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Physiological Indicators of Cell Function

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Summary

Successful high content screening (HCS) assays place large demands on the cell-based reagents used in their development and deployment. Fortunately, there is a wide range of fluorescent physiological indicators from which to choose that are continually increasing in size and variety. Ideal fluorescent reagents for cell-based assays exhibit optimal selectivity, signal intensity, and cell solubility, yet will be easily incorporated into assays across multiple detection platforms. The repertoire of existing fluorogenic and color changing dyes that indicate physiological changes in cells for live cell kinetic and fixed end-point assays are surveyed as well as newly developed reagents for the next generation of HCS assays.

Key Words: Apoptosis; assays; calcium; cell counts; cell biology; drug discovery; expression reporters; fluorescence; high content screening; high content analysis; HTS; high-throughput screening; imaging; membrane voltage; microplate; organelle; pathway analysis.

1. Introduction

The growing potential of fluorescent reagents for cell-based discovery is most apparent in the scaled up imaging approach offered by high content screening *(1–3)*. The sacrifice in throughput compared with nonimage-based high-throughput screening (HTS) is offset by both the quality and content of the data and ability to query multiple parameters per well. Coupled with RNAi or more routine gene transfections, it is now feasible to study the cellular function of hundreds of genes in one well-conceived assay *(4)*—a staggering leap in technology from just 5 yr ago. Gone are the legions of radioactivity based applications, which were limited by costs, single analytes, and cumbersome disposal and health risks. Bright field and chromogenic dyes with their poor dynamic range, low sensitivity, nonquantitative nature, and limited targets are being superceded by fluorescent biosensing reagents. In a recent survey, novel reagents and probes were identified as having the biggest potential impact on the field of high content screening (HCS) over the next few years *(1)*. Clearly, work remains.

2. The Automated Imager's Innovator Tool Box

Automated imaging, in which the information and conclusions are extracted through software analysis, demands much of the protocol and reagent, as well as the instrument. Thresholding requirements, contrast settings, cell segmentation, background binding all confound the process, as discussed in the section on Informatics and Bioinformatics. Add in the diversity of instruments out there, which differ in magnification, light source, confocality, capture device, analysis software, and then cell type, and it is nearly impossible to insure that any dye or protocol will work in all

From: *Methods in Molecular Biology, vol. 356:*
High Content Screening: A Powerful Approach to Systems Cell Biology and Drug Discovery
Edited by: D. L. Taylor, J. R. Haskins, and K. Giuliano © Humana Press, Inc., Totowa, NJ

systems. In addition to the range of instruments and cells types, reagent choice is further constrained by spectral limits—for example, green is often crowded with fluorescent protein (FP)-based sensors.

However, the motivated assay designer will find solace in the range of options in color and probes from which to solve their individual problem. To aid assay developers, this chapter provides an overview of the dyes that have been successfully qualified for whole-cell, microplate assays, while anticipating others that might prove useful in the future. Of practicing HCS users, assay development was listed as the most restrictive limitation ***(1)***. Although this creates an opportunity for reagent companies, it is impossible to make kits for all potential targets. Fortunately, the ease of exploring the available options through the web and the many outstanding tech services departments make the search easier.

Molecular Probes, a part of the growing family of Invitrogen-owned discovery companies since 2003, has been principally a chemistry-driven company. The applications in this space have been developed to a large degree by others. Companies like Cellomics (now a part of Fisher Scientific International, Hampton, NH) and Molecular Devices (Sunnyvale, CA) have pioneered the use of many of the Molecular Probes dyes in the HCS/HTS space. Although others might be using Molecular Probes technology in these applications, Probes has traditionally qualified reagents in simple single-cell applications. It has always been up to others to establish their suitability in any HCS/HTS type protocol. Scalability is addressed internally primarily by testing the uniformity of the response, the intensity of the signal, wash steps required, media compatibility, fixability, and general ease-of-use. Clearly a response that is crisp and bright with zero background will work on any device—including those with ×10 dry optics. Those applications with the need for automation, loosely adherent cells, and general cost reduction, minimizing wash steps are critical—benefits also enjoyed in any protocol. In the end, dedicated reagent kits for one device are not sought, rather, dyes that might enjoy widespread utility on all instruments are the focus.

This chapter will highlight the dyes that indicate physiological changes in cells. Although many are traditionally used in HTS mode, through plate readers that integrate the entire well response, these reagents can also be used in any imaging based protocols. For the purpose of this chapter, reagents that are traditionally used in HTS on live intact cells will be covered, provided they could also be used in imaging based HCS modes as well. In designing any physiological probes, three parameters are considered: selectivity, signal intensity, and cell permeability. The perfect probe can be used across platforms—from cells, to wells, to multiwells, to gels.

