological surgery contrast
Prussian Blue	Antidote for cesium and thallium poisoning
Rose Bengal Free Acid	Ophthalmic contrast
Toluidine Blue	Visual diagnostic aid on mucosal lesions
Trypan Blue	Ophthalmic contrast

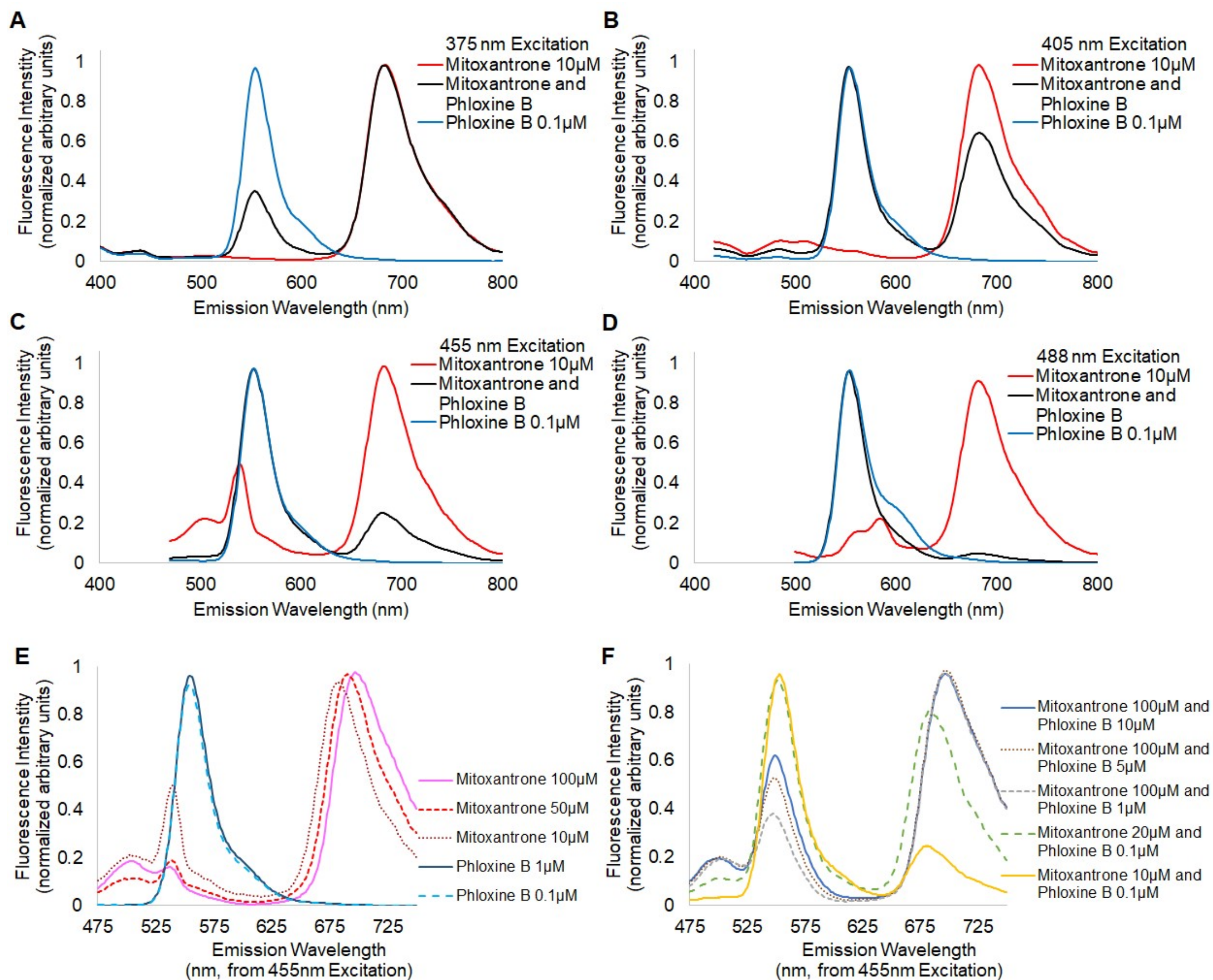
Supplemental Table S3: FDA-approved chromogenic/fluorescent drugs used *in vivo*. Data are as of mid-2019 from DrugBank.ca database.



