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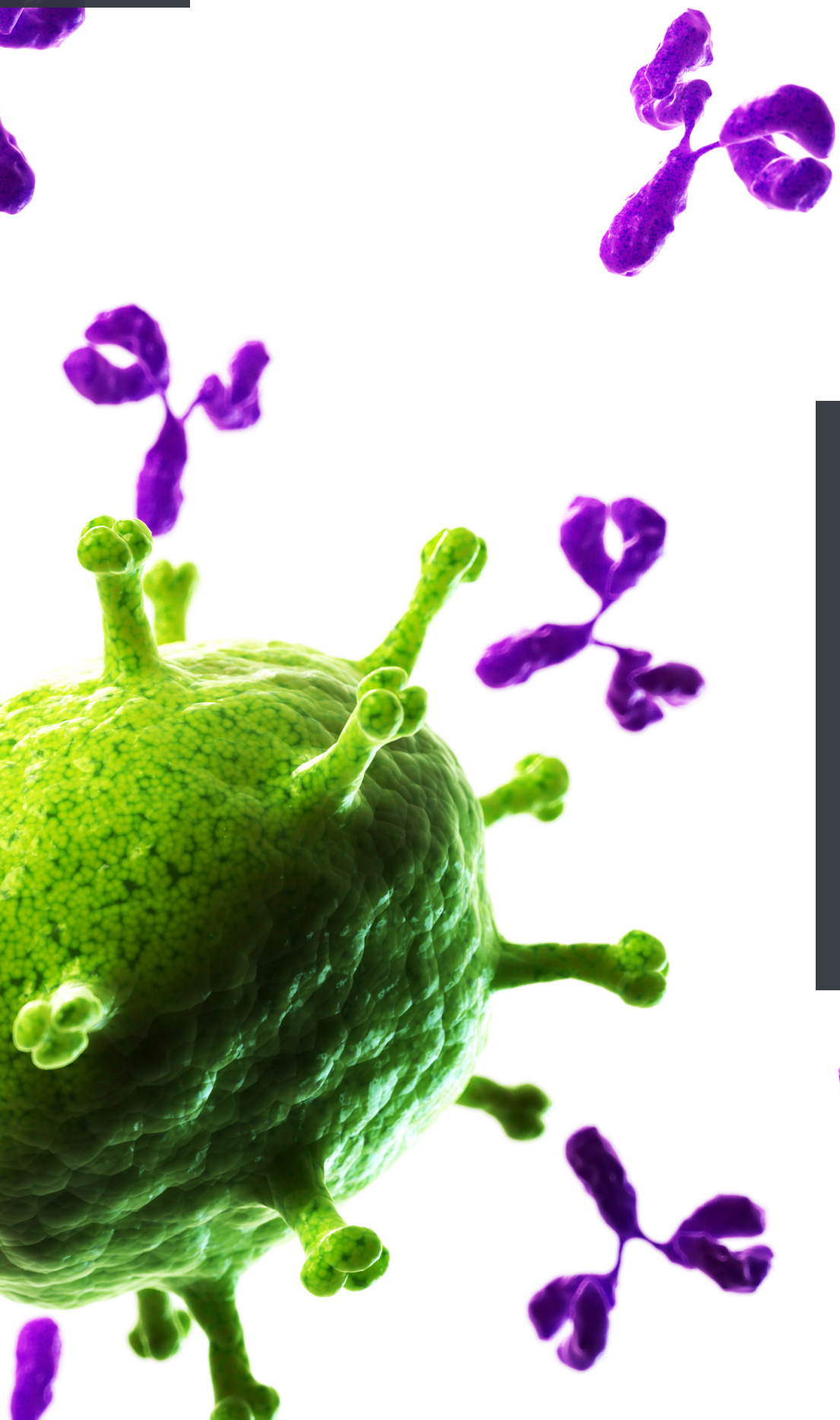

Featuring

Exploration of SYTO® 9 Variabilities of Labeling Live Bacterial Cells and Analysis of MycoLight™ Fluorophore Alternatives for Live Bacterial Labeling and Viability Assessment

RATIOWORKS™ pH 165, AM: A Dual Excitation/Emission Fluorescence Probe For Imaging Live Cells

Therapeutic Monoclonal Antibody Fluorescent Labeling Tools for Imaging and Flow Cytometry

Transfectamine™ 5000: an efficient and reliable DNA delivery system

AAT Bioquest®

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Exploration of SYTO® 9 Variabilities of Labeling

Live Bacterial Cells and Analysis of MycoLight™ Fluorophore Alternatives for Live Bacterial Labeling and Viability Assessment

Abstract

The proprietary cell-permeable nucleic acid probe SYTO® 9 is commonly used in microbiology as either a standalone dye or in combination with propidium iodide (PI) in bacterial viability assays. The usefulness of SYTO® 9 in general overcomes the negative aspects such as high cytotoxicity, but cell-permeable fluorophores such as MycoLight™ Green JJ98 and MycoLight™ Red JJ94 provide alternatives with equivalent sensitivity and fewer downsides. MycoLight™ Green JJ98 in particular shows near-ideal results in combination with PI in bacterial viability assays, with highly similar spectra to SYTO® 9 and slightly improved performance with common instrumentation settings of both flow cytometers and fluorescence microscopes.

Introduction

The small size of bacterial cells (typically 1 µm wide and up to 5 µm in length)[1] is a major factor contributing to the difficulty of fluorescent imaging of living bacterial cultures. This small size means that the genetic modification of bacteria to express GFP (a traditional method of labeling going back decades) can be difficult to impossible due to the size of GFP (25-30 kDa) and its derivatives[1]. Significantly smaller fluorescent probes such as MycoLight™ Green JJ98 or MycoLight™ Red JJ94, with molecular weights of ~.579 kDa and ~.739 kDa, respectively, are a more sizeable option. This vastly decreased size minimizes proteomic interference and damage to cell behavior. Additionally, the ease of labeling and minimized trauma of the simplified protocol allows wider applications to different bacterial strains, as many species are not suitable for genetic manipulation[3].

Many popular nucleic acid (NA) stains including SYTO® 9 (SYTO® 9 is the trademark of Invitrogen™) have an inhibitory effect on cell growth and viability. For endpoint assays, this is an insignificant drawback. However, this common label perturbs cellular processes, skewing results in live cell experiments. Although new bacterial imaging procedures are being continually introduced, such as live cell kinetic imaging and nanoparticle usage, fluorescence remains the dominant methodology in current bacteriological studies[1]. A growing trend within bacteriology is the usage of flow cytometry. As the instrumentation has become more affordable, the efficiency of the procedure has lent itself perfectly to the need for fast, hyper-accurate assays. Other factors include bleaching, variation in fluorescence intensity (binding affinity) in live vs. dead bacterial cells, variations in staining in gram-positive or gram-negative bacteria, and double-staining due to the compound binding to extracellular nucleic acids (eNA), can either increase background signal or obscure accurate readings when attempting multicolor imaging.

The use of cell-permeable NA probes such as SYTO® 9 in conjunction with membrane-impermeable probes such as propidium iodide (PI) is a widely used method in microbiology for testing bacterial cell viability[4]. Ideally, living bacteria will only take in the cell-permeable stains but not the impermeable

one, whereas membrane-compromised dead bacteria will be readily accessible to both stains. Fluorescent imaging will then show clear differentiation of living and dead cells. In practice, this technique has three main issues, often due to limitations of the commonly used cell-permeable fluorophore.

PI Is Known to Label Both Living and Dead Adherent Bacterial Cells

In adherent cells (such as biofilms), bacterial viability has been shown[5] to be severely underestimated when assessed by these tests, due to a subpopulation of double-stained cells. Gram-negative E. coli have been shown to fluoresce 96% PI-positive, though 82% of cells were successfully cultivated after imaging, indicating that they were alive and viable. A common gram-positive species, Staphylococcus epidermidis, fluoresced 76% PI-positive, with a full 89% of those cells being subsequently cultivable. Extracellular nucleic acids are often present in adherent colonies and will readily be stained by impermeable probes, forming a false coating-indicating 'dead' cells-surrounding viable bacterial cells[5]. These skewed results may still provide rough information or demonstrate comparative trends when combined by validation via an alternative method, but the additional time and expense of doing so means that the initial, potentially significantly incorrect assessment is accepted as-is.

SYTO® 9 Has Different Binding Affinities for Live and Dead Cells in Gram-Positive and Gram-Negative Bacteria

Gram-positive bacteria such as Staphylococcus aureus show ideal equal signal intensity when staining live and dead cells. However, gram-negative bacteria such as Pseudomonas aeruginosa show 18x higher signal in dead cells over living ones. Even after PI counterstaining, the SYTO® 9 signal for dead cells is still markedly higher than for living bacteria[6]. This variation not only needs to be taken into account in regards to interpretation of viability assay results, but also for counterstain selection.

SYTO® 9 Suffers Severe Bleaching and Diminishing Signal

SYTO® 9 was observed to suffer near-immediate bleaching effects when observed using a laser confocal microscope and fluorescence microscope[6]. For testing small numbers of cells, photobleaching is not as much of a detractor, but for extensive imaging, for high-volume viability assessments, and for ease of use for the end-user, a wider imaging window without diminishing signal is of high importance.

To investigate these variabilities and others, comparisons of MycoLight™ Red JJ94 and SYTO® 9 effects on bacterial growth against a negative control were run. The effectiveness of a bacterial viability kit using MycoLight™ Green JJ98 and PI was tested with each of the bacterial cultures referenced above as well as others. Photostability and brightness of all of the MycoLight™ dyes were explored, and a full spectral comparison of MycoLight™ Green JJ98, SYTO® 9, and PI was made, including peak emission intensities at the popular excitation laser wavelength of 488 nm.

Materials and Methods

As with other NA probes, including SYTO® 9, the MycoLight™ dyes have minimal intrinsic fluorescence, which increases exponentially when bound to nucleic acids. MycoLight™ Red JJ94 preferentially labels gram-positive bacteria. MycoLight™ Green JJ98 labels both gram-positive and gram-negative bacteria. For NA probes, avoid using phosphate-containing buffers, as they hinder accurate measurement, due to the DNA phosphate backbone. This is particularly relevant when investigating bacterial cells, due to the variable nature of the DNA structure, such as the phosphorothioate modification, where a phosphate oxygen atom is replaced by a sulfur atom[7]. HEPES or other compatible buffers are recommended.

MycoLight™ Red JJ94 and SYTO® 9 Bacterial Cytotoxicity Comparison Methodology

Three healthy, logarithmically growing cultures of E. coli were supplemented with either MycoLight™ Red JJ94 or SYTO® 9 at a concentration of 2.5 µM for 10 minutes, or with a control of 1% DMSO. Both fluorophores use DMSO as a base solvent, so the low dose of DMSO was evaluated to be a fair control. Although DMSO can have a toxic effect, at 1% concentration the bacterial inhibition is minimal3. To measure changes in bacterial

populations, optical density (OD) readings at 600 nm were taken using a spectrophotometer over a period of 7 hours. Higher readings demonstrate higher populations of bacteria indicating growth.

