High-throughput screens for fluorescent dye discovery

Vebjorn Ljosa and Anne E. Carpenter

Broad Institute of MIT and Harvard

7 Cambridge Center

Cambridge, MA 02142

Corresponding author: Carpenter, A.E. (anne@broad.mit.edu)

Abstract: A recent screen of a combinatorial library of fluorescent compounds discovered fluorescent dyes that were able to distinguish myoblasts from differentiated myotubes. New fluorescent dyes that respond to biologically relevant changes in cell state or type are useful as stains in a wide variety of biological experiments, including high-throughput screens for chemical and genetic regulators. Combining this approach with microscopy imaging is likely to be even more powerful and might lead to the discovery of new dyes with interesting and useful properties.

1 The challenge of dye discovery

The process of using fluorophore-labeled antibodies to mark an antigen has become one of the most common staining techniques for fluorescence microscopy because of its versatility: fairly specific antibodies can be developed for most proteins. If enough is known about a biological pathway, an investigator can readily create antibodies to proteins known to be expressed differently in the cellular compartment, state, or cell type of interest. For instance, an antibody to a well-known marker of mitosis, phosphorylated histone H3, was chosen to screen for mitosis regulators [1]. Often, however, there are no such clear candidate target proteins. Antibodies have other disadvantages, including intricate staining protocols, inability to penetrate cell membranes, and generally high costs. Labeling proteins with GFP is an alternative approach that has the important advantage of being compatible with live cells, but which also has disadvan-

tages: there are concerns of perturbing the protein's function, as well as having to engineer or modify cell lines. In both cases, a protein that identifies a cellular compartment or differentiates a cell state must be known, as screening antibodies or GFP-labeled proteins is mostly impractical.

Small-molecule dyes have many experimental advantages by comparison. The most important advantage, namely that a target need not be known, is also a disadvantage in that designing dyes for a particular purpose is difficult. Rational design techniques, which modify the molecule according to what is known or suspected about how structural elements affect it, have been very successful at improving dyes' fluorescent properties and target specificity [2], and have even lead to some novel probes [3, 4, 5, 6]. However, many useful dyes have been discovered essentially by accident. Nile red, for example, was discovered when trace amounts appeared in a Nile blue preparation and, unexpectedly, stained lipids [7].

Our ability to discover new dyes may be improved dramatically by the combination of two new technologies, combinatorial chemistry and high-throughput screening. Combinatorial chemistry produces large, diverse libraries of structurally related compounds [8]. High-throughput screens employ automation to test a large number of chemicals or other perturbations at reasonable cost and accuracy [9]. Both techniques are already widely used in drug discovery, and high-throughput screens have led to notable successes in screening for chemical activators and inhibitors [10], but their application to fluorescent dye discovery is still at an early stage [11].

2 Screening combinatorial libraries of dyes

In their paper in the April issue of the *Journal of the American Chemical Society*, Wagner *et al.* [12] apply the screening approach to search for a dye specific for myogenesis, i.e., cellular differentiation of muscle cells. Their strategy is illustrated in Figure 1. Myoblasts (muscle stem cells) and myotubes (differentiated muscle fibers) from mouse (*Mus musculus*) were prepared in parallel sets of 384-well plates. Both myoblasts and myotubes were treated with each member of two combinatorial libraries comprising 1606 fluorescent compounds (rosamine [13] and styryl dye [11, 14] derivatives). After 1 h, the samples were fixed, and the amount of fluorescence in each well was measured with a plate reader. The authors discovered six fluorescent dyes whose fluorescent signals differed between myoblasts and myotubes.

A small molecule capable of distinguishing myoblasts from myotubes is a significant advance over antibodies to myotube-specific proteins [15], for it enables screens that have been impractical because the cost of the antibody is substantial for multiplate screens, and because the antibody staining protocol consists of multiple steps over a period of two days (B. Wagner, personal comm.). Wagner *et al.* use E26, one of their newly discovered dyes, to perform a pilot screen of kinase inhibitors for inhibitors of myogenesis. Eighty-four compounds were screened, including known inhibitors of myogenesis, and 17 of them inhibited differentiation significantly. These results suggest that this method could be used in the future to screen for enhancers or inhibitors

of differentiation, which may be relevant to cancer and regenerative medicine.

The rosamine and styryl compound libraries that have been used in this study are not myogenesis-specific. They can therefore be readily used to screen for markers for other cellular events or compartments. In fact, while the Wagner *et al.* paper was the first to report screening for differentiation markers, some of its authors have previously (along with others) screened the rosamine library for probes that monitor cellular glutathione (GSH) levels *in vivo* [13]. They have also screened the styryl dye library for fluorescent dyes that are able to label specific subcellular compartments [14] or amyloids [11]. These previous screens demonstrated that screening dyes for a specific purpose is feasible. That Wagner *et al.* were able to re-screen the libraries for a different application, namely myogenesis, indicates that combinatorial libraries based on one or a few known fluorescent scaffolds have wide utility.