Supplemental Figure S1: Fluorescence excitation and emission spectra of nuclear drug-dye candidates *in vitro*. (**A**) 10μM Dauorubicin. (**B**) Methylene blue at 100μM. Mitoxantrone at (**C**) 10μM and (**D**) 100μM which demonstrated concentration-related variance in fluorescence. (**E**) 10μM Proflavine. (**F**) 10μM Pyrvinium pamoate. Spectragraphs are representative of 2-3 repeated acquisitions.

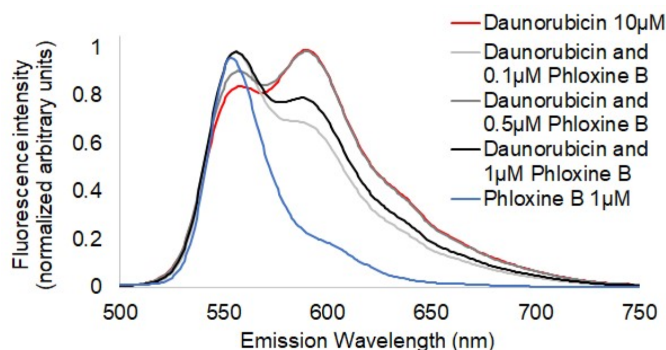


Supplemental Figure S2: Fluorescence excitation and emission spectra of protein drug-dye candidates *in vitro*. (**A**) Congo red at 10μM. (**B**) Fluorescein at 1μM. (**C**) Indigo carmine at 100μM. (**D**) Phenol red at a concentration of 100μM at pH 5. (**E**) Phloxine B at 10μM. (**F**) Rose bengal free acid at 0.1μM. Spectragraphs are representative of 2-3 repeated acquisitions.

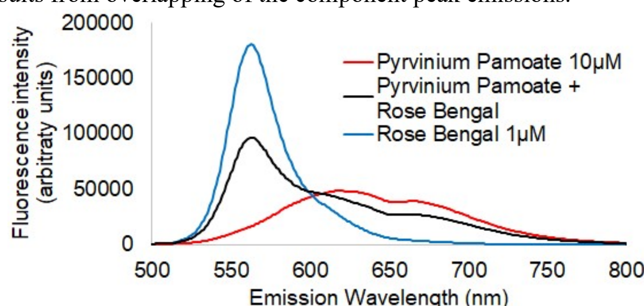


Supplemental Figure S3: Optimizing the excitation wavelengths of DDAs. (**A-D**) Mitoxantrone as a hematoxylin-alternative candidate at 10μM, with phloxine B at 0.1μM as an eosin-altrenative candidate drug-dye, excited at different wavelengths: (**A**) 375nm, (**B**) 405nm, (**C**) 455nm, and (**D**) 488nm. (**E-F**) Concentration-dependent fluorescence of mitoxantrone but not phloxine B results in various concentration ratios of the two resulting in similar fluorescence intensity peaks, all excited at 455nm. (Of note, smoothing after normalizing to peak intensity resulted in peaks not reaching exactly 1.0.)

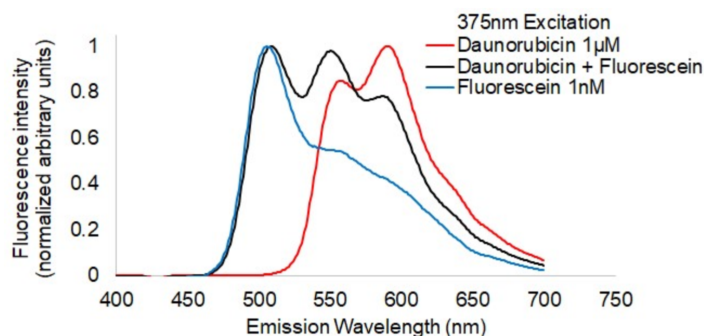
The resultant combinations of DNA and protein dyes were next evaluated *in vitro*. In developing DDAs, titrations were done to grossly match either the peak fluorescence emission intensity or emission area under the curve from the component DNA and protein dyes for a given single wavelength of excitation. The molar ratios varied primarily based on excitation wavelength (Supplemental Figure S3A-D), although some dyes demonstrated concentration-dependent change in fluorescence (Supplemental Figure S3E) which resulted in unexpected changes in the molar ratio required for similar peak emission intensity. Specifically, mitoxantrone as a nuclear dye mixed with phloxine B as a non-specific protein dye at 100:1 molar ratio resulted in primarily mitoxantrone from the resultant fluorescence emission signal at 375nm excitation, but nearly all the fluorescence emission signal from phloxine B at 488nm excitation (Supplemental Figure S3F). Additionally, there was overlap of emission spectra (Supplemental Figure S4) and the combination of certain DNA and protein dyes decreased the emission intensity relative to the same concentration of the component dye alone (Supplemental Figures S4 and S5). Congo red and indigo carmine were excluded from further evaluation as protein dyes based on peak fluorescence in the UV range.