Bacterial Viability Kit Using MycoLight™ Green JJ98 Effectiveness Testing Protocol

A logarithmically growing culture of E. coli HST08 was diluted to an average density of ~107 cells/mL in a 0.85% NaCl solution and then divided equally. One culture was left untouched and served as a positive control of 100% living bacterial population. The other culture was treated with ethanol until the population was composed of 70% dead bacteria to serve as a comparison. One third of each of these cultures were removed and combined into a 3rd colony to represent a mixed population. MycoLight Green™ JJ98 and PI were mixed in equal volumes and added to the cultures at a ratio of 1:250 of dye to bacteria (approximately 4 µL of dye per 1 mL of bacterial solution). The solutions were mixed thoroughly and then incubated in the dark at room temperature for 15 minutes before imaging.

Fluorescent Imaging of MycoLight™ Green JJ98 and MycoLight™ Red JJ94 in Live Bacterial Cells

MycoLight™ Green JJ98 was added to a culture of logarithmically growing E. coli at a concentration of 5 µM, vortexed to mix, and then incubated in darkness for 30 minutes prior to imaging. MycoLight™ Red JJ94 was added to a logarithmically growing culture of *Rhodococcus qingshengii* at a concentration of 2.5 µM, vortexed to mix, and then incubated in darkness for 20 minutes prior to imaging.

Spectrum Comparison of MycoLight™ Green JJ98, SYTO® 9 and Propidium Iodide

The spectrum comparison of the excitation and emission curves of each dye, along with intensity calculations and added excitation sources and filters was generated using the AAT Bioquest Fluorescence Spectrum Viewer tool.

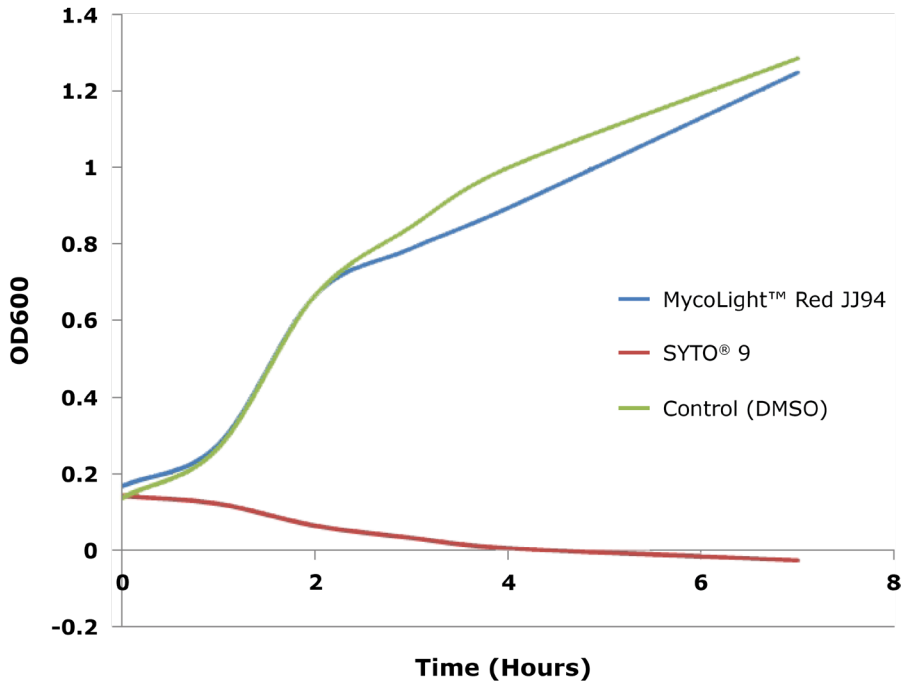

Figure 1. Optical density (OD600 nm) of E. coli LB cultures supplemented with 1% DMSO (control), MycoLight™ Red JJ94 or SYTO® 9 over time. SYTO® 9 inhibits bacterial growth substantially while MycoLight™ Red JJ94 is fully compatible with normal bacterial growth.

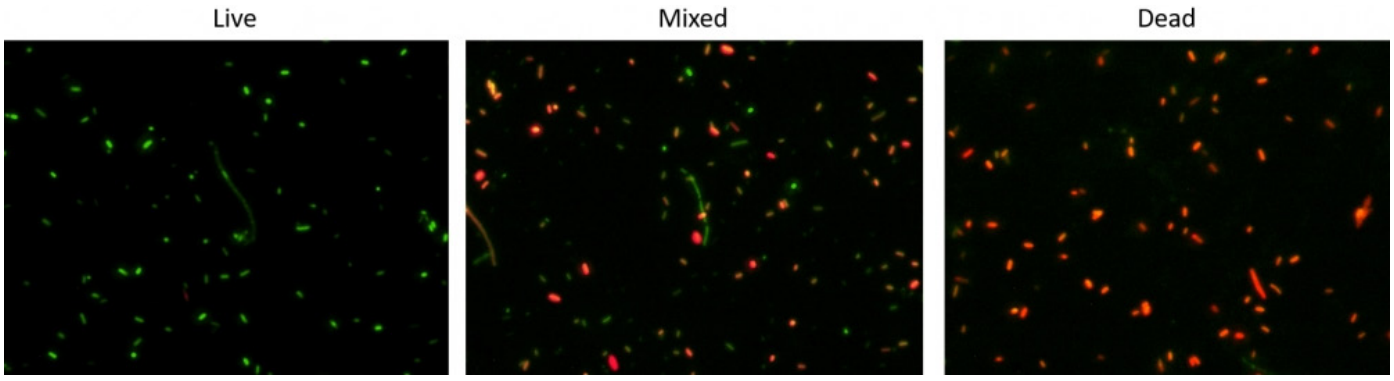

Figure 2. Fluorescence images of E. coli HST08 stained with MycoLight™ Bacterial Viability Assay Kit. Live bacteria with intact cell membranes showed green fluorescence (Left), while 70% alcohol-killed dead bacteria (Right) with compromised membranes showed red fluorescence. Live and dead E. coli bacterial cells were also visualized in a mixed population (Middle). Images were taken using a Keyence fluorescence microscope equipped with FITC/**Texas Red filter sets.**

Results and Discussion

The aggregated results of the four areas of comparison and effectiveness of the MycoLight™ fluorophores are shown and discussed in the same order as they were listed in the methods section above.

MycoLight™ Red JJ94 and SYTO® 9 Bacterial Cytotoxicity Comparison

As shown in Figure 1, SYTO® 9 is toxic to cells over less than half of the experimental time, whereas MycoLight™ Red JJ94 showed very minor variation from the control population. MycoLight™ Red JJ94's compatibility with bacterial growth not only allows further characterization of bacteria, but allows for normal cellular behavior. Particularly in experiments investigating antibiotics and changes in activity over time, this compatibility is essential.

Bacterial Viability Kit Effectiveness Using MycoLight™ Green JJ98

The results of the bacterial viability tests using the 3 E. coli colonies were precisely indicative of the known ratios of live and dead bacterial cells, indicating that the potential problems of PI double-labeling were of minimal concern. By employing MycoLight™ Green JJ98 instead of SYTO® 9 as the chosen cell-permeable NA probe, the problems of conflicting fluorescence with PI were minimized.

Fluorescent Images of MycoLight™ Green JJ98 and MycoLight™ Red JJ94 in Live Bacterial Cells

The sensitivity of MycoLight™ Green JJ98 is shown well in Figure 3, with the clear outlines of each bacterial cell indicating a good ratio of signal from background noise. Sensitive dyes are useful for quantifying samples which may be low-density, and the brightness is useful for applications which may require more elaborate adjustments and difficult cell lines requiring very low dye concentrations.

Gram-positive *R. qingshengii* was shown with a good signal-to-noise ratio when labeled by MycoLight™ Red JJ94. Other bacterial strains may require dye-loading protocol adjustments, but MycoLight™ Red JJ94 was compatible with all of the strains tested, with minor alterations based on instrumentation, cell density, and other factors.

Spectrum Comparison of MycoLight™ Green JJ98, SYTO® 9 and PI

Excitation laser at 488 nm included, as well as the common Green Channel filter as referenced by Stocks et al.[8] in their paper on spectral overlap and DNA binding affinities of SYTO® 9 and PI. Excitation curves are shown as dotted lines and emission curves are shown as solid lines filled in with each fluorophore color.

Demonstrating a mild improvement of 4% fluorescence intensity, MycoLight™ Green JJ98 more importantly is more thoroughly within the wavelength range of the green filter

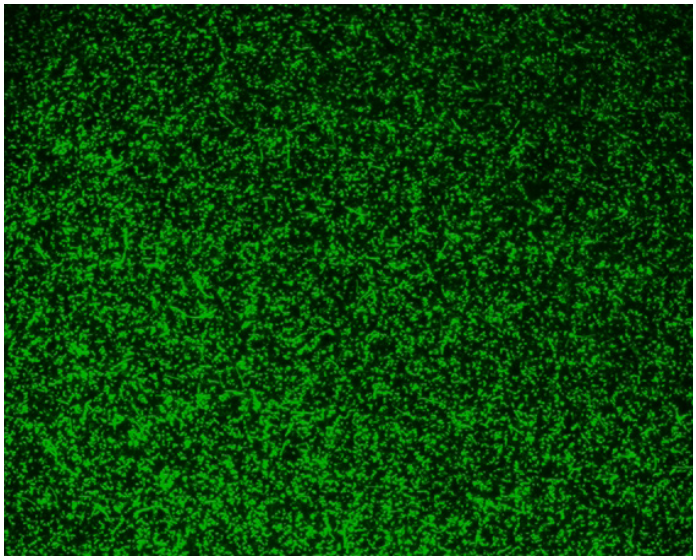

Figure 3. E. coli were stained with 5 μM of MycoLight™ Green JJ98 for 30 minutes and imaged with FITC channel with a Keyence fluorescence microscope at 20x.