Libraries not designed to be fluorescent may also be useful. A recent study [16] measured the autofluorescence of 70,000 compounds in regions of the spectrum commonly used in high-throughput screens. The primary purpose of this experiment was to characterize the autofluorescence in order to eliminate false positives in screens. Several novel fluorophores were also discovered, however, including two that did not have a direct overlap with any core moiety common to known fluorophores, and would therefore not have been found in a combinatorial library designed around known fluorescent scaffolds.

3 Future possibilities

We expect that the general approach of screening combinatorial libraries of fluorescent compounds will yield a number of other useful dyes. Some of these may elucidate processes for which no marker was previously available, while others will simply provide an alternative to existing antibodies. Because antibody labeling of intracellular proteins normally requires fixation and permeabilization procedures [17], new cell-permeable dyes will enable live-cell experiments. Screens of the styryl dye library have already led to the discovery of novel, cell-permeable DNA dyes [18] and RNA probes [19].

New dyes will benefit traditional small-scale experiments, but will have an even more profound effect on large-scale experiments, such as high-throughput screens. In practical terms, the high cost and cumbersome protocols of antibodies are a more serious impediment to large-scale experiments. Furthermore, new dyes may enable screens for conditions or cell states for which no markers are currently known. For example, in cases in which a cell line engineered to contain a pathological genetic alteration yields no obvious phenotype, researchers could search for dyes that would be able to distinguish the engineered cell line from the wild-type. Also, dyes that preferentially label primary cells from patients affected by a particular disease compared to healthy individuals could be used in a screen for novel therapeutics that impinge upon pathways that have not otherwise been elucidated.

Discovery of new dyes also opens up new frontiers in image-based screening of chemical or genetic (RNAi, overexpression) libraries [20, 21]. Whereas a plate reader or flow cytometer measures the total fluorescence emitted from a sample or a cell, respectively, image-based screens employ a robotic microscope to image each sample. Therefore, using image processing software, visually discernable features of cells can be measured, such as shape of cells/organelles and subcellular staining patterns, rather than merely the overall staining intensity. Compared to other high-throughput screening modalities, image-based screens particularly benefit from chemical dyes as stains because high-quality stains with high signal-to-noise ratio are needed for accurate measurements of morphology. In this respect, chemical dyes are often much more reliable than antibody staining procedures, and Wagner *et al.*'s approach may provide useful new reagents enabling previously challenging image-based screens.

Beyond these practical benefits, integrating the dye discovery process directly with image-based screening may enable previously intractable types of screens (Figure 2). As mentioned before, there are in some cases no obvious choices for labels that mark the cellular state of interest. The Wagner screen looked for dyes whose fluorescent staining intensity alone could distinguish between positive and negative controls, the perfect readout for dyes intended to be used in a plate-reader–based screen. But for assays where such robust intensity responders cannot be found, image-based screening of fluorescent compounds may find dyes whose intensity *and* localization respond to the cellular differentation state of interest. The combination of different image-based

cellular features may provide higher potential for distinguishing cell states as compared to fluorescence intensity alone.

4 Conclusion

In conclusion, Wagner *et al.*'s article together with other recent work demonstrate that screening of relatively small combinatorial libraries of fluorescent compounds can yield new dyes for diverse targets. These dyes will enable new small-scale experiments as well as large-scale chemical and genetic screens, including complex image-based screens.

Competing interests statement

The authors declare that they have no competing financial interests.

References

[1] Moffat J., *et al.* (2006) A lentiviral RNAi library for human and mouse genes applied to an arrayed viral high-content screen. *Cell* 124, 1283–1298

[2] de Silva A.P., *et al.* (1997) Signaling recognition events with fluorescent sensors and switches. *Chem. Rev.* 97, 1515–1566

[3] Grynkiewicz G., *et al.* (1985) A new generation of ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260, 3440–3450

[4] Minta A. and Tsien R. (1989) Fluorescent indicators for cytosolic sodium. *J. Biol. Chem.* 264, 19458–19467

[5] Gonzalez J. and Tsien R. (1997) Improved indicators of cell membrane potential that use fluorescence resonance energy transfer. *Chem. Biol.* 4, 269–277

[6] Zaccolo M., *et al.* (2000) A genetically encoded, fluorescent indicator for cAMP in living cells. *Nature Cell Biol.* 2, 25–29

