

# High-throughput screens for fluorescent dye discovery

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**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.**

## 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 green fluorescent protein (GFP) is an alternative approach that has the important advantage of being compatible with live cells but that also has disadvantages: 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 led to some novel probes [3–6]. 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].

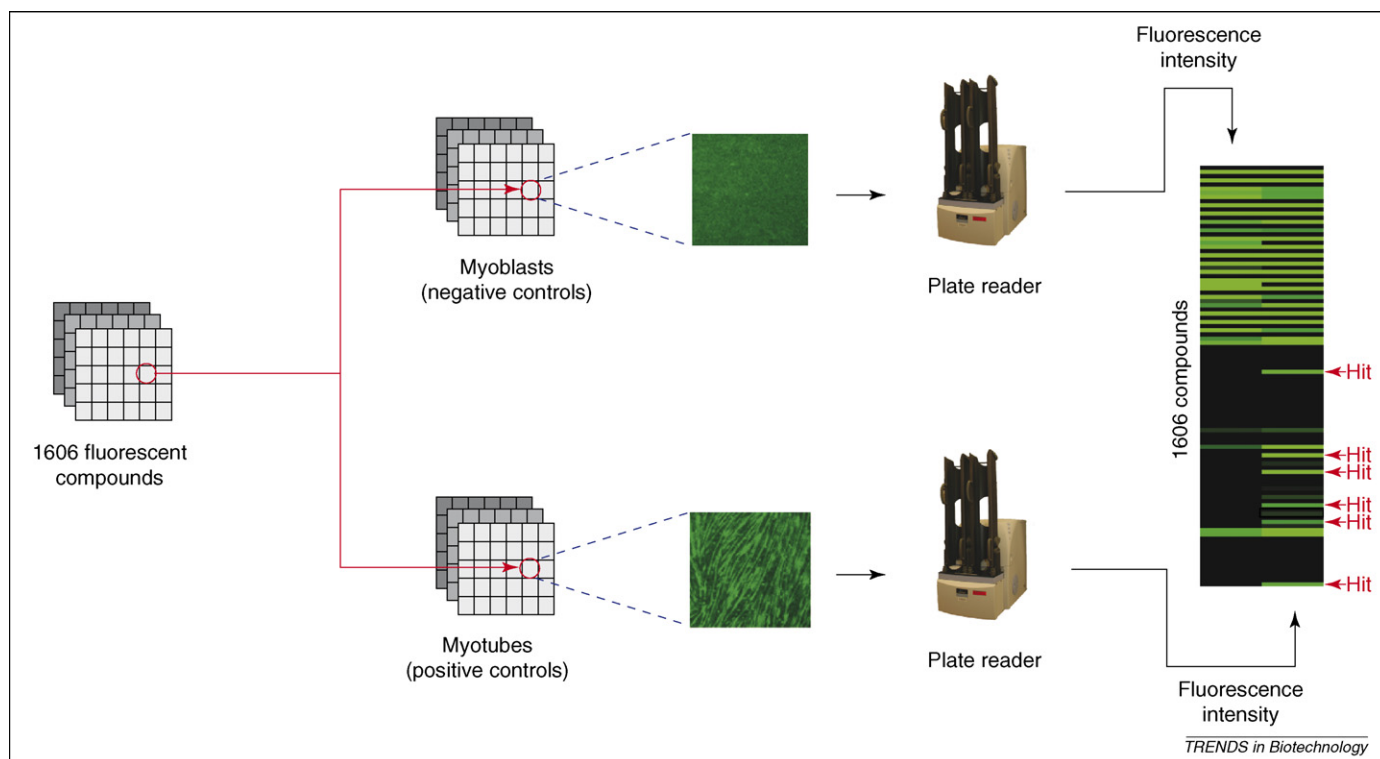
Our ability to discover new dyes could be improved dramatically by the combination of two new technologies, combinatorial chemistry and high-throughput screening. Combinatorial chemistry produces large, diverse libraries of structurally related compounds [8]. High-throughput screens employ automation 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].

## 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 for myotube-specific proteins [15] as it enables screens that have been impractical because the cost of antibodies is substantial for multi-plate screens and because the antibody staining protocol consists of multiple steps over a period of two days (B. Wagner, personal communication). Wagner *et al.* used 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 might be relevant to cancer and regenerative medicine.