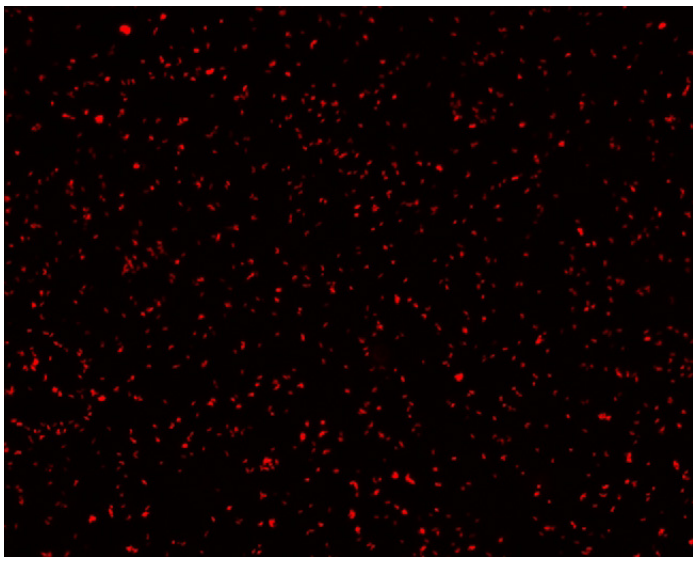

Figure 4. Rhodococcus qingshengii was stained with 2.5 μM of MycoLight™ Red JJ94 for 20 minutes. Image was taken by a Keyence fluorescence microscope with a Cy5 filter set.

commonly installed on flow cytometers and fluorescence microscopes. This ~10% improvement (51% versus SYTO® 9's 42%) represents a dependable, stable signal even in low-density samples. When MycoLight™ Green JJ98 is used as a standalone probe, this sensitivity allows the end-user to optimize dye concentrations for ideal signal levels. This flexibility allows for easier multiparameter imaging, where targets may be of varying levels of expression and mismatched dye intensities may swamp weaker signals.

Conclusion

SYTO® 9 is useful for applications as an NA probe in pure bacterial cultures and samples with low density (Applied and Environmental Microbiology citation, Sensitive determination), but its cytotoxicity, variable binding affinities for differing bacterial groups, and high bleaching necessitate the use of alternative cell-permeable fluorophores for bacterial viability assays and other experiments with live bacterial cells. For flow cytometers or fluorescence microscopes, MycoLight™ Green JJ98 is a useful alternative fluorophore for bacterial viability testing in combination with traditional PI counterstaining. MycoLight™ Green JJ98's improved excitation by the 488 laser and resistance to bleaching allow for better and easier imaging with a wider useful visualization window. For flow cytometry especially, steady signal is crucial for generating dependable counts. Bacterial viability tests using PI and permeable NA probes are the workhorse of microbiology, and an updated version that doesn't require extensive validation are key to preserving the efficacy of the test. For customized tests and experiments with gram-positive bacterial species such as Staphylococcus epidermidis referenced earlier in this paper, MycoLight™ Red JJ94 can be incubated long-term with no negative effects on bacterial growth, and generates excellent signal. Both of these aspects minimize hands-on time, and the red color emission spectrum allows pairing with common green fluorescent probes for multiparameter imaging.

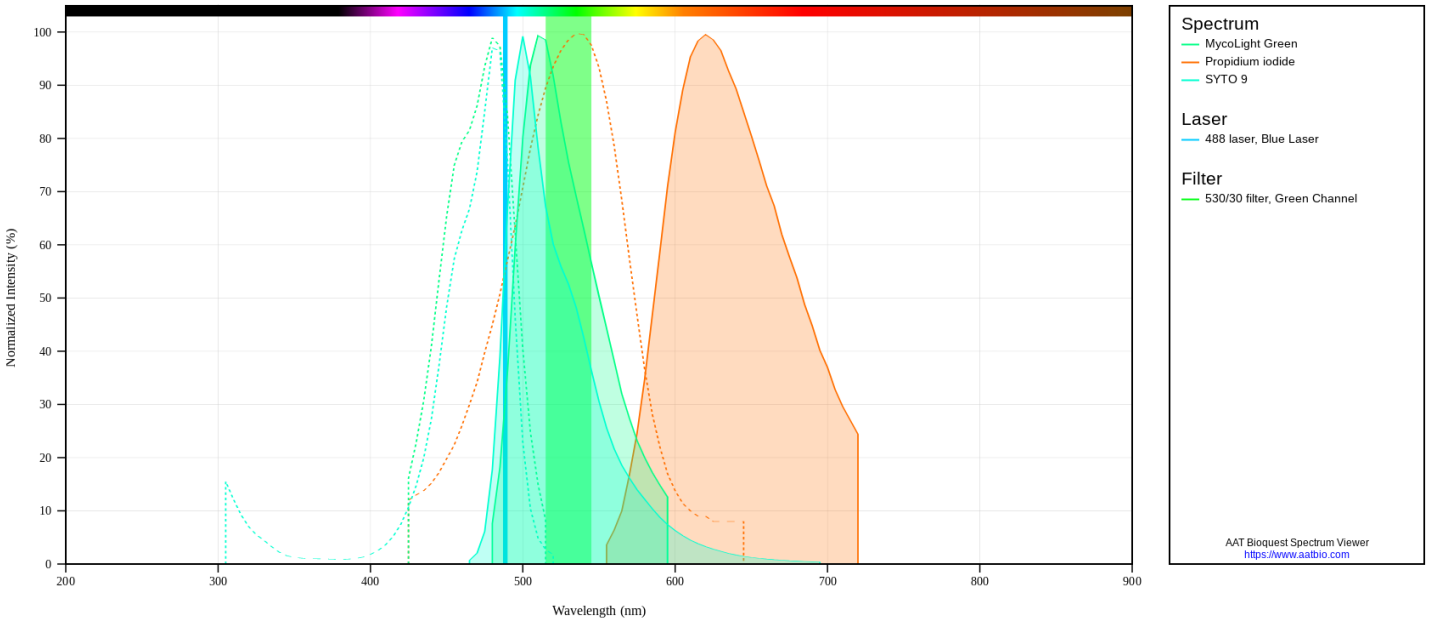

Figure 5. Excitation and emission curves of MycoLight™ Green JJ98, SYTO® 9, and PI.

Table 1. Peak intensity percentage comparisons of fluorophores.

Fluorophore	Type	Peak (nm)	Peak Intensity (Ex max)	Peak Intensity (488 nmlaser)	Spillover (530/30 filter)
Propidium Iodide	excitation	537			
Propidium Iodide	emission	618	100%	55%	0%
MycoLight™ Green JJ98	excitation	482			
MycoLight™ Green JJ98	emission	512	100%	91%	51%
SYTO® 9	excitation	483			
SYTO® 9	emission	500	100%	87%	42%

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Product	Unit Size	Cat No.
MycoLight™ Green JJ98	100 µg	23998
MycoLight™ Green JJ98	1 mg	23999
MycoLight™ Green JJ98 *5 mM in DMSO*	100 µL	24000
MycoLight™ Green JJ99 *5 mM in DMSO*	100 µL	24001
MycoLight™ Red JJ94 *2.5 mM in DMSO*	100 µL	24006
MycoLight™ Ratiometric Bacterial Membrane Potential Kit *Red/Green Fluorescence*	200 Tests	22401
MycoLight™ Rapid Fluorescence Gram-Positive Bacteria Staining Kit	100 Tests	22415
MycoLight™ Rapid Fluorescence Bacterial Gram Stain Kit	100 Tests	22413
MycoLight™ Live Bacteria Fluorescence Imaging Kit	100 Tests	22409
MycoLight™ Fluorescence Live/Dead Bacterial Imaging Kit	100 Tests	22411
MycoLight™ Flow Cytometric Live Bacteria Assay Kit	100 Tests	22407
MycoLight™ Bacterial Viability Assay Kit	200 Tests	22400
MycoLight™ Fluorimetric CTC Live Bacteria Quantification Kit	100 Tests	22405

RATIOWORKS™ pH 165, AM

A Dual Excitation/Emission Fluorescence Probe For Imaging Live Cells

Abstract

A novel dual excitation/emission fluorescent probe has been developed for specific and sensitive determination of intracellular pH in cellular processes. The present study explores the effectiveness of RatioWorks™ pH 165, AM as a pH-sensitive fluorescent indicator by analyzing the spectral characteristics in HeLa cell lines with pH measurements using a fluorescence microplate reader. Standard buffers in the range of pH 4-10 were used in analyzing the excitation/emission spectrum of the dye. The pH measurements were determined using the ratio of the emission and excitation intensities of the dye at dual wavelengths (Ex/Em: 497nm/594nm and 578nm/654nm, respectively). Cell cytotoxicity was evaluated using CCK-8 assay at an incubation period of 120 min post WST-8 addition. The fluorescence response of RatioWorks™ pH 165, AM obtained with Cy3/TRITC and Cy5 filters at different pH regions provide evidence for accurate pH measurements of intracellular organelles as well as low cytotoxic nature of the probe. Furthermore, in contrast to other fluorescein derivatives, RatioWorks™ pH 165, AM exhibits response in the red and far red spectrum, making the probe an ideal dual excitation/emission fluorescence pH sensor for multiplex immunoassay technologies involving GFP. RatioWorks™ pH 165, AM proves to be a competitive pH indicator among other fluorescent-based technologies.

Introduction

Fluorescent pH indicators are of pivotal relevance in measuring changes to intracellular proton concentrations. These are selected based on their pKa values and similarity to the pH of the investigated system. Under physiological conditions, the pH of intracellular fluid ranges between 6.8 and 7.4, in which case a fluorescent probe with a pKa around 7 is required to make quantitative pH measurements. However, this neutral pH range is not characteristic of all cellular components, as some organelles like lysosomes function optimally only under acidic conditions. A variation in pH will affect the different resonance forms of the dye, altering the stability of the excited state and consequently, the associated spectrum shifts of the species.