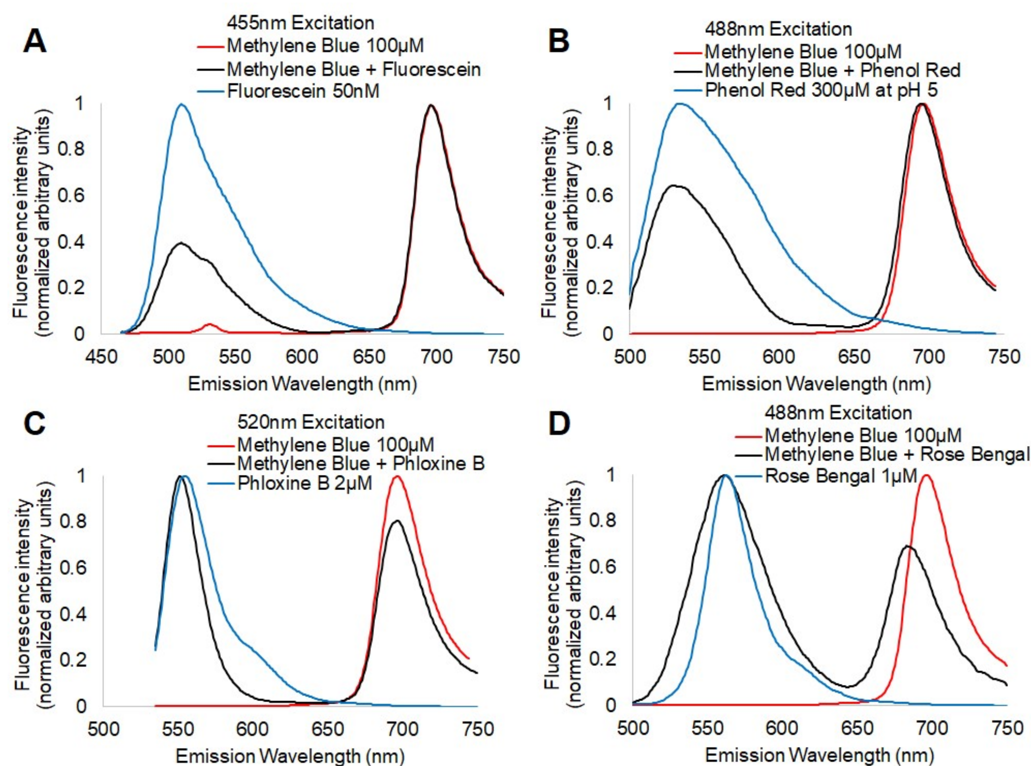
Supplemental Figure S4: Example of fluorescence incompatibility. At 488nm excitation, increasing concentrations of phloxine B (a proposed eosin-like dye) resulted in progressive decrease of fluorescence emission from daunorubicin (a proposed hematoxylin-like dye) fluorescence peak around 590nm. Additional incompatibility results from overlapping of the component peak emissions.



