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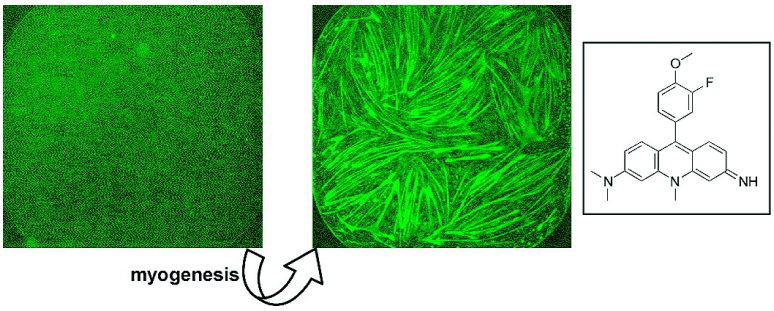

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Small-Molecule Fluorophores To Detect Cell-State Switching in the Context of High-Throughput Screening

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The identification of small molecules capable of detecting specific cellular states would facilitate high-throughput screening.[1] Many such probes are in current use, but the intended cell-state difference is usually within the same cell type, with detection often dependent on either enzymatic or metabolic activity.[2] A small molecule capable of distinguishing the distinct states resulting from cellular *differentiation* would be of enormous value, for example, in efforts aimed at regenerative medicine. One example is the use of hydrophobic dyes targeting the lipid droplets that accumulate during adipocyte differentiation.[3] Other cell models of differentiation require more complex measurements of cell state, such as gene expression.[4] In the case of myogenesis, a probe capable of distinguishing myoblasts from differentiated myotubes would represent a significant advance over current detection techniques, which typically involve immunofluorescence of myotube-specific proteins, and would facilitate screening for small molecules involved in the differentiation process.

The design and synthesis of fluorescent compound libraries[5] was inspired by the desire to screen for probes of cellular metabolites.[6] We extended these studies by performing parallel cell-based screenings on a collection of 1606 optically active compounds (see Supporting Information (SI)), using both murine C2C12 myoblasts and myotubes differentiated using 2% horse serum. After incubating cells with compounds in PBS for 1 h at 37 °C, we washed the assay plates and measured fluorescence using a multimode plate reader at two common wavelength pairs (485 nm/530 nm and 530 nm/580 nm). Using a scoring algorithm dependent upon the distribution of raw values from mock-treated wells,[7] we arrived at a normalized score for each compound-treated well, representing the number of standard deviations from the mean of that distribution. During primary screening, the majority of compounds were positive outliers for cellular fluorescence at one wavelength in both cell states (see SI), suggesting either that these compounds have no cell-state selectivity or that the concentration of the compound used was so high as to mask selectivity by signal saturation.

In order to distinguish these possibilities, we treated both myoblasts and myotubes with a collection representing an 81-fold dilution of the original compounds, achieved by four serial 3-fold dilutions. Cell-state selectivity was assessed by automated microscopy. At this screening concentration (~250 nM), many of the compounds were no longer fluorescent in either cell state. Alternatively, a few compounds continued to fluoresce in both cell states.

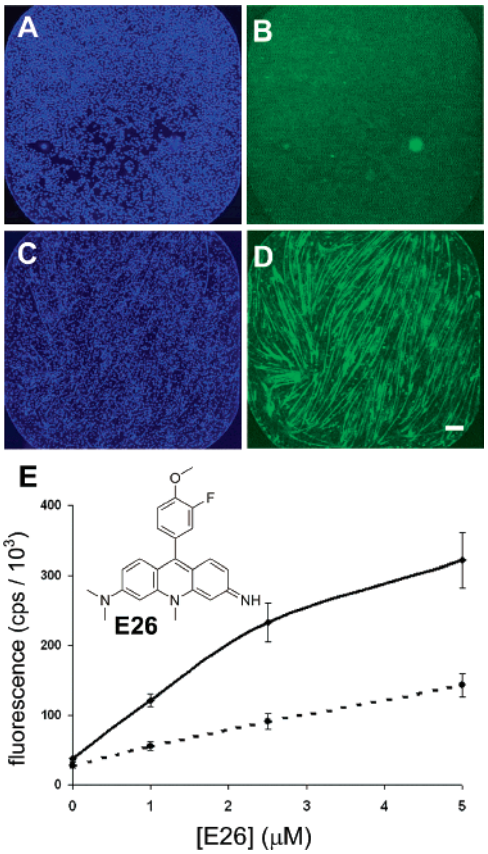

Figure 1. Discovery of a myotube-specific fluorescent probe. C2C12 cells were seeded at 4000 cells/well and differentiated, and either myoblasts (**A**, **B**) or myotubes (**C**, **D**) were treated with 250 nM **E26** for 1 h and imaged using an automated microscope. DNA was stained with Hoechst dye (**A**, **C**) to confirm cell adherence and imaged for **E26** fluorescence (**B**, **D**). Treatment of C2C12 myoblasts (dashed line) or myotubes (solid line) with increasing concentrations of **E26** results in fluorescence detectable using a plate reader, displayed as thousands of counts per second (**E**). The mean and standard deviation of 48 wells for each condition are shown. Scale bar = 150 μm.

However, six of the compounds had the desired result, with observable fluorescence exclusively in myotubes (Figure 1A−D and Figure S1).