Fluorescent probes can be sophisticated sensors, but the diffusion properties and specificity of each probe vary profoundly with the structural characteristics of the associated fluorescent molecules. Several techniques have been developed for pH measurements in cells, including partitioning of weak acids and bases, pH selective microelectrodes, nuclear magnetic resonance, etc. Nevertheless, pH sensitive fluorescent probes continue to remain a powerful technique with their increased sensitivity and greater sampling capability when compared to microelectrodes and other fluorescence-based assays for quantifying intracellular pH in cellular processes. The most frequently used indicators for near neutral pH conditions are fluorescein and fluorescein derivatives, like BCECF AM, BCFL AM, and SNARF, SE. Despite the advantages of these pH indicators,

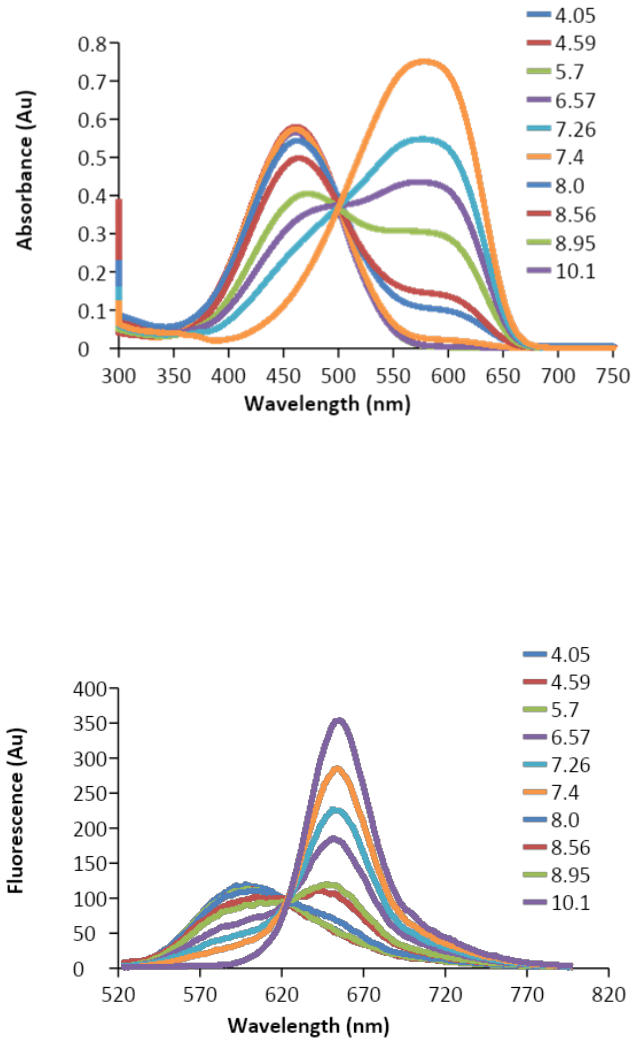

Figure 1. Optical responses of RatioWorks™ pH 165 (15 μM) at various pH values with buffers in DMSO. (Left) Absorbance Spectra (right) Emission Spectra.

there still exist limitations in their emission spectra that result in compromised image resolutions, rendering them ineffective for certain multiplex immunoassay techniques with GFP. In an attempt to overcome these shortcomings, AAT Bioquest has developed a novel pH sensitive fluorescent probe, RatioWorks™ pH 165, AM which not only reflects all the advantages of the fluorescein based pH indicators listed above, having essentially identical basic structural characteristics, but also addresses the issue of compromised image resolution by providing well-resolved dual emissions at two discrete wavelengths.

RatioWorks™ pH 165, AM is an indispensable tool for intracellular pH measurements in living organisms. The emission spectrum of the probe undergoes a pH-dependent wavelength shift, making it a long wavelength fluorescent probe. This allows for the ratio of the fluorescence intensities from the dye at two emission wavelengths to be used for quantitative pH measurements in near-neutral regions. The indicator operates by exciting the dye at two regions (497 nm and 578 nm), while also monitoring the fluorescence emission at dual wavelengths (594 nm and 654 nm). At low pH values, RatioWorks™ pH 165, AM is weakly fluorescent in the far red spectrum and highly fluorescent in the red spectrum. As pH increases, it demonstrates higher fluorescence in the far red spectrum and lower fluorescence in the red spectrum. Attributing to its cell permeability (pKa ~ 7.1), RatioWorks™ pH 165, AM is particularly well suited to quantifying cytosolic pH between 4-9 and is compatible with instrumentation platforms involving any visible-light fixed-wavelength excitation source, such as confocal laser scanning microscopes, flow cytometers, and fluorescence microplate readers. The dual excitation/emission property along with its ratiometric analysis tool makes RatioWorks™ pH 165, AM useful not only for studies requiring accurate pH determinations in cells, but also those employing multiplex immunoassay technologies where background fluorescence can be introduced at shorter wavelengths.

The present study aims to explore the development of RatioWorks™ pH 165, AM, a proprietary fluorescent pH sensor from AAT Bioquest for near-neutral pH conditions in intracellular organelles. The role of RatioWorks™ pH 165, AM as an effective pH indicator is evaluated by analyzing spectral characteristics in

HeLa cell lines through pHi measurements with a fluorescence microplate reader. Based on the fluorescence response obtained with Cy3/TRITC and Cy5 filters at different pH regions, it is evident that RatioWorks™ pH 165, AM is an ideal dual excitation/emission fluorescence pH sensor for quantitative pH measurements in intracellular processes. The probe proves to be a competitive pH indicator among other fluorescent-based technologies used in bioassays and applications of these assays for food safety, quality, and efficacy.

Materials and Methods

Materials

For the preparation of dye solutions the following buffers were used: Component A (pH 4.5), Component B (pH 5.0), Component C (pH 5.5), Component D (pH 6.0), Component E (pH 6.5), Component F (pH 7.0), Component G (pH 7.5), Component H (pH 8.0), Component I (Nigericin free acid), Component J (DMSO). All the chemicals were of spectroscopic grade quality and were used as received without further purification.

Instrumentation

UV-VIS absorption spectra were recorded on a Shimadzu UV-1800 spectrometer. Fluorescence emission spectra were performed on a Varian Cary-Eclipse spectrofluorometer. The fluorescence images of cells were obtained using a Keyence BZ series fluorescence microscope with a Cy3/TRITC-Cy5 filter sets and cells plated in black wall/clear bottom plate. The pH values were measured with a Denver Instruments UB-10 pH meter using a combined glass-calomel electrode. Key parameters for the fluorescence microplate reader (FlexStation 3) include bottom read mode with black wall/clear bottom plate. Signals were examined in the excitation and emission wavelength of 497-578 nm and 594-654 nm, respectively, with a cutoff wavelength between 570-630 nm.

Sample Preparation

Stock solutions (1-5 mM) of RatioWorks™ pH 165, AM were prepared with DMSO. Each test solution (20-50 µM) for fluorescence measurement was prepared from the

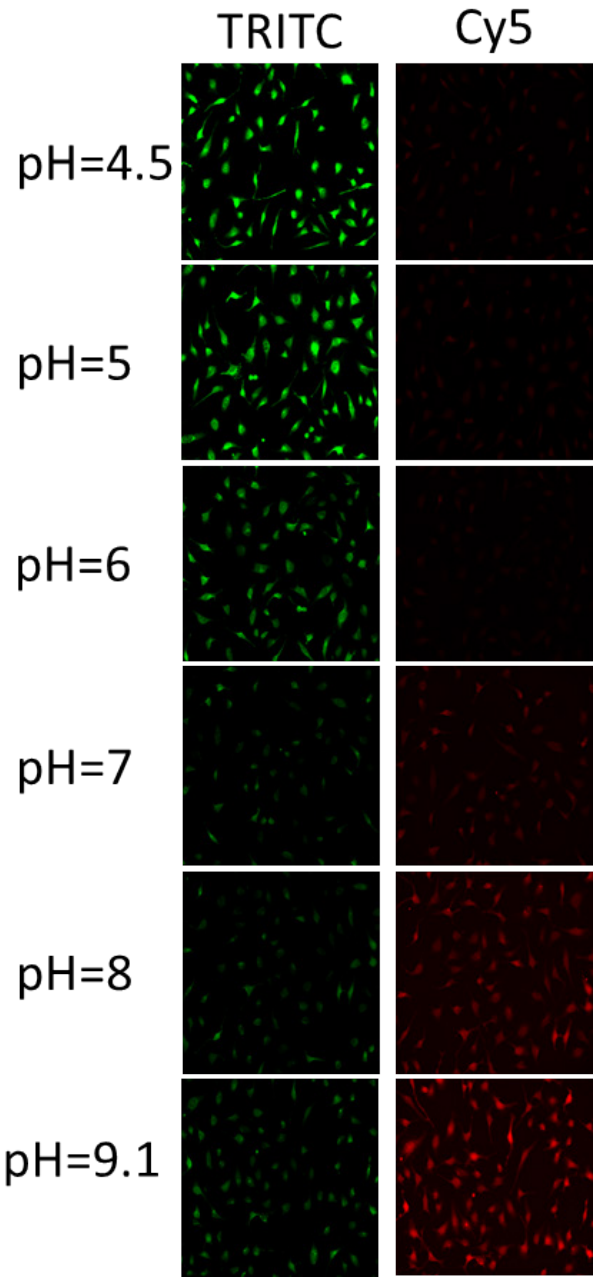

Figure 2. Fluorescence images of live HeLa cells with RatioWorks™ pH 165 at various pH values (4 to 9) with buffers in DMSO. (a) Green channels (Pseudo Color) with Cy3/TRITC filter were collected in 570-640 nm (b) Red channels with Cy5 filter were collected in 662-737 nm.

corresponding stock solution by diluting in 20 mM Hanks and Hepes buffer (HHBS), so that the absorbance of the final solutions at Ex was lower than 0.1. For fluorescence experiments, six dye solutions were prepared in their respective buffers with the following pH values: 4.5, 5, 6, 7, 8, and 9.1.

Cell Culture

HeLa cells were cultured in a DMEM growth medium supplemented with 10% Bovine serum, penicillin (100 µg mL-1), streptomycin (100 µg mL-1) and L-glutamine (2.5 x 10-4 M) at 37°C in a 5:95 CO2-air incubator. The fluorescence images of cells were obtained with a fluorescence microscope. The green emission filter (Pseudo Color), Cy3/TRITC, was used with a range of 570-640 nm, while the red emission filter, Cy5, was in the range of 662-737 nm. The HeLa cells were incubated with RatioWorks™ pH 165, AM dye solutions (10 µL, 120 min). Upon removing the cell culture medium, the cells were washed with phosphate-buffered saline (PBS) and transferred to their respective pH solutions (4.1 to 9.0) with Nigericin (2 µg mL-1). For the purpose of cell imaging and ratiometric analysis, the signal intensity was measured at Ex1/Em1 = 497/594 nm with a cutoff at 570 nm and Ex2/Em2 = 578/654 nm with a corresponding cutoff wavelength of 630 nm. The ratio was calculated by dividing Ex1 over Em1 and Ex2 over Em2.

Cytotoxicity Assay

Cell toxicity was determined using CCK-8 assay. HeLa cell lines were seeded in 96-well microplates in 100 µL of Bovine medium for a stationary culture. After 24 hours of cell attachment, the plates were washed with 100 µL per well PBS. In a fresh medium, different concentrations of RatioWorks™ pH 165 dye solutions (10 µM, 20 µM, 50 µM, and 100 µM) were mixed, added to the cells, and incubated for 6 hours. Cells in a culture medium without fluorescent dyes were used as the control. Subsequently, WST-8™ Solution (10 µL) was added to each well. After 120 min of incubation in a 5% CO2 humidified incubator at 37°C and protected from light, the working solution was pipetted out of the cells, which were then washed and replaced with an HHBS buffer. The fluorescence signal in the cells was observed using a fluorescence microscope with a Cy3/TRITC filter for the acidic pH readings and Cy5 filter for the basic pH readings. The absorbance was determined with a microplate reader at 460 nm. Cell viability was evaluated by monitoring signal intensity at different dye concentrations. The absorbance intensity is directly proportional to the amount of formazan dye produced, which is indicative of the number of living cells in the medium, allowing quantification of cell viability.