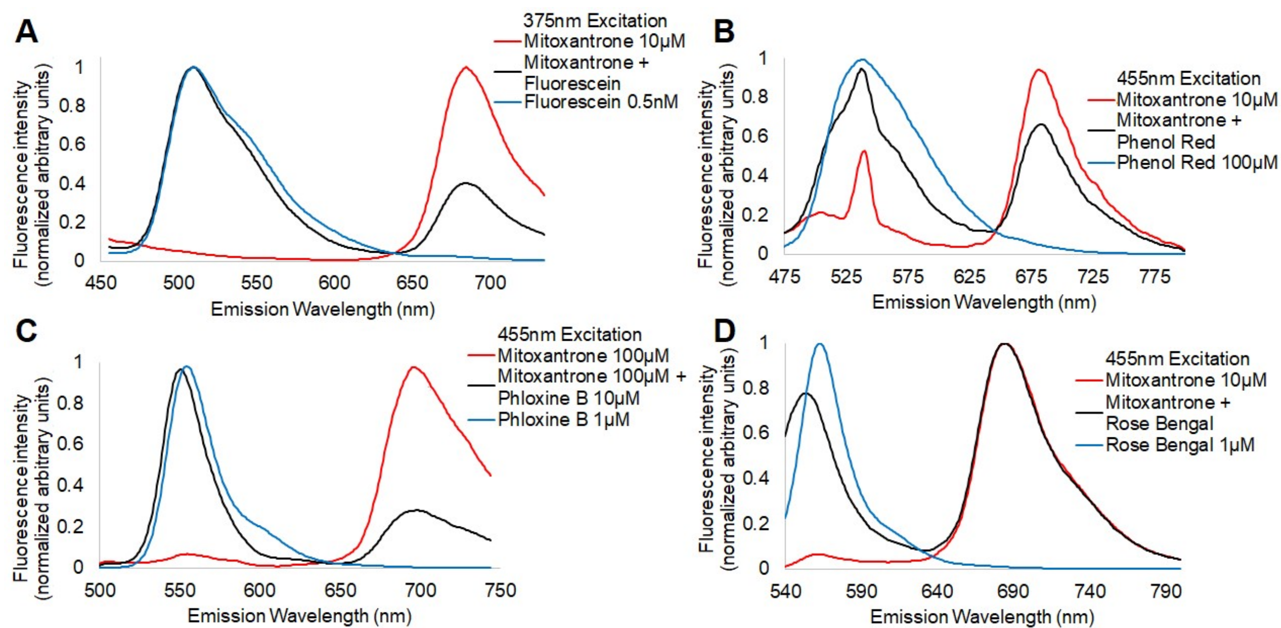
Supplemental Figure S5: Borederline compatibility of nucleic acid and cytoplasmic dyes. Actual (not normalized) fluorescence intensity of 10µM pyrvinium pamoate decreased in the presence of 1µM rose bengal, partially obscuring the pyrvinium pamoate component of the combined spectrum at 488nm excitation.



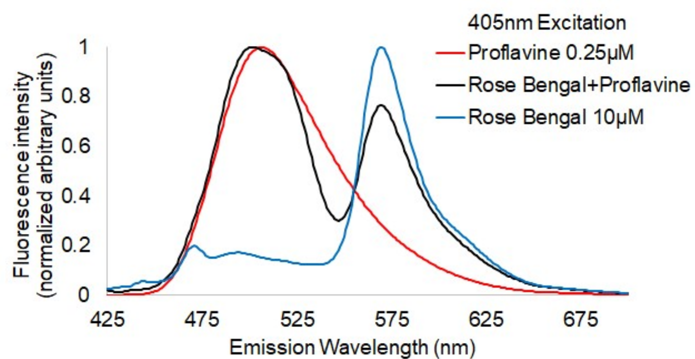
Supplemental Figure S6: Optimized fluorescence emission spectra compatibility *in vitro* of the possible protein drug-dye fluorescein with nucleic acid drug-dye daunorubicin. 1µM Daunorubicin, 1nM fluorescein and the combination were excited at 375nm, with resultant fluorescence emission.