3. Collection Modality Determines Reagent Options

Fluorescent dyes and probes can be viewed as working in one of three modalities. First and the most challenging to the instrument are assays on live cells that are repeatedly imaged live (kinetic). Second and the most challenging to the reagents are assays that can be used in live-cell protocols but whose response can be preserved or retained with standard fixation. The third and most common are assays in which the cells are fixed then labeled, primarily with antibodies, or expression tags. These latter two are typically described as end-point analysis. With the range of instruments available, it is sensible for any reagent provider, unconstrained by their own proprietary box design, to qualify their reagents in these differing modalities. **Tables 1–3** shows lists of dyes and the general conditions for their use.

The live-cell experiments can be further broken down into (1) single time-point, (2) continuous time-series, and, finally, (3) staggered time-series in which each well of multiplate is imaged sequentially and time-lapse series extracted later. When considering the diversity of physiological events that can be induced in phenotypic screens of RNAi screens, or any cellular event that is represented in only a fraction of a cells' daily life, the need for multiple time domains is apparent. These live cell refinements are the cutting edge of instrument features, requiring environmental,

Table 1
Small Molecule-Based Ion Indicators

Calcium	fluo-4 AM, fluo-4 NW, fura-2,AM, Calcium Green™-1, rhod-2 AM, Indo1 AM[a]
Magnesium	mag-fluo-4 AM, mag-fura-2 AM
Sodium	SBFI (crown ether), Sodium Green, CoroNa Red
Potassium	PBFI (crown ether)
Chloride	SPQ (6-methoxy-*N*-(3-sulfopropyl) quinolinium, MQAE (*N*-(ethoxycarbonylmethyl) -6-methoxyquinolinium bromide), MEQ (6-methoxy-*N*-ethylquinolinium iodide)
pH	SNARF®-1, BCECF-AM (2′,7′-bis(2-carboxyethyl) -5-(and-6)-carboxyfluorescein), fluorescein, CypHer™ 5 (GE/Amersham)
Heavy metals (lead and zinc)	FluoZin™-1, Phen Green™, Leadmium™ Green AM dye
Membrane voltage sensors	
Fast	Di-4- and Di-8-ANEPPS, (pyridinium, 4-(2-(6-(dibutylamino)-2-naphthalenyl) ethenyl)-1-(3-sulfopropyl)-, hydroxide)
	VSP [voltage sensing probe (oxonol and coumarin based)]
Slow	$DiSBAC_2(3)$ bis-(1,3-diethylthiobarbituric acid)trimethine oxonol
	$DiBAC_4(3)$ bis-(1,3-dibutylbarbituric acid)trimethine oxonol

[a]For complete list please *see* The Handbook of Fluorescent Probes and Research Chemicals (probes.invitrogen.com).

control liquid handling, and extensive memory. Such factors have limited their acceptance and driven the popularity of many end-point assays, such as translocation assays in which movement of a reporter molecule is detected either green fluorescent protein (GFP) ***(5)***, or an antibody ***(2)*** often to the nucleus. These include the TransFluor assay by NORAK ***(6)*** and nuclear factor of activated T-cells (NFAT), NF-κβ, PKC-α activation, signal transducers and activators of transcription STAT ***(2,7–10)*** in which events that required several hours to days to appear, are captured by the permanent movement of a reagent. Yet, the number of physiological events that are covered by this approach remain rare. Live cell reagents represent the greatest hope of parsing out these more complex systems biology types of interactions, and are the primary focus of this chapter on physiological indicators.

4. Physiological Reagents for Automated Imaging Platforms Including HCS

4.1. Calcium Sensing

A rise in intracellular Ca^{2+} can occur directly or indirectly from a variety of critically important drug targets. Ca^{2+} signaling is vital to processes as diverse as memory, cell proliferation, apoptosis, and muscle action ***(11–16)***. Indirect release is produced with stimulation of any of the G protein coupled receptors linked to adenylate cyclase, phospholipase C, or some ion channels. Direct release of Ca^{2+} occurs through the many ligand gated channels ***(17)***. Nature and chemist have shown that this divalent cation is the most tractable of ions for designing affinity scaffolds—with the added benefit of eliciting large structural changes on binding. The high affinity chelation complexes formed create long lasting structural changes useful in transmitting signaling cascades in nature and fluorogenic changes in dyes for discovery scientist.

The first set of calcium indicators monitor changes in their peak excitation or emission maxima with calcium binding ***(18)***. For fura-2, by scanning with excitation wavelengths in the UV, a shift in absorption toward the blue can be seen when Ca^{2+} rises, whereas indo-1 shifts its emission toward the blue with Ca^{2+} loading ***(19)***. Although these ratiometric dyes produce the most accurate absolute values of Ca^{2+} concentrations, their ratiometric and UV excitation make them

difficult to use in high-throughput imaging screens. Dedicated beam splitters, fast filter wheels, UV sources, and other technologies are needed.