Results and Discussion

The pH titration experiments of RatioWorks™ pH 165, AM at 15 µM were performed in various buffer solutions containing 5% DMSO. With a decrease in pH from 10.1 to 4.05, the maximum absorbance at 578 nm was blue shifted to obtain an absorption

Table 1. Ratiometric Analysis of RatioWorks™ pH 165, AM at pH 4.5, 5, 6, 7, 8, and 9.

pH	497/594	578/654	Ratio
4.5	43.7	9.8	4.5
5	41.9	9.3	4.5
6	30.0	7.9	3.8
7	21.7	6.1	3.5
8	19.3	7.5	2.6
9	17.7	10.6	1.7

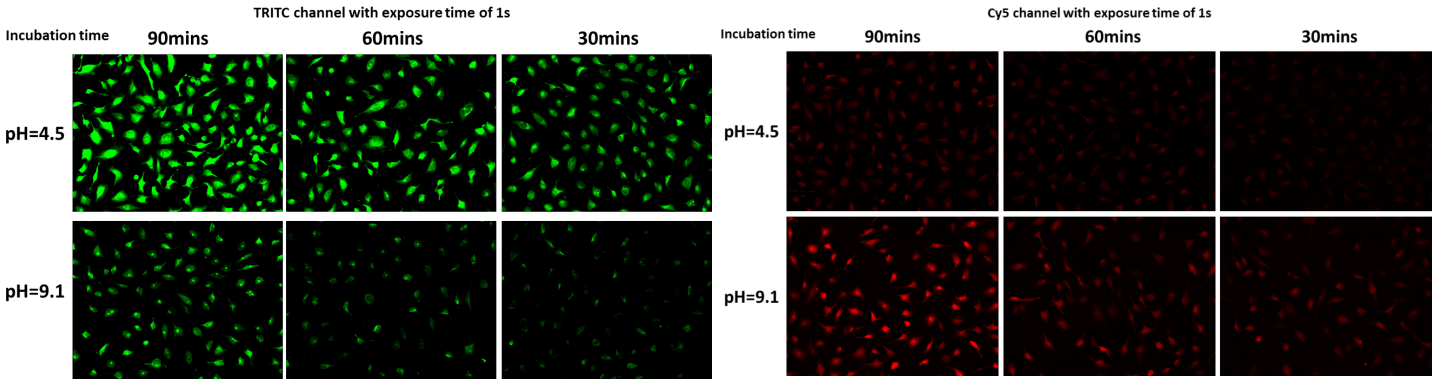

Figure 3. Effect of incubation time (30, 60, and 90 min) on fluorescence images of live HeLa cells with RatioWorks™ pH 165, AM at an exposure time of 1 sec and pH of 4.5 and 9.1, respectively. (a) Green channels (Pseudo Color) with Cy3/TRITC filter were collected in 570-640 nm (b) Red channels with Cy5 filter were collected in 662-737 nm.

peak at 478 nm (Figure 1a). A well-defined isosbestic point at around 497 nm was observed in the absorption spectra. The fluorescence emissions of the dye for pH 10.1 to 4.05 obtained by excitation at this point were recorded. At pH 10.1 solution, the probe exhibits only one emission peak at about 654 nm. With an increase in proton concentration, the fluorescence intensity of the probe at 654 nm was reduced and a new emission band at 594 nm concomitantly emerged (Figure 1b).

The above results provide evidence for how ratiometric fluorescent measurements, which are based on the ratio of two fluorescent bands instead of the absolute intensity of one band, make quantitative analyses more accurate and sensitive by minimizing background signals. The fluorophores display dual emission with a change in pH from basic to acidic conditions. This is caused by a combination of excited state intramolecular proton transfer (ESIPT) and an intramolecular charge transfer (ICT), which allows these fluorophores to demonstrate unique sensitivity to subtle variations in their immediate environment that are independent of the probe concentration and other variables. A pKa value of 6.80 was obtained by analyzing the fluorescence ratios at 594 nm and 654 nm. Further observations include color changes (violet to yellow) under visible light, indicating stable equilibrium states. Lastly, the RatioWorks™ pH 165, AM fluorescent indicator shows large Stokes shifts. This improves detection sensitivity by reducing self-quenching that can cause measurement errors.

Cell imaging was initially performed on HeLa cell lines containing a dye concentration of 5-20 µM incubated at 37°C for 30 min and observed at different pH values (4 to 9) using a fluorescence microscope with Cy3/TRITC and Cy5 filter. As pH in the cells increases, the red fluorescence (Cy5 channel) of the dye goes up, while the green fluorescence (Cy3/TRITC channel) goes down. Although the dye works as intended, high concentration of the dye along with a longer incubation time was recommended for consideration in future experiments.

Consequently, in a follow up study, a higher dye concentration of 35 µM incubated at 37°C for 30 min was analyzed keeping every other parameter constant. The results demonstrate a significant improvement in the fluorescence signal intensity in both channels (Figure 2). To quantitatively determine pH values, ratiometric analysis at the dual excitation and emission wavelengths (Ex/Em = 497/594 nm and 578/654 nm, respectively) was carried out (Table 1). The calculated data was in trend with the results obtained from cell image analysis i.e. the ratio of the intensities tend to diminish with an increase in intracellular pH.

Various incubation time periods (30, 60, and 90 min) were explored to optimize optical properties of RatioWorks™ pH 165, AM (Figure 3a, b). Keeping the dye concentration constant (30-50 µM), the same procedure was applied at pH values 4.5 and 9.1. The results demonstrate an increase in signal intensity in both Cy3/TRITC and Cy5 channels, indicating that longer

incubation time improves image quality.

Cytotoxicity of RatioWorks™ pH 165, AM dye in HeLa cells was studied using CCK-8 assay. Figure 4 shows the toxicity measurements after two hours of cellular internalization of the probe at concentrations of 10 µM, 20 µM, 50 µM, and 100 µM against a control (DMSO) at its respective concentrations. The results indicate low toxicity of the dye up to a concentration of 50 µM where the cell viability remained more than 90% with respect to DMSO. While at a dye concentration of 100 µM, the cell viability was reduced significantly as cells tend to exhibit adverse effects. Based on the findings, it can be concluded that a tolerable dye concentration will go up to 50 µM, based on the cell type and application. The low cytotoxic nature of RatioWorks™ pH 165, AM indicator makes it an ideal tool for handling pH fluctuations in live cell fluorescence imaging.

Conclusions

A novel dual excitation/emission fluorescent pH indicator has been developed by AAT Bioquest for sensitive and selective determination of intracellular pH in cellular processes. RatioWorks™ pH 165, AM displays a distinguished ratiometric fluorescent response with well separated emission bands and a large stokes shift that favors resolution fluorescence imaging.

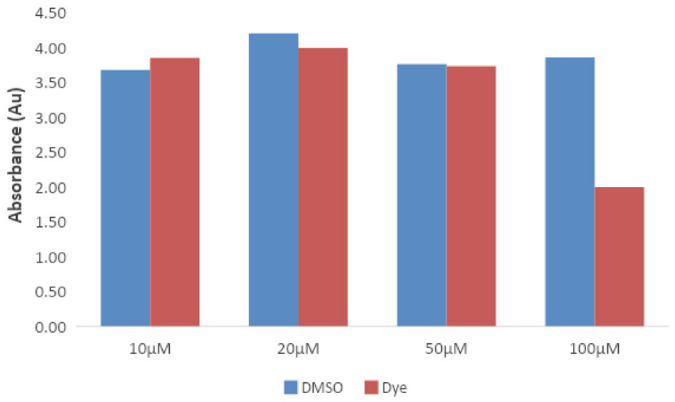

Figure 4. Cell cytotoxicity measurement at various concentrations of RatioWorks™ pH 165, AM (10, 20, 50, and 100 µM) using CCK-8 assay at a wavelength of 460 nm and an incubation period of 120 min post WST-8 addition.

With its ratiometric fluorescence imaging abilities, the probe is able to eliminate the influences of the microenvironment, localized probe distribution, and instrumental parameters. This explains the enhanced accuracy of RatioWorks™ pH 165, AM in monitoring molecular events by responding to near-neutral pH environments in live cells. In contrast to other fluorescein derivatives, RatioWorks™ pH 165, AM dye exhibits responses in the red and far red spectrum, making it ideal for multiplex immunoassays with GFP, where the dye prevents spectrum interference and saturation. This feature is a clear distinction of RatioWorks™ pH 165, AM from other fluorescent based assay technologies used in similar applications.

References

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2. Colorimetric and Ratiometric pH responses by the protonation of henolate within hemicyanine. J. Miao, C. Fan, X. Shi, R. Sun, Y. Xu, and J. Ge. Analyst, 2014, 139, 6290-6297.

Product	Unit Size	Cat No.
RatioWorks™ PH165, AM	10 x 50 µg	21212
RatioWorks™ PH165	10 x 50 µg	21213
RatioWorks™ PH165, NHS ester	1 mg	21214

Therapeutic Monoclonal Antibody Fluorescent

Labeling Tools for Imaging and Flow Cytometry

Abstract

Therapeutic monoclonal antibodies (mAbs) represent a promising class of biopharmaceutical tools revolutionizing the treatment landscape of immunological disorders, reversal of drug effects, and cancer therapy. Since the late 1990s to 2018, the total number of approved therapeutic mAbs by the US FDA reached 64, with eleven being approved in 2017. This growing interest in therapeutic mAb technologies has sparked a noteworthy need to enhance and expand the range of available tools for diagnostic and therapeutic evaluation of mAbs in pre-clinical models. To satisfy these demands, scientists at AAT Bioquest have developed a plethora of ReadiLink™ conjugation technologies to assist researchers in developing custom-labeled fluorescent mAb conjugates for use in imaging and flow cytometric target identification and validation studies, as well as, determining viable lead candidates.

Introduction

In recent decades, there has been an intensifying interest in therapeutic monoclonal antibodies (mAbs) as a form of immunotherapy to treat autoimmune diseases, neurological disorders, cancer, cardiovascular disease, infection, organ transplantation, and so on. Since the 1986 commercialization of the first therapeutic mAb, Orthoclone OKT3 an immunosuppressive agent used in liver and kidney transplants, therapeutic mAbs and their derivatives (e.g. Fc-fusion proteins, antibody fragments, and antxibody-drug conjugates) have grown to become a dominant class of tools within the biopharmaceutical sector and academia. Today, a reported 570 therapeutic mAbs have been studied in clinical trials, and approximately 79 therapeutic mAbs have been approved by the United States Food and Drug Administration (US FDA) for commercialization.