[7] Greenspan P., *et al.* (1985) Nile red: A selective fluorescent stain for intracellular lipid droplets. *J. Cell Biol.* 100, 965–973

[8] Lam K.S. and Renil M. (2002) From combinatorial chemistry to chemical microarray. *Curr. Opin. Chem. Biol.* 6, 353–358

[9] Inglese J., *et al.* (2007) High-throughput screening assays for the identification of chemical probes. *Nat. Chem. Biol.* 3, 466–479

[10] Rausch O. (2006) High content cellular screening. *Curr. Opin. Chem. Biol.* 10, 316–320

[11] Li Q., *et al.* (2004) Solid-phase synthesis of styryl dyes and their application as amyloid sensors. *Angew. Chem. Int. Ed.* 43, 6331–6335

[12] Wagner B.K., *et al.* (2008) Small-molecule fluorophores to detect cell-state switching in the context of high-throughput screening. *J. Am. Chem. Soc.* 130, 4208–4209

[13] Ahn Y.H., *et al.* (2007) Combinatorial rosamine library and application to in vivo glutathione probe. *J. Am. Chem. Soc.* 129, 4510–4511

[14] Rosania G.R., *et al.* (2003) Combinatorial approach to organelle-targeted fluorescent library based on the styryl scaffold. *J. Am. Chem Soc.* 125, 1130–1131

[15] Scherer P.E., *et al.* (1995) A novel serum protein similar to C1q, produced exclusively in adipocytes. *J. Biol. Chem.* 270, 26746–26749

[16] Simeonov A., *et al.* (2008) Fluorescence spectroscopic profiling of compound libraries. *J. Med. Chem.* 51, 2363–2371

[17] Giepmans B.N.G., *et al.* (2006) The fluorescent toolbox for assessing protein location and function. *Science* 312, 217–224

[18] Lee J.W., *et al.* (2003) Development of novel cell-permeable DNA sensitive dyes using combinatorial synthesis and cell-based screening. *Chem. Commun.* 1852–1853

[19] Li Q., *et al.* (2006) RNA-selective, live cell imaging probes for studying nuclear structure and function. *Chem Biol.* 13, 615–623

[20] Carpenter A.E. (2007) Image-based chemical screening. *Nat. Chem. Biol.* 3, 461–465

[21] Carpenter A.E. and Sabatini D.M. (2004) Systematic genome-wide screens of gene function. *Nat. Rev. Genet.* 5, 11–22