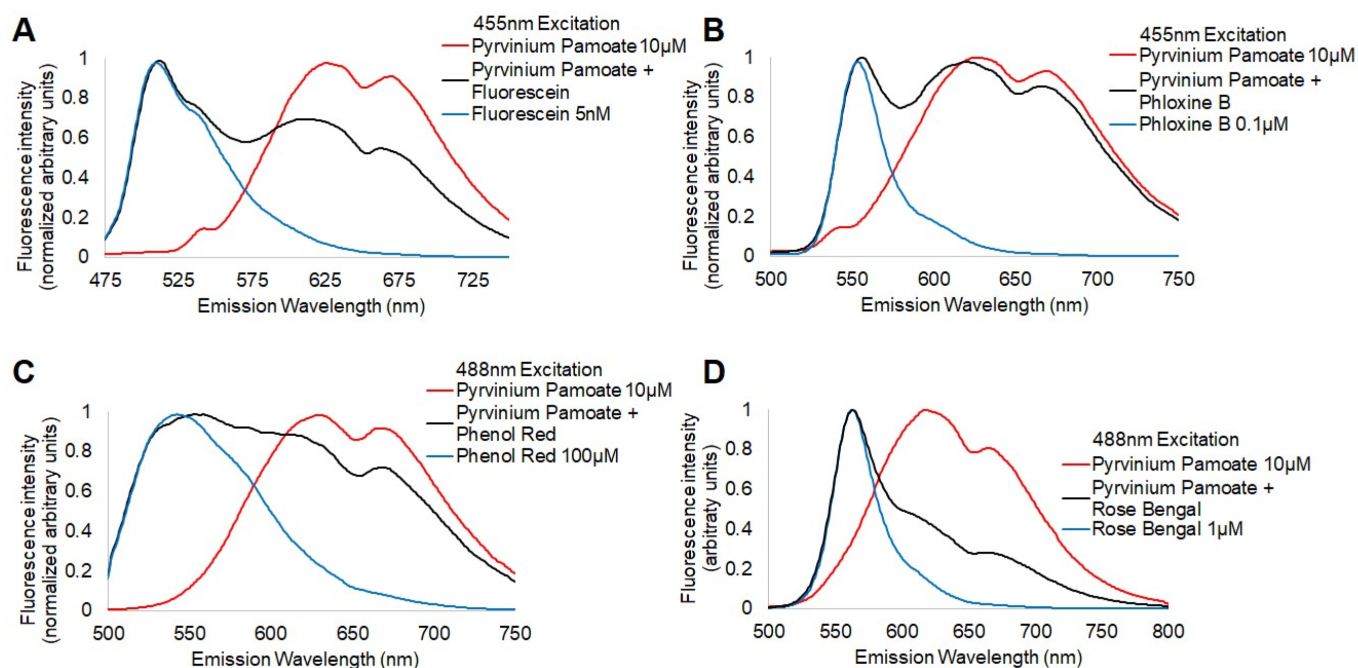
Supplemental Figure S7: Optimized fluorescence emission spectra compatibility *in vitro* of methylene blue as a nuclear drug-dye with (**A**) fluorescein as a protein/cytoplasmic dye at 455nm excitation, (**B**) 300µM Phenol red at pH 5 and 488nm excitation, (**C**) phloxine B at 2µM at 520nm excitation, (**D**) and 1µM rose bengal at 488nm excitation with their respective resultant fluorescence emission spectra, smoothed and normalized to peak for display.



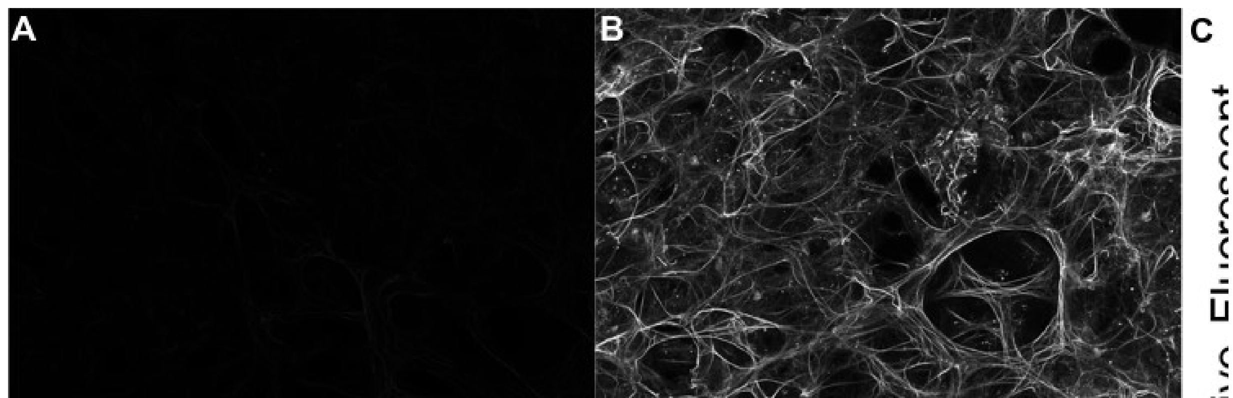
Supplemental Figure S8: Optimized fluorescence emission spectra compatibility *in vitro* of possible cytoplasmic drug-dyes with nucleic acid drug-dye mitoxantrone. (**A**) Mitoxantrone at 10μM, fluorescein at 0.5nM and the combination was excited at 375nm, with the resultant emission spectrum shown. (**B**) Mitoxantrone 10μM, phloxine B 10μM and the combination was excited at 405nm and resultant spectrum displayed. (**C**) Mitoxantrone 10μM, phenol red at 100μM, and the combination of 10μM mitoxantrone and 10μM phenol red at pH 5 was excited at 455nm; the resultant spectrum is shown. (**D**) Mitoxantrone 10μM, rose bengal 1μM and the combination was excited at 455nm and resultant spectrum displayed.