Why Therapeutic mAbs

The usefulness of therapeutic mAbs is three fold. First, is their monovalent affinity, which is their ability to recognize and target a specific antigen. This high level of specificity for a single target antigen improves consistency among experiments, and reduces cross-reactivity and the adverse effects that accompany it. Second, therapeutic mAbs have the capacity to elicit various mechanisms of action to promote a natural immune response, which enables them to be applied to a wide range of therapeutic targets. Third, improvements in genetic engineering and recombinant manufacturing technologies have made industrial scale manufacturing of therapeutic mAbs possible and cost-efficient.

Mechanisms of Action of Therapeutic mAbs

The underlying objective of therapeutic mAb immunotherapy is to stimulate and resture the patient's natural immune system functions, or to utilize the antibody as a drug delivery carrier (more commonly known as missile therapy). Natural immune system functions include neutralization, blocking, signaling, antibody-dependent cell mediated cytotoxic (ADCC) activity, or complement-dependent cytotoxic (CDC) activity (Table 1).

Significance of Fluorescent of Therapeutic mAbs

Before a mAb can be considered for therapeutic use it is funneled through a rigorous drug discovery process. During the initial drug discovery phase – target identification, validation and selection of lead candidates – it is often advantageous to fluorescently modify mAbs to facilitate detection in imaging and flow cytometric analysis. For in vivo target-specific cancer imaging, where low background and high target-to-noise ratios are critical (e.g. tumor targeting or whole-animal bio-distribution studies) near-infrared (NIR) and infrared (IR) fluorescence modification of mAbs is preferred. Within the NIR to IR spectrum (700 to 1000 nm) both light scattering and autofluorescence are drastically reduced due to the minimal absorption of biological molecules in this region.

Labeling mAbs for use as imaging reagents, however, presents its own unique challenges. For example, conjugation methods utilizing amine-reactive dyes, such as FITC succinimidyl esters, require large starting volumes (milligrams) of mAbs. Once labeled, conjugates must be purified via spin column or gel filtration media, which reduce conjugation yields to ~50 – 60%. More importantly the degree of labeling (DOL), which refers to the number of dye molecules per antibody, must be optimized to preserve the antibody's immunoreactivity and reproducibility in conjugation results. Under-labeled (DOL < 2) and over-labeled mAbs (DOL > 6) can experience a reduction in their fluorescence intensities. The latter is due to the conjugation of multiple fluorophore to a mAb and its effect on the fluorescence quantum yield (a factor that contributes to dye brightness). This effect is a result of self-quenching between neighboring dyes and between dyes and the mAb.

To address the aforementioned concerns, AAT Bioquest has developed an arsenal of ReadiLink™ Rapid Antibody Labeling Kits for microscale labeling (50 to 100 µg) of therapeutic mAbs for use in imaging and flow cytometry. Utilizing a streamlined labeling protocol in combination with amine-reactive fluorophores, ReadiLink™ kits produces mAb conjugates in two simplified steps at optimal DOL values. To assess the fluorescence brightness, a comparative analysis between CD4-iFluor™

Table 1. Ratiometric Analysis of RatioWorks™ pH 165, AM at pH 4.5, 5, 6, 7, 8, and 9.

Mechanism (Mode)	Principle	Therapeutic mAb (Target)
Neutralization	Therapeutic mAbs bind to a target ligand or cell surface receptor, thereby blocking that target's signaling pathway. The suppression of the signal through that particular antigen can result in loss of cellular activity, inhibition of proliferation, the activation of pro-apoptotic pathways, or the cell being re-sensitized to cytotoxic agents	Ibalizumab (CD3) Muromonab-CD3 (CD3) Bevacizumab (VEGF) Infliximab (TNF) Daclizumab (CD25)
ADCC activity	ADDC is initiated when the Fv binding domain of a therapeutic mAb binds to an antigen expressed on the surface of a target cell. The mAb subsequently recruits immune effector cells (e.g. Natural Killer cells and macrophages) to lyse the target cell.	Rituximab (CD20) Obinutuzumab (CD20) Trastuzumab (HER-2)
CDC activity	CDC occurs when C1q binds to the mAb-antigen complex. This activates a cascade of complement proteins resulting in the formation of a membrane attack complex (MAC) and ultimately the target cell lysis.	Alemtuzumab (CD52) Panitumumab (EGFR) Catumaxomab (CD3, EpCAM)
Drug Delivery Carrier	Therapeutic mAbs are conjugated to toxins, drugs, radioisotopes, or cytokines. This facilitates the direct delivery of cytotoxic agents to target cells at higher concentrations, without damaging healthy cells.	Gemtuzumab ozogamicin (CD33)

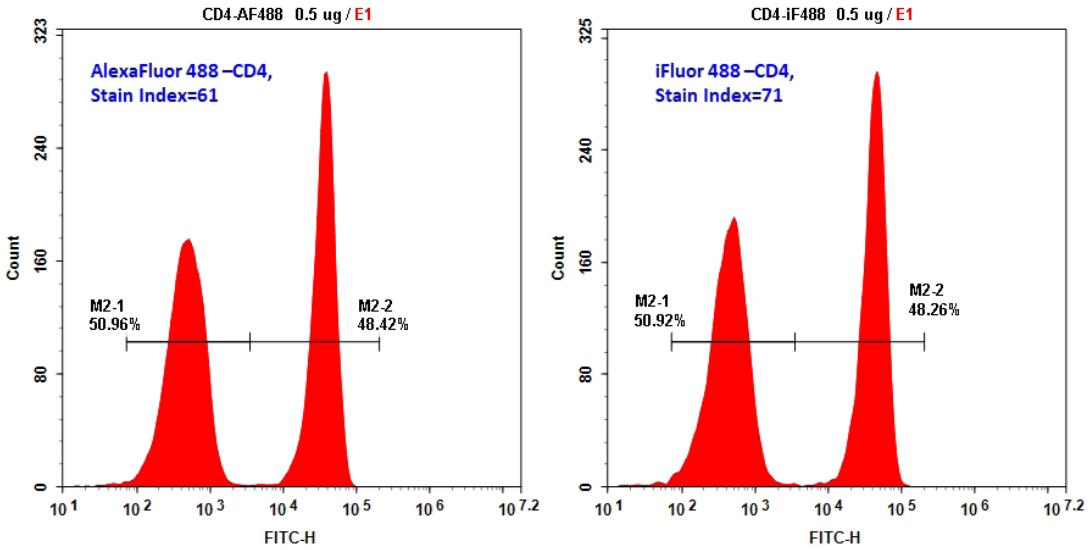

Figure 1. Flow cytometric analysis of Alexa Fluor® 488 or iFluor™ 488 anti-human CD4 on human lymphocytes. PBMC cells were stained with 0.5 µg Alexa Fluor® 488 anti-human CD4 or 0.5 µg iFluor™ 488 anti-human CD4 in each test. Flow cytometric analysis was performed on a ACEA flow cytometry system.

488 conjugates prepared with ReadiLink™ Rapid iFluor™ 488 Antibody Labeling Kit and commercially purchased CD4-Alexa Fluor® 488 was performed by lymphocyte immunophenotyping.

Materials and Methods

Cells

Cryopreserved human peripheral blood mononuclear cells (PBMCs) were obtained from iXCells Biotechnology (San Diego, CA). Cryopreserved PBMCs were thawed in a 37°C water bath with continuous agitation until completely melted, and then placed on ice for 2 minutes. Each 100 µL of thawed cell suspension was diluted with 9 mL Blood Cell Culture medium (iXCells Biotechnology) supplemented with 1 mL 10% fetal bovine serum. Cells were centrifuged (1000 rpm) at room temperature for 5 minutes and washed a second time with 10 mL of medium. Cell viability was quantified by trypan blue dye exclusion from AAT Bioquest (Sunnyvale, CA), counted on a hemocytometer and resuspended in medium required for lymphocyte immunophenotyping.

Antibodies

Cryopreserved human peripheral blood mononuclear cells (PBMCs) were obtained from iXCells Biotechnology (San Diego, CA). Cryopreserved PBMCs were thawed in a 37°C water bath with continuous agitation until completely melted, and then placed on ice for 2 minutes. Each 100 µL of thawed cell suspension was diluted with 9 mL Blood Cell Culture medium (iXCells Biotechnology) supplemented with 1 mL 10% fetal bovine serum. Cells were centrifuged (1000 rpm) at room temperature for 5 minutes and washed a second time with 10 mL of medium. Cell viability was quantified by trypan blue dye exclusion from AAT Bioquest (Sunnyvale, CA), counted on a hemocytometer and resuspended in medium required for lymphocyte immunophenotyping.

Results

Improved stain index with CD4-iFluor™ 488 conjugated by ReadiLink™ technology

Stain Index (SI) measurements were taken to assess the fluorescence brightness of CD4-iFluor™ 488 conjugates prepared using ReadiLink™ Rapid iFluor™ 488 Antibody Labeling Kit and commercially available CD4-Alexa Fluor® 488. This index provides a practical definition of conjugate brightness and enables side-by-side comparison of CD4- iFluor™ 488 and CD4-Alexa Fluor® 488. Flow cytometry analysis shows both CD4-iFluor™ 488 and CD4-Alexa Fluor® 488 conjugates provide

distinct discrimination of positive and negative populations. The separation between respective populations at conjugate saturation provides a measurable parameter for determining conjugate brightness. Using this model, CD4-iFluor™ 488 conjugates prepared using ReadiLink™ technology had a 16% improvement in the fluorescence brightness versus CD4-Alexa Fluor® 488 conjugates.

Discussion

Stain Index (SI) measurements were taken to assess the fluorescence brightness of CD4-iFluor™ 488 conjugates prepared using ReadiLink™ Rapid iFluor™ 488 Antibody Labeling Kit and commercially available CD4-Alexa Fluor® 488. This index provides a practical definition of conjugate brightness and enables side-by-side comparison of CD4- iFluor™ 488 and CD4-Alexa Fluor® 488. Flow cytometry analysis shows both CD4-iFluor™ 488 and CD4-Alexa Fluor® 488 conjugates provide distinct discrimination of positive and negative populations. The separation between respective populations at conjugate saturation provides a measurable parameter for determining conjugate brightness. Using this model, CD4-iFluor™ 488 conjugates prepared using ReadiLink™ technology had a 16% improvement in the fluorescence brightness versus CD4-Alexa Fluor® 488 conjugates.

Another quality to note is the dramatic increase in conjugation yield using ReadiLink™ labeling technologies. By replacing the spin column or gel filtration media (two common purification techniques used in conjugation methods) with a novel TQ™-Dyed Quencher Buffer (Component C, ReadiLink Kit), 100% of the conjugate is retained.

