Avoiding Fluorescence Assay Interference— The Case for Diaphorase

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ABSTRACT

Fluorescence is utilized as the output for a range of assay formats used in high-throughput screening (HTS). Interference with these assays from the compounds in libraries utilized in HTS is a well-recognized phenomenon, particularly for assays relying on UV excitation such as for direct detection of the oxidoreductase cofactors NADH or NADPH. In this study, we discuss these interference challenges and highlight the specific case of the diaphorase/resazurin system that can be coupled to enzymes utilizing NADH or NADPH. We review the utilization of this assay system in the literature and argue that the diaphorase/resazurin system is underutilized in assay development. It is the authors' hope that this Perspective and the accompanying Technical Brief in this issue will stimulate interest in a robust and sensitive coupling system to avoid assay fluorescence interference.

Fluorescence as a spectroscopic phenomenon is utilized as the output for a range of assay formats used in high-throughput screening (HTS). These include readouts such as fluorescence polarization (FP), analyte quantitation, cell viability, fluorescence resonance energy transfer, and high-content imaging applications. While the wavelength at which light emitted in many fluorescence-based assays can be “tuned”, many small molecules in a small molecule library are optically active and can interfere with assay performance. This interference can be either by absorbing and emitting light in the same window as the assay resulting in false-positive signal (fluorescing) or absorbing light at either the excitation or emission wavelengths resulting in attenuated intensity of signal (quenching).¹ An HTS can utilize library compound concentrations at the upper end of 20–50 μ M, well above the concentration of the fluorescent reporter in an assay (discussed by Dahlin *et al.*).² To account for these artifacts, assessment of compound interferences using orthogonal and counter assays is critical to ensure that HTS hits are not due to

the optical properties of the molecule. Following triage of false positives, “true” assay hits can be assessed with further biologically relevant assays.^{3,4}

Understanding the extent of compound fluorescence within a library, and the spectroscopic window in which it occurs, can inform assay design and aid in avoiding significant false positives during the HTS phase. For example, Simeonov *et al.* profiled a library containing >70,000 compounds across a number of spectral regions that are regularly utilized in HTS.⁵ Fluorescence was compared against a range of fluorophore standards. For example, the standard dyes 4-MU (4-methylumbelliferone, hymecromone) and Alexa Fluor 350 excite at 340 nm and emit at 450 nm (Fig. 1). Of those compounds assessed, 5% (about 3,500 compounds) of the library produced fluorescence equivalent to 10 nM 4-MU or Alexa Fluor 350, and almost 2% of compounds (about 1,000) produced signal equivalent to 100 nM of the fluorophore standards—these are concentrations regularly utilized in fluorescent-based assays. At longer wavelengths, a far lower proportion of library was fluorescent (0.01%–0.1%), demonstrating that “redshifting” the fluorophore reporter during assay development can aid in reducing the number of false positives that arise due to spectroscopic artifacts from test compounds.

The relevance of the data relating to 4-MU and Alexa Fluor 350 lies in the fact that it is the same optical window used to measure the fluorescence of the common substrates NADH and NADPH in biochemical assays. The seminal work of Britton Chance and colleagues from the 1950s onward demonstrated that tissues and cells excited by UV light emit blue fluorescence (termed tissue autofluorescence) and that this is due to NADH and NADPH (predominately in the mitochondria).⁶ NADH and NADPH (collectively termed NAD(P)H) share almost identical excitation (340 ± 30 nm) and emission (460 ± 50 nm) windows,⁷ and just as importantly, NAD^+ and NADP^+ are not optically active in the same window. As NAD(P)H are cofactors for a range of oxidoreductases, one can readily identify probes modulating enzymatic activity through the measurement of consumption or production NAD(P)H (depending on the direction of the biochemical reaction),⁸ and in kinetic mode or at an endpoint. A number of advantages to this strategy exist, including the fact that the native biochemical reaction can be performed without modification.

Fig. 1. Spectral profiling of library compounds excited at 340 nm and fluorescing at 450 nm (the 4-MU/Alexa Fluor 350 spectral window). **(A)** Cumulative spectroscopic profiling results for >70,000 compounds on a well-by-well basis, showing a strong concentration-dependent increase in fluorescent signal (*i.e.*, greater fluorescence at higher concentrations of a given compound). Fluorescent samples are shown in *purple*, inconclusive samples (weakly fluorescent) are shown in *orange*, and nonfluorescent samples are shown in *gray*. **(B)** Compound library SOM based on the structural similarity of compounds in the library, with fluorescent activity in the 4-MU window, is overlaid. The hexagons (clusters) in each SOM are colored by the enrichment level of fluorescent compounds in that cluster. A cluster with a dark color is significantly enriched in fluorescent compounds compared to the library average, and a *green* cluster has no fluorescent compounds. It can be seen that groups of structurally similar compounds in the library are likely to fluoresce, suggesting that the chemotype clustering in HTS hit analysis can point to “repeat offenders” in an HTS compound library. **(C)** A fluorescence profile across 39 quantitative HTS assays is shown, where the fraction of fluorescent compounds out of all actives identified in each screen is plotted. Reprinted with permission from Ref.⁵ Blue-fluorescent compounds represent about 5% of the library assessed, but in assays run in the blue window (4-MU window, excitation = 340 nm and emission = 450), nearly 50% of the active compounds were fluorescent, which is nearly a 10-fold enrichment. HTS, high-throughput screening; SOM, self-organizing map; 4-MU, 4-methylumbelliferone.