Supplemental Figure S9: Optimized fluorescence emission spectra compatibility *in vitro* of rose bengal as a cytoplasmic drug-dye candidates with the nucleic acid drug-dye proflavine. With 405nm excitation, proflavine at 0.25μM, rose bengal at 10μM, and the combination result in roughly the same order of magnitude peaks resulting from their respective fluorescence.



Supplemental Figure S10: Optimized fluorescence emission spectra compabitility *in vitro* of candidate cytoplasm-dyes with possible nucleic acid drug-dye pryvinium pamoate. (**A**) 10μM pyrvinium pamoate, 5nM fluorescein, and the combination emission spectra were interrogated with 455nm excitation. (**B**) At 455nm excitation, 10μM pyrvinium pamoate, 100nM phloxine B, and the combination emission spectra are shown. (**C**) At 488nM excitation at approximately pH 5, 10μM pyrvinium pamoate, 100μM phenol red, and the combination emission spectra thereof are shown. (**D**) 10μM Pyrvinium pamoate, 1μM rose bengal, and the combined emission spectra. Spectra are representative of 2-3 repeated experiments.

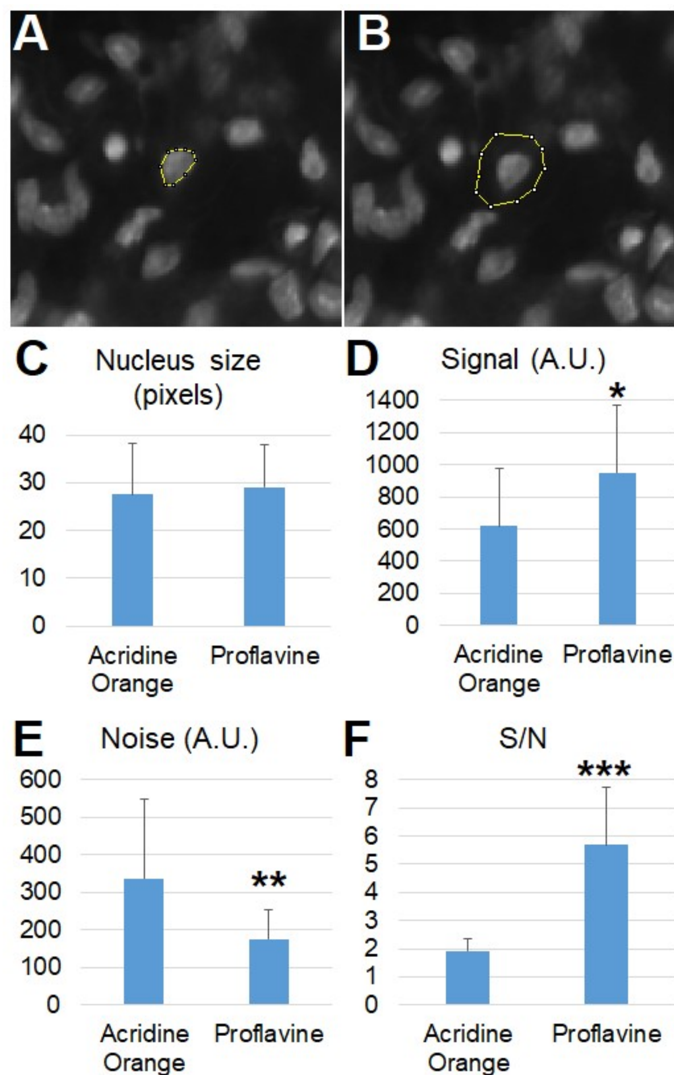


Supplemental Figure 11: Autofluorescence of *ex vivo* lung. (**A**) Autofluorescence of bovine lung tissue was negligible under the imaging conditions for Figure 1 & Supplemental Figure 12 comparing the research nuclear dye with its structurally-similar approved drug. (**B**) Autofluorescence signal with increased electronic gain from the 488nm excitation laser in Channel 1 (515/30nm). Brightness and contrast were uniformly adjusted after imaging, without change in gamma. (**C**) Autofluorescence emission spectra at 488nm excitation of minced ovine lung tissue for comparison to Figures 3-6. The blue line on the abscissa represents Channel 1, the green Channel 2, and red Channel 3 of the confocal microscope system used.

Nucleus dyes	Approximate peak fluorescence emission (nm)	Fluorescein	Phloxine B	Phenol Red	Rose Bengal
		505	550	535	565
Daunorubicin	555, 590	Fig. 3	N/C	N/C	N/C
Methylene Blue	695	Fig. 4A	Fig. 4B	Fig. 4C	Fig. 4D
Mitoxantrone	685	Fig. 5A	Fig. 5B	Fig. 5C	Fig. 5D
Proflavine	508	N/C	N/C	N/C	Fig. 6

(Column group header: Protein/Cytoplasm dyes)