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Product	Unit Size	Cat No.
ReadiLink™ Rapid mFluor™ Violet 450 Antibody Labeling Kit *Microscale Optimized for Labeling 50 µg Antibody Per Reaction*	2 Labelings	1100
ReadiLink™ Rapid mFluor™ Violet 420 Antibody Labeling Kit *Microscale Optimized for Labeling 50 µg Antibody Per Reaction*	2 Labelings	1105
ReadiLink™ Rapid mFluor™ Violet 510 Antibody Labeling Kit *Microscale Optimized for Labeling 50 µg Antibody Per Reaction*	2 Labelings	1110
ReadiLink™ Rapid mFluor™ Violet 540 Antibody Labeling Kit *Microscale Optimized for Labeling 50 µg Antibody Per Reaction*	2 Labelings	1114
ReadiLink™ Rapid mFluor™ Blue 570 Antibody Labeling Kit *Microscale Optimized for Labeling 50 µg Antibody Per Reaction*	2 Labelings	1120
ReadiLink™ Rapid mFluor™ Green 620 Antibody Labeling Kit *Microscale Optimized for Labeling 50 µg Antibody Per Reaction*	2 Labelings	1123
ReadiLink™ Rapid mFluor™ Red 700 Antibody Labeling Kit *Microscale Optimized for Labeling 50 µg Antibody Per Reaction*	2 Labelings	1130
ReadiLink™ Rapid mFluor™ Red 780 Antibody Labeling Kit *Microscale Optimized for Labeling 50 µg Antibody Per Reaction*	2 Labelings	1131
ReadiLink™ Rapid iFluor™ 350 Antibody Labeling Kit *Microscale Optimized for Labeling 50 µg Antibody Per Reaction*	2 Labelings	1220
ReadiLink™ Rapid iFluor™ 555 Antibody Labeling Kit *Microscale Optimized for Labeling 50 µg Antibody Per Reaction*	2 Labelings	1227
ReadiLink™ Rapid iFluor™ 594 Antibody Labeling Kit *Microscale Optimized for Labeling 50 µg Antibody Per Reaction*	2 Labelings	1230
ReadiLink™ Rapid iFluor™ 647 Antibody Labeling Kit *Microscale Optimized for Labeling 50 µg Antibody Per Reaction*	2 Labelings	1235
ReadiLink™ Rapid iFluor™ 680 Antibody Labeling Kit *Microscale Optimized for Labeling 50 µg Antibody Per Reaction*	2 labelings	1240
ReadiLink™ Rapid iFluor™ 700 Antibody Labeling Kit *Microscale Optimized for Labeling 50 µg Antibody Per Reaction*	2 Labelings	1245
ReadiLink™ Rapid iFluor™ 750 Antibody Labeling Kit *Microscale Optimized for Labeling 50 µg Antibody Per Reaction*	2 Labelings	1250
ReadiLink™ Rapid iFluor™ 488 Antibody Labeling Kit *Microscale Optimized for Labeling 50 µg Antibody Per Reaction*	2 Labelings	1255
ReadiLink™ Rapid iFluor™ 633 Antibody Labeling Kit *Microscale Optimized for Labeling 50 µg Antibody Per Reaction*	2 Labelings	1260
ReadiLink™ Rapid iFluor™ 790 Antibody Labeling Kit *Microscale Optimized for Labeling 50 µg Antibody Per Reaction*	2 Labelings	1265
ReadiLink™ Rapid Cy3 Antibody Labeling Kit *Microscale Optimized for Labeling 50 µg Antibody Per Reaction*	2 Labelings	1290
ReadiLink™ Rapid Cy5 Antibody Labeling Kit *Microscale Optimized for Labeling 50 µg Antibody Per Reaction*	2 Labelings	1292
ReadiLink™ Rapid Cy7 Antibody Labeling Kit *Microscale Optimized for Labeling 50 µg Antibody Per Reaction*	2 Labelings	1294
ReadiLink™ Rapid FITC Antibody Labeling Kit *Microscale Optimized for Labeling 50 µg Antibody Per Reaction*	2 Labelings	1299
ReadiLink™ Rapid trFluor™ Eu Antibody Labeling Kit *Microscale Optimized for Labeling 50 ug Antibody Per Reaction*	2 Labelings	1300
ReadiLink™ Protein Conjugation Stop Buffer	1 mL	5400
ReadiLink™ BSA Conjugation Kit	1 Kit	5501
ReadiLink™ KLH Conjugation Kit	1 Kit	5502
ReadiLink™ Protein Biotinylation Kit	2 Labelings	5520
ReadiLink™ Protein Biotinylation Kit *Powered by ReadiView™ Biotin Visionization Technology*	1 Kit	5521
ReadiLink™ xtra Rapid AF488 Antibody Labeling Kit	2 Labelings	1978
ReadiLink™ xtra Rapid AF555 Antibody Labeling Kit	2 Labelings	1980
ReadiLink™ xtra Rapid AF594 Antibody Labeling Kit	2 Labelings	1982
ReadiLink™ xtra Rapid AF647 Antibody Labeling Kit	2 Labelings	1985
ReadiLink™ xtra Rapid AF750 Antibody Labeling Kit	2 Labelings	1988
ReadiLink™ xtra Rapid FITC Antibody Labeling Kit	2 Labelings	1970
ReadiLink™ xtra Rapid iFluor™ 350 Antibody Labeling Kit	2 Labelings	1950

Product	Unit Size	Cat No.
ReadiLink™ xtra Rapid iFluor™ 488 Antibody Labeling Kit	2 Labelings	1955
ReadiLink™ xtra Rapid iFluor™ 555 Antibody Labeling Kit	2 Labelings	1958
ReadiLink™ xtra Rapid iFluor™ 594 Antibody Labeling Kit	2 Labelings	1960
ReadiLink™ xtra Rapid iFluor™ 647 Antibody Labeling Kit	2 Labelings	1963
ReadiLink™ xtra Rapid iFluor™ 750 Antibody Labeling Kit	2 Labelings	1965
Buccutite™ Peroxidase (HRP) Antibody Conjugation Kit *Optimized for Labeling 1 mg Protein*	1 Kit	5504
Buccutite™ Peroxidase (HRP) Antibody Conjugation Kit *Optimized for Labeling 100 ug Protein*	1 Kit	5503
Buccutite™ Peroxidase (HRP) Antibody Conjugation Kit *Optimized for Labeling 25 ug Protein*	2 Labelings	5505
Buccutite™ Rapid APC Antibody Labeling Kit *Microscale Optimized for Labeling 100 ug Antibody Per Reaction*	2 Labelings	1311
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Buccutite™ Rapid APC-Cy5.5 Tandem Antibody Labeling Kit *Microscale Optimized for Labeling 100 ug Antibody Per Reaction*	2 Labelings	1320
Buccutite™ Rapid APC-Cy5.5 Tandem Antibody Labeling Kit *Microscale Optimized for Labeling 25 ug Antibody Per Reaction*	2 Labelings	1350
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Buccutite™ Rapid APC-iFluor™ 700 Tandem Antibody Labeling Kit *Microscale Optimized for Labeling 100 ug Antibody Per Reaction*	2 Labelings	1319
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Buccutite™ Rapid PE-Cy7 Tandem Antibody Labeling Kit *Microscale Optimized for Labeling 100 ug Antibody Per Reaction*	2 Labelings	1317
Buccutite™ Rapid PE-Cy7 Tandem Antibody Labeling Kit *Microscale Optimized for Labeling 25 ug Antibody Per Reaction*	2 Labelings	1342
Buccutite™ Rapid PE-Texas Red Tandem Antibody Labeling Kit *Microscale Optimized for Labeling 100 ug Antibody Per Reaction*	2 Labelings	1318
Buccutite™ Rapid PE-Texas Red Tandem Antibody Labeling Kit *Microscale Optimized for Labeling 25 ug Antibody Per Reaction*	2 Labelings	1343
Buccutite™ Rapid PerCP Antibody Labeling Kit *Microscale Optimized for Labeling 100 ug Antibody Per Reaction*	2 Labelings	1325
Buccutite™ Rapid PerCP Antibody Labeling Kit *Microscale Optimized for Labeling 25 ug Antibody Per Reaction*	2 Labelings	1353
Buccutite™ Rapid Protein Crosslinking Kit *Microscale Optimized for Crosslinking 100 ug Antibody Per Reaction*	2 Labelings	1315

Transfectamine™ 5000

An Efficient and Reliable DNA Delivery System

Abstract

The impact of any protein in cells is widely studied by over expressing a protein of interest and investigating the biomolecular alterations that occur due to the overexpression of that protein. These studies are undertaken to better understand the functionality of vital proteins, such as tumor suppressor proteins or oncogenes. The process of overexpressing proteins is mainly facilitated using a lipid based DNA delivery system which passively delivers DNA into the cells for subsequent translation into protein. In this proposed study, the transfection efficiency of Transfectamine™ 5000, Lipofectamine™ 2000 and Lipofectamine™ 3000 were compared. Comparative analysis revealed a 2-3 fold increase in transfection efficiency with much lower cytotoxicity when using Transfectamine™ 5000 over its counterparts. Additionally, agonist-stimulated calcium responses with Transfectamine™ 5000 transfected cells was much more pronounced than Lipofectamine™ 2000 and Lipofectamine™ 3000 transfected cells.

Introduction

Over the past 30 years, DNA delivery has become a powerful tool for understanding gene structure, regulation and function. Traditionally, it is divided into two main systems: viral vector mediated systems and nonviral mediated systems. Viral vector mediated systems are by far the most efficient for both delivery and expression, and thus highly used in the clinical settings. Despite its high efficiency, it presents caveats such as toxicity, production and packaging issues, and high cost.[1, 2] The potential for increased toxicity and immunogenicity of viral vector mediated systems further limits its use in basic research laboratories. For these reasons, nonviral vector mediated systems have become increasingly desirable in both research laboratories and clinical settings.[3]

DNA delivery systems rely on three main functions: DNA condensation, endocytosis and nuclear entry. DNA molecules, which are negatively charged, are complexed with a cationic transfection reaction system. These complexes then get endocytosed by cells, facilitating the entry of DNA through nuclear pores. Once inside the nucleus, the DNA gets translated and converted into its respective protein. There are several methods through which nonviral DNA delivery system function. This includes a mechanical approach such as microinjection or particle bombardment, an electrical approach such as electroporation or a chemical approach such as artificial lipids, DEAE-dextran, proteins, dendrimers and other polymers.[4]

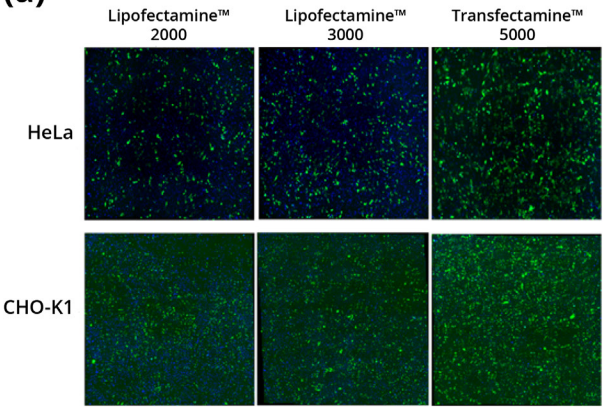
Artificial lipids such as lipofectamines have been widely popular for the transfection of foreign DNA molecules into cells. In this proposed study, transfection efficiency and toxicity comparisons were made between Transfectamine™ 5000, Lipofectamine™ 2000 and Lipofectamine™ 3000.

