High Throughput Fluorescence Polarization Screening Assays Using the IsoCyte™ Laser Scanning Platform

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Abstract

Fluorescence polarization (FP) assays are used in high-throughput screening environments for monitoring the activity or inhibition of kinases, phosphatases, nuclear receptors and for measuring many other binding events. In a standard FP assay single binding events are monitored via change of one fluorophore's emission's polarization. Extension to multiplexed FP assays in a high-throughput format is of great interest to the screening community. The IsoCyte™ laser scanning platform (Molecular Devices, Inc.) is a microplate imaging instrument ideally suited for screening of many cell-based assays with the added capability of measuring FP in homogeneous solution, cell and bead-based assay formats. The IsoCyte can simultaneously detect polarization at two separate wavelengths, enabling multiplexing of two fluorophores in a single well. Data will be presented showing FP assay results in glass and polystyrene clear-bottom plates in 384 and 1536 well densities. Commercially available polarization assays were successfully run in both the competitive and direct formats and with green and orange emitting fluorophores. The effect of reducing the IsoCyte sampling rate, and by correlation the scan time, was studied. It was shown that a scan requiring 2.5 minutes and a scan requiring only 30 seconds (regardless of plate well density) yielded results that were similar to each other in dynamic range, signal to noise, and IC_{50}. Polarization assay data acquired on the IsoCyte was shown to be similar to data acquired on other benchtop FP instruments.

Introduction

Molecular Devices has developed a high throughput laser scanning platform, the IsoCyte™, that can also perform anisotropy or fluorescence polarization (FP) measurements in solu tions, cells or bead-based systems. The ability to perform FP measurements is a standard feature in both the hardware and software. Here we report on our validation of IsoCyte for FP using model systems and commercially-available reagent kits. The IsoCyte provides extension to higher-density formats (1536 wells) and 2 color FP while maintaining a rapid read speed (1 min/plate).

Materials and Methods

Reagents. A model system was used to measure the binding of streptavidin to Alexa Fluor® 488 biocytin or Alex Fluor® 546 biocytin from Molecular Probes (**Figure 1**). To prevent non-specific binding, the protein bovine gamma globulin was used at a concentration of 0.1% in all assay buffers. Calibration curves were run using the TKXtra Tyrosine Kinase Kit from Molecular Devices Corp. Final volumes in each well were 40 µL in 384 well plates and 6 µL in 1536 well plates.

Microplates. Black-walled, glass-bottom 384 and 1536 well microplates from Matrical were used for the majority of the assays. Black-walled, clear-bottom 384 well Greiner polystyrene plates were also validated for the FP application.

Detection Instrumentation. All assay plates were read on IsoCyte™ laser scanning platform and for comparison to a well-established fluorescence polarization instrument, Analyst GT Multi-mode reader from Molecular Devices was used. The IsoCyte™ used for these experiments utilized a 488 nm excitation laser and the following band pass filters: Channel 1 and Ch2: 510-540 nm (green) and Ch3 and Ch4: 560-610 nm (red). The image acquisition was done at 20 x 10 micron sampling unless otherwise noted.

Analysis. Signal from the center of each well was averaged and background corrected using buffer wells. Anisotropy values were calculated from parallel and perpendicular signals using a row-specific G factor. G factor was determined using calibration wells containing unbound tracer. For the purposes of direct comparison to other microplate readers, the anisotropy values (units=r) have been converted to polarization (units=mP) using the equation mP=1500*r.

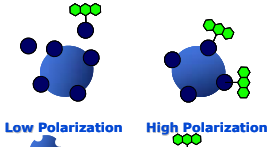

Figure 1. Streptavidin binds to either labeled or unlabeled biocytin If the labeled biocytin is bound, the polarization value is high. If unbound, due to competition with unlabeled biocytin, the polarization value is low.

Results

Effect of Read Speed/Scan Resolution

A 384 well microplate was scanned at different resolutions and the scan times were measured. In the first experiment, a model system for fluorescence polarization (FP) binding was used. Biocytin labeled with Alexa Fluor 488 was bound to streptavidin to yield a high polarization value in all wells. Data was analyzed for uniformity of signal and expected polarization value. Results in **Table 1** show similar signal to background ratios and mean mP values with a slight increase in CV at lower resolution. Note that data acquisition with a scan resolution of 40 um takes 4 times less than at 10 um resolution.

In another experiment the effect of scan resolution was determined in a 384 well plate using a commercially available fluorescence polarization kit for measuring tyrosine kinase activity. A competitive calibration curve was set up following the kit's package insert. The results in **Figure 2** show comparable calibration curves regardless of scan resolution or read time.

Scan Resolution	Parallel Signal: Bkd	Perp Signal: Bkd	mean (mP)	Std. Dev. (mP)	Plate Read Times (min:sec)
10 um	7.5	10.1	129	11	1:57
20 um	7.6	10.5	129	12	1:00
40 um	7.5	10.4	123	13	0:31

Table 1. A 31 second scan time yielded comparable data to a 2 minute scan time. Standard 384 well plate read times for other commercial FP readers range from 1 ½ to 2 ½ minutes.