Based on the issue with blue-shifted screening library probes discussed above, one might anticipate that directly measuring NAD(P)H in an HTS produces a significant number of false-positive hits, and just as worrying, might produce false-negative data that results in loss of potentially tractable hits. This is indeed the case. In their analysis, Simeonov *et al.* examined 39 screens that had previously been performed at their center and assessed the fraction of active compounds likely to have been fluorescent, which was particularly significant for fluorescence-based assays that excited in the UV range.⁵ For example, an oxidoreductase biochemical assay that measured the production of NADH (gain of fluorescence) produced a series of 28 compounds that shared a core structure. However, it was found that 23 of these hits (80%) were fluorescent upon excitation with UV light. Similar compounds ranked as inactive in the HTS were not fluorescent (or were weakly fluorescent), and orthogonal assays against the same target revealed that the initial fluorescent assay hits were indeed inactive false positives. This is a significant issue when one considers the range of oxidoreductases screened using this assay strategy. A sample list of dehydrogenases and enzymes coupled to dehydrogenases is provided in *Table 1*.

In this issue, Davis *et al.* provide a detailed protocol for coupling a given NAD(P)H-dependent enzyme of interest to a second enzyme reporter system, diaphorase/resazurin, to quantify the concentration of NAD or NADPH present in a biochemical assay (*Fig. 2*).⁹ In effect, this strategy redshifts the

assay, avoiding the optical activity of a significant proportion of the screening library. The term “diaphorase” is a generic term for any oxidoreductase enzyme that can utilize either NADH or NADPH to catalyze the reduction of dyes (chromogenic electron acceptors),¹⁰ including commonly used cell-based viability reporters such as MTT and methylene blue. The form of diaphorase commonly utilized in HTS assays is purified from *Clostridium kluyveri*, although other diaphorases have been purified and reported also.¹¹ Diaphorase was first reported to reduce the weakly-fluorescent resazurin (also known as Alamar Blue) to the highly fluorescent resorufin as a means of measuring the activity of dehydrogenases by Guibault and Kramer in 1964 and 1965.^{12,13} One possible advantage of coupling diaphorase in an oxidoreductase HTS is that if NAD(P)H is the product it is consumed by diaphorase and this prevents the enzyme running in the “reverse” direction. Despite the advantages of redshifting NAD(P)H detection, the diaphorase/resazurin assay does not appear to have been greatly utilized over the past 50 years (only 29 articles appear in PubMed with the terms “diaphorase” and “resazurin”).

The diaphorase/resazurin assay has been adopted for HTS in a number of ways, and the first example in HTS appears to have been reported in 2005.¹⁴ Direct coupling of diaphorase was reported in an HTS for inhibitors of *Plasmodium falciparum* glucose-6-phosphate dehydrogenase 6-phosphogluconolactonase to measure production of NADPH as a result of the conversion of glucose-6-phosphate to

Table 1. Reported High-Throughput Screening Campaigns and Assays that Incorporated the Diaphorase/Resazurin Reaction for the Assay Readout

Enzyme	Reaction direction	Couple	Reference
Isocitrate dehydrogenase (IDH1)	NADP ⁺ to NADPH	Diaphorase	16,25
Mutant isocitrate dehydrogenase (mIDH1)	NADPH to NADP ⁺	Diaphorase	16,25
<i>Plasmodium falciparum</i> glucose-6-phosphate dehydrogenase 6-phosphogluconolactonase	NADP ⁺ to NADPH	Diaphorase	15
<i>Giardia lamblia</i> fructose-1,6-bisphosphate aldolase (FBPA)	NAD ⁺ to NADH	Glyceraldehyde-3-phosphate dehydrogenase, triose phosphate isomerase, Diaphorase	26
<i>Mycobacterium tuberculosis</i> dihydrofolate reductase (DHFR)	NADPH to NADP ⁺	Diaphorase	27
Glucose-6-phosphate dehydrogenase (G6PD)	NADP ⁺ to NADPH	Diaphorase	28
Glucokinase (GCK)	NADP ⁺ to NADPH	Glucose 6-phosphate dehydrogenase, Diaphorase	17
Lactate dehydrogenase (LDH)	NAD ⁺ to NADH	Diaphorase	29
Aldehyde dehydrogenase 5A1 (ALDH5A1)	NADP ⁺ to NADPH	Diaphorase	30
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	NAD ⁺ to NADH	Diaphorase	31
Phosphomannose isomerase	NADP ⁺ to NADPH	Phosphoglucose isomerase, Glucose 6-phosphate dehydrogenase, Diaphorase	32

For each enzyme target, the reaction direction of the coupling enzyme is listed (reaction direction). The "Couple" for each assay lists any enzymes used for coupling between the target enzyme and the final fluorescence readout.