Supplemental Table S4: Top 4 nuclear and protein/cytoplasmic drug-dye candidates and peak emission. Peak wavelengths are approximate due to variance in concentration- and excitation-dependence. N/C, not compatible.



Supplemental Figure 12: Semi-quantitative comparison of research-dye, acridine orange (AO), to structurally-similar drug-dye, proflavine. 50µl of 20µM AO or proflavine were added to *ex vivo* bovine lung tissue and examined at 488nm excitation with identical camera settings. (**A**) Manual regions of interest (ROIs) were drawn around the presumed nuclei and then (**B**) adjacent cytoplasm/background in 10 cells per image for 3 images. (**C**) There was no difference in the size of the nuclei between groups, suggesting similar sized nucleus/no bias in manual ROIs (One-way ANOVA for independent measures $F(1,59)=0.39$, $p=0.53$). (**D**) With an alpha of 0.01, there was a slight difference in signal intensity, a relative but arbitrary measure (*, $F(1,59)=10.80$, $p=0.0017$). (**E**) There was a difference in the background, suggesting differences in non-nucleus uptake (**, $F(1,59)=14.98$, $p=0.00023$), and (**F**) an even stronger difference in signal/background ratio (***, $F(1,59)=96.15$, $p<0.00001$). Data are presented as means plus standard deviation.

Drug-dye	Common clinical indication	Typical clinical dosing	Route	Typical dose*	Proposed intralesional dose (concentration and volume)	Proposed dose (% of typical)
Daunorubicin	Leukemia	45mg/m2	IV	76.5 mg	0.132mg (50µM x 5 mL)	0.172549
Methylene Blue	Methemoglobinemia	1mg/kg	IV	70 mg	0.160mg (100µM x 5mL)	0.2285714
Mitoxantrone	Leukemia	12mg/m2	IV	20.4 mg	0.222mg (100µM x 5mL)	1.0882353
Proflavine	Antiseptic	1.14mg	TOP	1.14 mg	0.105mg (100µM x 5mL)	9.2105263
Fluorescein	Fluorescence angiography	7.7mg/kg	IV	500 mg	0.001mg (0.5µM x 5 mL)	0.0002
Phloxine B	Colorant, dental disclosing agent	1.25mg/kg	PO	87.5 mg	0.041mg (10µM x 5mL)	0.0468571
Phenol Red	Phenolsulfonphthalein test	1mg/kg	IV	70 mg	0.177mg (100µM x 5mL)	0.2528571
Rose Bengal	Ophthalmic diagnostic	1.3mg	OU	1.3 mg	0.487mg (100µM x 5mL)	37.461538

Supplemental Table S5: Drug-dyes and proposed concentrations relative to typical clinical dose. *, using 70kg, 1.7m^2 body-surface area, or a maximum dosage. IV, intravenous. PO, per os (by mouth). OU, oculus uterque (applied to eyes). TOP, topical.