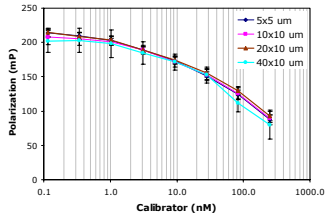

Figure 2. A comparison of plate data acquired at different scan resolutions shows similar signal/background ratio, curve shape, and delta mP although the IC_{50} and z factor were slightly affected at a 40 um scan resolution (30 sec read speed). Data shown is an average of 4 wells.

Green vs. Red emission

The same streptavidin/biocytin model system was used to show performance with the red-shifted Alexa Fluor 546 and the ability of the IsoCyte to simultaneously measure two color FP. Each well contained a constant concentration of labeled biocytin and streptavidin but with varying levels of unlabeled biocytin to show a competition curve. **Figure 3** shows a comparison between the green and orange labeled biocytin.

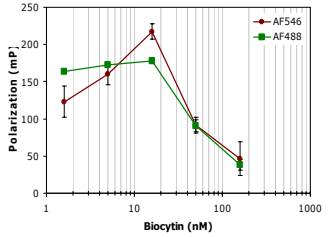

Figure 3. The signal to background (wells containing 5 nM labeled biocytin divided by wells with only buffer) was 26 for AF488 and 10 for AF546. The polarization values were quite comparable and the IC_{50} for biocytin was 50 nM using AF488 and 48 nM using AF546. Each data point represents 4 wells.

Results – cont'd

Comparison of Microplates

Glass-bottom vs. Polystyrene. The uniformity of polarization measurements in 384 well glass-bottom plates was compared to traditional polystyrene plates. One column of the plate contained only buffer (background), one contained unbound AF 488 (for the purposes of calculating a G factor), and the remaining 22 columns contained AF 488 bound to streptavidin. The results are illustrated in **Figure 4**.

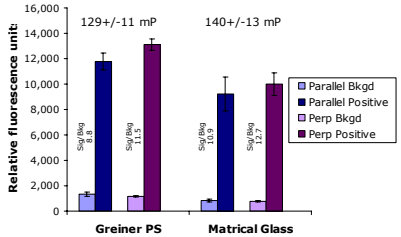

Figure 4. At the same PMT gain settings, polystyrene plates showed higher signal in both background and positive sample wells. The signal-to-background ratio, however, was slightly larger in glass-bottom plates. It was also found that glass plates give higher polarization values than polystyrene.

Plate well density. A tyrosine kinase calibration curve was set up in 384 and 1536 well glass-bottom microplates. Total well volume was 40 µL and 6 µL respectively. Results of plates pipetted by hand and run on two separate days are shown in **Figure 5**. The two calibration curves, while offset from each other, have the same shape and yield similar IC_{50}s.

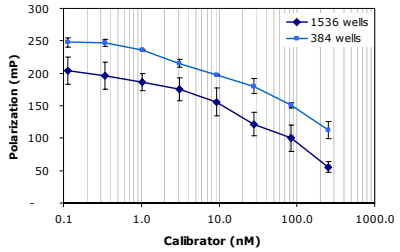

Figure 5. The mean signal to background ratio was 10 in the 384 well plate and 14 in the 1536 well plate. Both plates were scanned in under 1 minute. Data shown is an average of 4 wells.

Results – cont'd

Performance compared to a benchmark reader

The FP performance of the IsoCyte laser scanning platform was compared to that of a high-throughput screening industry-standard fluorescence polarization instrument, Analyst GT from Molecular Devices Corp. Clear-bottom glass 384 well plates were read using both instruments with optimum optical settings. Analyst reads plates from the top, while IsoCyte reads through the glass bottom. A tyrosine kinase calibration curve was used for the evaluation. A graph of summarized data is shown in **Figure 6**.

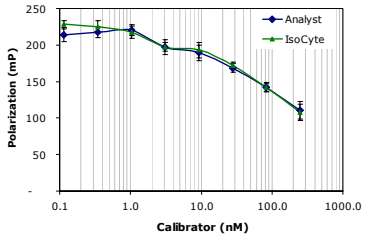

Figure 6. The results of a 384 well plate directly compared on the two instruments showed the same dynamic range (delta mP of 167 vs. 172), IC_{50} (100 vs. 102 nM), and signal to noise ratios (z=0.70 vs. 0.69) listing Analyst and IsoCyte results respectively. Data shown is an average of 4 wells.

Summary

In this report we have shown the suitability of the IsoCyte™ laser scanning platform for reading fluorescence polarization assays.

- Evaluation was completed in 384 and 1536 well microplates, with both glass and polystyrene bottoms, at varying scan speeds, and validated against another fluorescence polarization instrument.
- Superior performance was found in glass-bottom plates but polystyrene plates also yielded acceptable results.
- Valid polarization results were obtained in volumes as low as 6 µL/well.
- Since data is acquired and processed simultaneously, results can be obtained in under 1 minute for any plate density.
- IsoCyte in the standard instrument configuration can be used for determination of FP with two different fluorophores simultaneously.
- The platform is available with an integrated plate handler (Twister II) for walk-away operation (up to 160 plates).

Molecular Devices, Inc. invites you to contact us to discuss your specific applications for the IsoCyte™ platform.