6-phosphoglucono- δ -lactone produced.¹⁵ Conversely, mutant isocitrate dehydrogenase (IDH1 R132H) consumes NADPH in converting α -ketoglutarate to 2-hydroxyglutarate, measuring the loss of NADPH over time monitored using diaphorase.¹⁶ Diaphorase can also be used as a reporter for a

nondehydrogenase biochemical assay, by enzymatic coupling to the enzyme of interest. For example, glucokinase (GCK) is a diabetes target that converts glucose to glucose-6-phosphate (G6P), and its activity is tightly regulated by glucokinase regulatory protein (GKRP). To link to diaphorase, glucose-6-phosphate dehydrogenase (G6PDH) that converts G6P and NAD⁺ to 6-phosphogluconate and NADH, respectively, was used. The NADH produced is then detected using the diaphorase/resazurin system.¹⁷

In each of the three HTS cases mentioned above, orthogonal assays with HTS hits screened against the diaphorase/resazurin alone were needed to triage false-positive compounds that inhibited/interfered with the reporter assay. While few compounds in a high-quality screening library would share resorufin's optical properties, this can be easily assessed when following up on hits by performing a "preread" after pin-transferring compound, but before initiating the biochemical reaction. The opposite risk is that hits are quenching fluorescence signal (through absorbing, exciting, or emitting light), and assessing the hit compounds in the presence of resorufin alone can identify these false-positive compounds. Anecdotal, it appears that the false-negative hit rate is relatively low compared with direct measurement of NAD(P)H levels. A

Fig. 2. Schematic conveying diaphorase/resazurin coupling in HTS assays. A generic dehydrogenase converts substrate to product, converting NAD⁺ to NADH or NADP⁺ to NADPH. Diaphorase utilizes the resulting NADH or NADPH (excitation 340 nm, emission 460 nm) as the electron source for reducing resazurin (weakly fluorescent) to the fluorescent product resorufin (excitation 550 nm, emission 585 nm), allowing the excitation and emission to be "redshifted."

systematic profiling effort of an HTS library against diaphorase/resazurin needs to be carried out, similar to those reported for other assay readouts such as luciferase and fluorescence,^{5,18} to fully understand library interference for this assay.

Other fluorescence assay artifacts also exist that are equally confounding and not so readily resolved through assay modification. Library compounds can interfere with fluorescence signal through the “inner filter effect” by absorbing emitted light, particularly at higher concentrations. The library compound inner filter effect can be mitigated by performing a “preread” measuring the absorbance of the compounds at the excitation and emission wavelengths of the fluorophore used in the assay to identify possible interference.¹⁹ Fluorophores with conjugated planar systems can also confound assay design through dimer (and multimer) formation.¹ The rhodamine dyes are such a family that can form dimers through noncovalent self-association.^{20,21} Dimerization results in a spectral shift, with a consequent change in fluorescence intensity. FP assays also meet with interferences,²² including spectral interference (interference with the detection of fluorescence polarized light signal), particularly when low concentrations of labeled ligand are used in an assay. FP assays can be redshifted during assay development by assessing dyes that avoid blue-shifted assay library interference. For example, a study developing inhibitors for the interaction between Vitamin D Receptor and Steroid Receptor Coactivator 2 utilized an Alexa Fluor 647-conjugated peptide specifically to avoid library fluorescence interference.^{23,24} It is important to emphasize that fluorescent compounds may indeed be active in an HTS; an FP study seeking inhibitors of calcium-bound S100B reexamined fluorescent hits that were initially triaged, and found that they were in fact active in orthogonal assays.

All fluorescence assays have their own unique challenges for assay design that can generally be accounted for thorough careful consideration of counter screens and orthogonal assays. In our experience, the diaphorase/resazurin system for measuring NAD(P)H levels appears to be a highly useful coupling strategy that improves assay quality by decreasing library interference and improving sensitivity. It should be pointed out that while diaphorase is now being incorporated into HTS protocols, no systematic data exists to make the conclusion that the diaphorase assay is superior to the direct NAD(P)H assay in practice, although the accompanying protocol in this issue does partially address this issue. Furthermore, the reagents are not exceedingly costly, and so can be adopted relatively easily. It is our hope that the information in this perspective and the accompanying protocol will encourage its utilization in other screening centers.

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DISCLOSURE STATEMENT

The authors declare no conflicts of interest.

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Abbreviations Used

4-MU = 4-methylumbelliferone
 FP = fluorescence polarization
 HTS = high-throughput screening
 SOM = self-organizing map