The rosamine and styryl compound libraries that were 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, although 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



**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 are shown for illustration. Micrographs reprinted with permission from [12], American Chemical Society, copyright 2008.

*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 might 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 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.

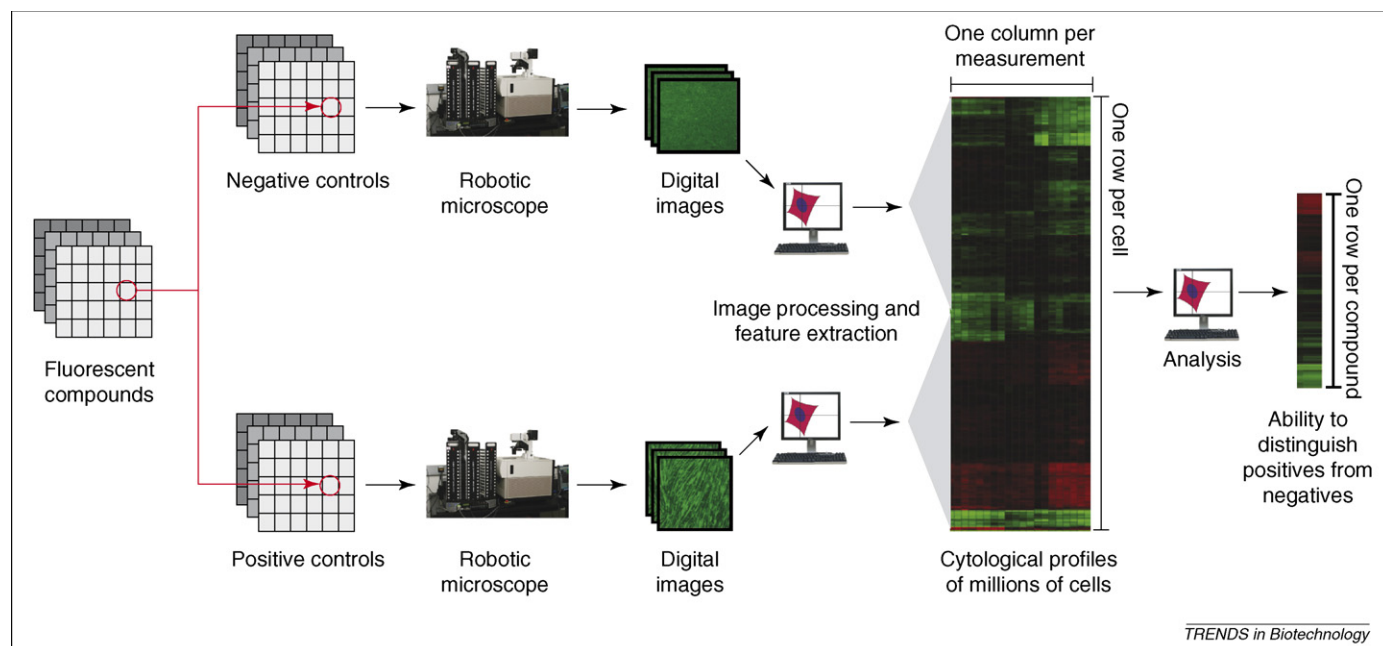
### Future possibilities

We expect that the general approach of screening combinatorial libraries of fluorescent compounds will yield several other useful dyes. Some of these might elucidate processes for which no marker was previously available, and 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 might 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 (RNA interference, 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, by using image processing software, visually discernable features of cells can be measured, such as subcellular staining patterns and the shape of cells and organelles, rather than merely the overall staining intensity. Compared to other high-throughput screening



**Figure 2.** Integrating fluorescent dye discovery with image-based screens. In a future application, the screening approach might 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, such screens might find dyes that are selective with regard to both intensity and localization, even when intensity alone is not sufficient, thereby enabling otherwise intractable screens. Schematic data are shown for illustration. Micrographs reprinted with permission from [12], American Chemical Society, copyright 2008.

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 might provide useful new reagents enabling previously challenging image-based screens.

Beyond these practical benefits, integrating the dye discovery process directly with image-based screening might 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 might locate dyes whose intensity and localization both respond to the cellular differentiation state of interest. The combination of different image-based cellular features might provide more potential for distinguishing cell states than fluorescence intensity alone.

## Conclusion

In conclusion, Wagner *et al.*'s article, together with other recent work, demonstrates 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.

## Disclosure statement

The authors declare that they have no competing financial interests.

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