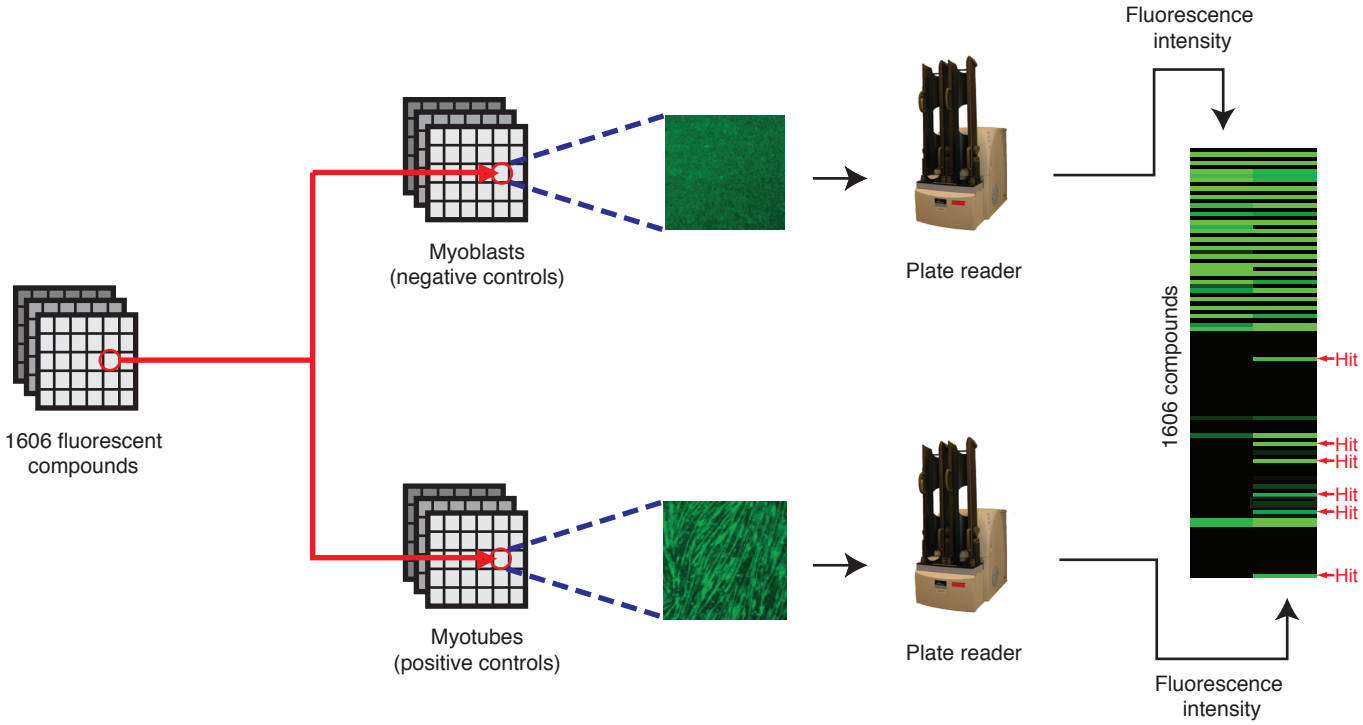

Figure 1: Screening fluorescent compounds. Wagner *et al.* used myoblasts (muscle stem cells) and myotubes (differentiated muscle fibers) in parallel sets of 384-well plates. Both myoblasts and myotubes were treated with each member of a combinatorial library of 1606 fluorescent compounds. After 1 h, the samples were fixed, and the fluorescence in each well was measured with a plate reader. Six dyes were found to differentiate myoblasts and myotubes. Schematic data shown. Micrographs reprinted, with permission, from Wagner *et al.* [12], American Chemical Society, copyright 2008.

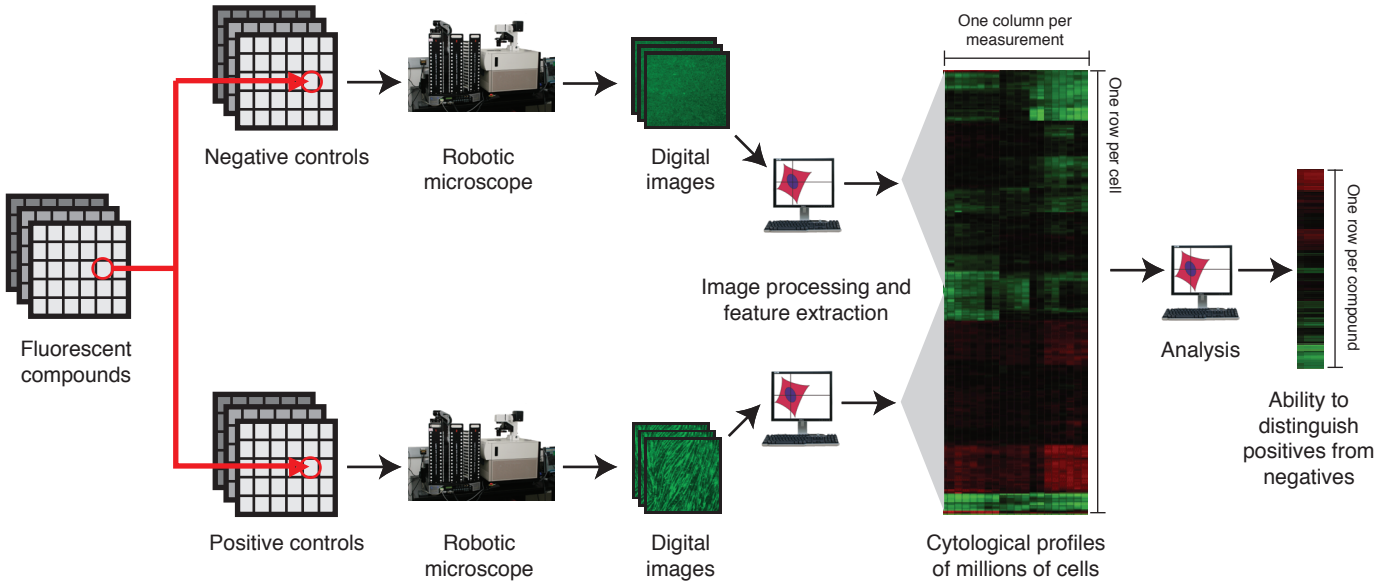

Figure 2: Integrating fluorescent dye discovery with image-based screens. In a future application, the screening approach may be extended to include software tools that compute a cytological profile for each cell, consisting of hundreds of measurements. Based on the resulting cytological profiles, the dyes are evaluated with regard to their ability to distinguish negative and positive controls. Compared to the approach in Figure 1, this may find dyes that are selective when both intensity and localization are considered, even when intensity alone is not sufficient, thereby enabling otherwise intractable screens. Schematic data shown. Micrographs reprinted, with permission, from Wagner *et al.* [12], American Chemical Society, copyright 2008.