One of the goals of determining the cell-state selectivity of compound fluorescence is to use these compounds as probes for high-throughput screening. In order to demonstrate the utility of these compounds, we confirmed differential cellular fluorescence using a multimode plate reader (Figure 1E) after treating 384-well plates of cells with varying concentrations of the compounds for 1

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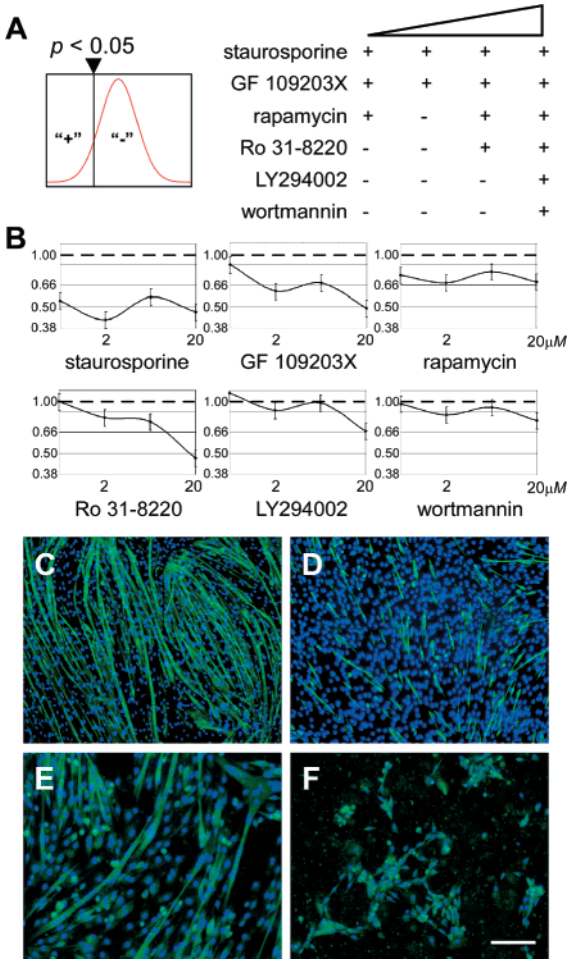

Figure 2. Use of **E26** in cell-based screening for inhibitors of muscle differentiation. C2C12 myoblasts were treated with a collection of diverse kinase inhibitors for 48 h and stained with **E26** after 5 days in cell culture. Selected compounds resulted in significantly decreased fluorescence ($p < 0.05$; indicated by "+") at increasing compound concentrations (**A**). Full screening results are available in the Supporting Information. Fold decrease in fluorescence as a result of treatment with selected kinase inhibitors was calculated as an arithmetic mean of per-plate ratios of a compound-treatment well (indicated as μM) and the mean of mock-treatment wells (**B**). Error bars represent the standard deviation of mock-treated wells (48 replicates), indicating the noise in the assay system. The dashed line in each graph represents a ratio of 1.0, in which fluorescence is unchanged from the mock treatments. Myosin heavy chain (MHC) immunofluorescence (**C**, **D**) or 1 h **E26** treatment (**E**, **F**) of C2C12 myotubes (**C**, **E**) or myoblasts treated with 100 n*M* (**D**) or 600 nM (**F**) rapamycin during differentiation. Scale bar = 150 μm.

h. Treatment with 1 μM **E26** resulted in a *Z*-factor score of 0.34 and a signal-to-noise ratio (*S*/N) of 14.1. Although the former statistic was borderline low for a superior assay, we felt the *S*/*N* was sufficiently high for HTS and thus decided to use this compound condition as a probe for myogenesis in a pilot screen of 84 kinase inhibitors, plated at four doses each in a 384-well stock plate. We treated two 384-well plates of C2C12 myoblasts with these compounds in differentiation media containing 2% horse serum (see SI) for 2 days, followed by three further days of culture in differentiation media without the compound. We treated these plates of cells with **E26** in the same manner as that during assay development (*vida supra*). Of the 84 compounds, including known inhibitors of myogenesis,[8] 17 significantly ($p < 0.05$; see SI) inhibited differentiation at one or more concentrations (Figure 2A). Kinase inhibitors known to induce apoptosis in myoblasts, such as staurosporine,[9] also reduced the fluorescent signal to a significant extent (Figure 2B). We compared the results from compound **E26** with immunofluorescent staining of the myosin heavy chain (MHC) for the ability to detect the effects of rapamycin on myogenesis. In both treatments, myotubes fluoresced brightly (Figure 2C, 2E). Rapamycin is capable at concentrations as low as 10 nM of inhibiting differentiation of C2C12 myoblasts,[10] and we observed a similar decrease in cellular fluorescence with either MHC antibody-based staining (Figure 2D) or **E26** treatment (Figure 2F). Detergent extraction resulted in a decrease in cellular fluorescence (see SI), suggesting that **E26** does not bind to the cytoskeleton. Further, **E26** is not fluorescent in another cell model of differentiation, 3T3-L1 adipogenesis (see SI). These results suggest that **E26** is a reliable fluorescent probe for use in cell-based screening for myogenesis. Cell-state selectivity of this compound may be achieved by binding to one of the many proteins expressed more highly in the myotube state;[11] alternatively, there may be an environmental effect within the myotube, such as a cellular metabolite that the compound can bind that promotes compound retention or fluorescence. While further study is necessary to determine the mechanism of cell-state selectivity, our observations are sufficient to confirm that such selectivity exists and can be leveraged for screening purposes. We suggest that the strategy of screening for screening agents reported here may be extended more broadly in the future.

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Supporting Information Available: Experimental procedures, high-throughput and high-content screening data. This material is available free of charge via the Internet at http://pubs.acs.org.

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