Materials and Methods

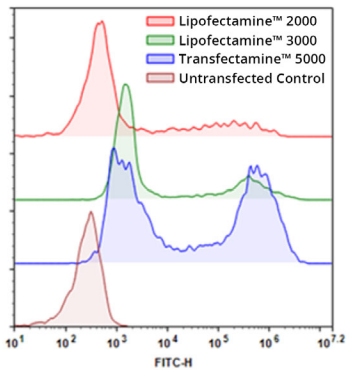
Artificial Lipids

Transfectamine™ 5000 reagent was obtained from AAT Bioquest (Sunnyvale, CA). Lipofectamine™ 2000

(a)



(b)



(c)

Transfection Reagent	Transfection Efficiency (%)
Lipofectamine™ 2000	28.64
Lipofectamine™ 3000	63.78
Transfectamine™ 5000	83.30

Figure 1. Comparison of Lipofectamine™ 2000, Lipofectamine™ 3000 and Transfectamine™ 5000 using various platforms. HeLa cells and CHO-K1 cells were transfected with a GFP expressing plasmid using Lipofectamine™ 2000, Lipofectamine™ 3000 and Transfectamine™ 5000 following their corresponding protocols. Fluorescence images were acquired using the FITC filter (a). Flow cytometry was performed on HeLa cells using 530/30 nm filter (b) and Transfection efficiency was measured base on flow cytometry data (c).

and Lipofectamine™ 3000 reagents were obtained from ThermoFisher (Waltham, MA).

Cell Culture

HeLa cells were cultured at 37 °C in DMEM containing heat inactivated serum (10% Fetal Bovine Serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin), under a humidified atmosphere containing 5% CO2. CHO-K1 cells were cultured at 37 °C in F12-K Nutrient mixture containing heat inactivated serum (10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin), under a humidified atmosphere containing 5% CO2.

Transfection Protocol

Cells were seeded in 6-well plates 18 hours prior to transfection. At the time of transfection, cells were grown around 90% confluency in the plate. (Note: Higher confluency is recommended.) Cell culture medium was removed and replaced with 2 mL of fresh culture medium. Transfectamine™ 5000-DNA working solution (2.5 µg GFP DNA, 200 µL Serum-free medium, 7.5 µL Transfectamine™ 5000) was added to each well and incubated at room temperature overnight. GFP transfected cells using Lipofectamine™ 2000 and Lipofectamine™ 3000, were prepared using the manufacturer's recommended transfection protocol. This mix was added to cells and incubated overnight. Next day, cells were transferred to fresh cell culture medium and kept for 48 hours. The expression of GFP was recorded after 72 hours post transfection. Images were acquired using FITC filter set on a fluorescence microscope. Flow cytometry was recorded with 488 nm laser excitation and 530/30 nm filter set (FITC channel).

WST-8 Viability Test

Transfection was performed on cells (as previously described). Viability was determined by adding 10 µL of WST-8™ solution (AAT Bioquest, Sunnyvale, CA) to each well and incubated at 37 °C for 1 hour. Absorbance was measured at 460 nm using absorbance plate reader.

Calcium Kinetic Assay

Cells were plated in 100 µL culture medium in 96-well bottom black plates (Greiner Bio-One, Kremsmunster, Austria) at 50,000 cells per well. The next day, equal volumes of prepared Calbryte™ 520-loading solutions were added to each well and incubated at 37 °C for 1 hour, under a humidified atmosphere containing 5% CO2. Calcium kinetic assays were performed on FlexStation® (Molecular Devices, Sunnyvale, CA) using the built-

in liquid handler to add the calcium flux stimulants (Vasopressin) and the kinetic reading mode to capture the changes in fluorescence signal over time. The kinetic calcium assay data were simultaneously collected by SoftMax®Pro (Molecular Devices, Sunnyvale, CA).

Results and Discussion

Increased transfection efficiency with Transfectamine™ 5000

First, we did transfection of green fluorescence protein (GFP) containing plasmid in various cell lines such as HeLa and CHO-K1 cell lines using various treatments including Transfectamine™ 5000 was and compared the transfection efficiency by numerous applications such as flow cytometry and fluorescence microscopy. We observed that cells transfected with Transfectamine™ 5000 have the highest level of GFP expression (Figure-1a). We calculated the transfection efficiency using the flow cytometry and observed significant increase in GFP expressed cells with Transfectamine™ 5000 upon comparison with their counterparts. (Figure-1b and 1c). Overall, we show much improved transfection efficiency with Transfectamine™ 5000.

Low cytotoxicity with Transfectamine™ 5000

Since viability of the transfected cells has been a huge issue, we next determined viability of the CHO-K1 cells transfected with different reagents in the presence/absence of plasmid via Cell Meter™ Colorimetric WST-8 Cell Quantification Kit (AAT Bioquest, Sunnyvale, CA) (Figure-2a and b). We observed that cells incubated with Transfectamine™ 5000 have very low cytotoxicity when compared with other transfection reagents. The viability of the cells was further down when transfected with DNA using Lipofectamine™ 2000 and Lipofectamine™ 3000 reagents but no major change was observed in viability with Transfectamine™ 5000.

Application of GPCR activation

We next tested the calcium response with CHO-K1 cells transfected with G-protein Ga16 and G protein coupled receptor V2R construct using various transfection reagents via vasopressin stimulation.[5] Calcium flux analysis was performed using Calbryte™ 520 AM (AAT Bioquest, Sunnyvale CA) with FlexStation3 (Figure-3). We observed significant change in calcium flux in cells transfected with Transfectamine™ 5000 upon comparison with Lipofectamine™ 2000 and Lipofectamine™

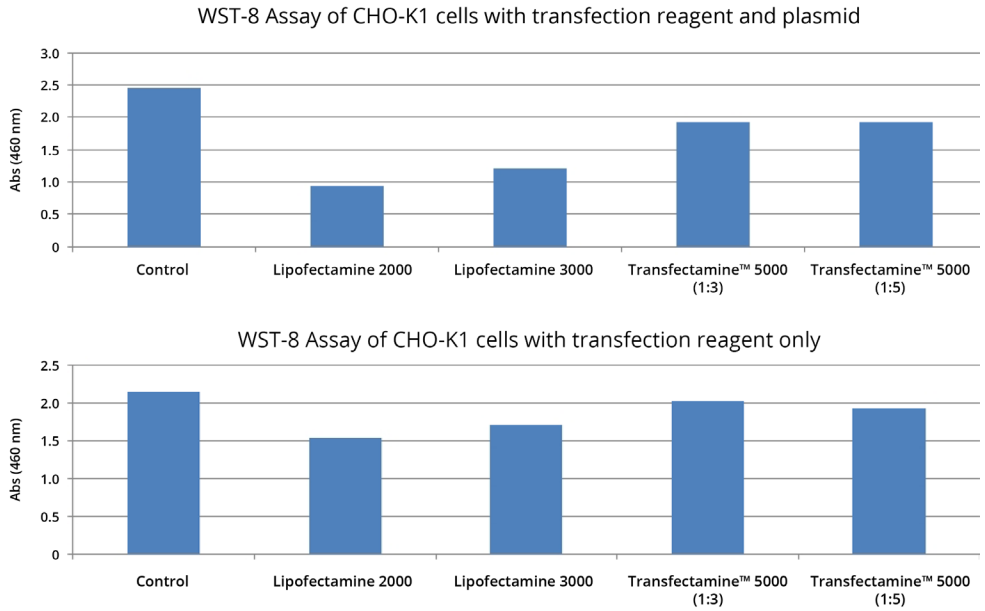


Figure 2. Cytotoxicity assessment of Transfectamine™ 5000. CHO-K1 cells were incubated with or transfection reagent and GFP plasmid (top) or transfection reagent only (bottom). Viability of cells was assessed using WST-8 cell quantification kit.

3000, confirming our previous findings that Transfectamine™ 5000 helps achieve maximum transfection efficiency for in vitro studies.

Conclusion

In this assay wise letter, for the transfection process, we have addressed two key issues, one is to improve the transfection efficiency and the other is to minimize the cytotoxicity associated with performing transfection process. We have successfully shown that cells transfected with Transfectamine™ 5000 provides much higher transfection efficiency with very little cytotoxicity.

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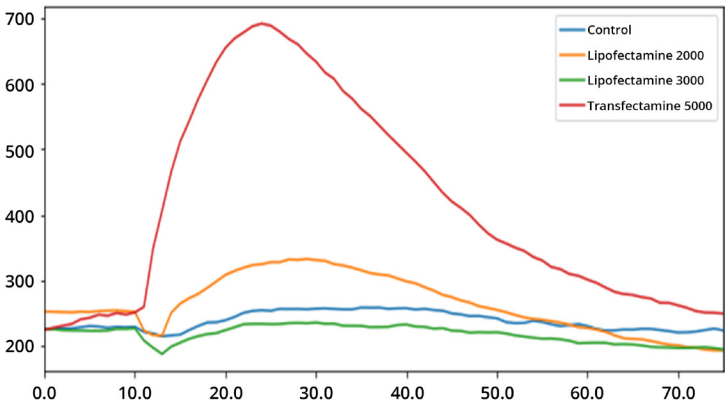

Figure 3. GPCR activation of G protein coupled receptor V2R construct transfected cells with Vasopressin using FlexStation 3. The promiscuous G-protein Ga16 and G protein coupled receptor V2R construct were co-transfected in CHO-K1 cells following each transfection reagent's protocol. Intracellular calcium flux assay were performed ~36 hours post transfection. Cells were treated with vasopressin to induce rapid calcium flux and were observed using the calcium sensitive fluorescent dye Calbryte™ 520 AM.

Product	Unit Size	Cat No.
Transfectamine™ 5000 Transfection Reagent	0.5 mL	60020
Transfectamine™ 5000 Transfection Reagent	1 mL	60021
Transfectamine™ 5000 Transfection Reagent	5 mL	60022
Transfectamine™ mRNA Transfection Reagent	500 µL	60030
Transfectamine™ mRNA Transfection Reagent	5 mL	60031

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