The nonratiometric, visibly light excited fluo-3, fluo-4, and most recently fluo-4NW (NW for no-wash) are ideal HCS/HTS/microplate substitutes. These dyes exhibit up to a 10-fold signal increase upon binding Ca^{2+}. Although non-ratiometric and, therefore, not able to determine absolute levels, some HTS and HCS companies have aggressively adopted these dyes (Molecular Devices, Perkin Elmer [Shelton, CT], Hamamatsu [Okayama City, Japan] for HTS, Cellomics, Evotec, Atto/Becton Dickson [San Jose, CA], and many others for HCS). Currently a popular reagent for calcium sensing is the Calcium-3 kit from Molecular Devices (Sunnyvale, CA), which includes a quencher dye in solution to reduce background emission and to obviate the need to wash the dye-loading medium prior to analysis.

The fluo-4 NW improves on Calcium 3 and standard fluo-4 reagents by better delivery formulation and by achieving robust S/N changes without the need for quencher dyes that can often confound pharmacology. Although fluo-4 NW backgrounds are slightly higher in whole media one initial media removal step with no subsequent washing steps will produce even better results. Efficient delivery of the dye solution into a broad range of cell types with the new delivery vehicle has been confirmed as well. The reduction in work flow and the absence of quenchers that might confound pharmacological interpretations make fluo-4 NW an ideal reagent for automated protocols. fluo-4 NW is typical of an ideal add and read reagent, in which fluorogenic, cell-permeant, robust signals are produced with minimal wash steps and few associated components.

4.2. Protein Expression Reporter Based Calcium Sensors

A number of expression reporter based calcium sensors have been described *(20–22)*. Requiring no reagent addition these expression tags are ideal for creating stable reporter lines. Like fluo-4, and other nonratiometric dyes, absolute values for calcium concentration remain a challenge. To address this, Miyawaki et al. *(23,20)* joined two GFP colored variants together with a composite linker sequence containing calmodulin and calmodulin-binding peptide. On Ca^{2+} binding a structural change is induced that bends the complex, increasing the fluorescence resonance energy transfer (FRET) signal. Based on rises in green emission with Ca^{2+} binding, this radiometric fluorescent protein-based cameleon reporter can be used directly or possibly targeted to a particular subcellular domain with targeting vectors to measure absolute Ca^{2+} flux values.

β-lactamase (BLA)-based Ca^{2+} sensors are also available (Invitrogen LiveBLAzer™ FRET B/G assay kit). This cell permeable substrate for BLA is a β-lactamase cleavable dye pair complex that before cleavage transfers the blue emission from a donor dye to a green emitting acceptor dye *(24,25)*. Cells with uncleaved substrate retain the FRET pairing and continue to glow green. Cells expressing LiveBLAzer™ BLA and therefore β-lactamase activity, cleave the substrate, uncoupling FRET and glow blue. The BLA construct has been made with two promoter elements that are downstream of GPCR activation and Ca^{2+} entry: the CRE-BLA (cAMP responsive element) *(26)* and NFAT *(9,10)*. Both of these transcription factors are expressed when a stable increase in Ca^{2+} has occurred, either through Ca^{2+} influx or efflux from intracellular stores.

Although distal to the calcium sensing and voltage sensor response reagents, the CRE-based reporter provides more information about the pathway that triggered the Ca^{2+} release. Moreover, the bright signals produced are long lived. Neither construct would be considered the top choice for kinetic or dose–response studies, because the response tends to have limited dynamic range. In their favor, they can be done in live or live-fixed samples, are very robust, changing a brief calcium transient into a permanent signal. These features make it a perfect choice in studies where fluidics or live cell imaging are not available but an understanding that a pathway was activated during a certain time period is sought.

Like all of the assays described so far in this section, these can be scaled from single cells to multiwells, read on either microplate readers- or imaging-based systems. Detection methods are selected based on the overall uniformity of the response. Variable expression patterns, as can happen in transient expression experiments is a key attribute in switching analysis from HTS to HCS. For example, with image-based HCS analysis, nonexpressing or nonresponding cells are ignored entirely (not merely subtracted as background) and expressing cells used to determine response patterns. One drawback or challenge in using GFP is the fixability and sensitivity of the signal. Antisera to *Aequorea victoria*-derived GFPs (Molecular Probes/Invitrogen) have been used successfully either to detect low levels of GFPs or to enhance signal lost following fixation. For extremely rare expression reporter based event detection Tyramide Signal Amplification (TSA, Molecular Probes/Invitrogen) can enhance signal strength manyfold. In this method secondary antibodies with HRP are used to detect the primary antibody then the TSA reagent is added and dyes are immediately complexed to any tyrosine in the vicinity. So unlike many enzymatic amplifications, TSA retains a crisp localization signal.

4.3. Voltage Sensing Dyes

Other ion indicators are listed in **Table 1** along with indicators that read out changes in membrane potential brought about by ion channel open and closing. Drugs affecting conductance of ion channels account for approx 5% of all drugs currently on the market ***(27,28)***. There are number of dyes that can sense voltage changes in whole cells and are therefore compatible with HTS and HCS formats ***(29–31)***. The voltage sensing dyes read out slow (seconds to minutes) and faster (subsecond) time frames. For fast sensing, there are the styryl dyes like di-4- and di-8-ANEPPS ***(32)***. Their fast response, need for ratiometric excitation sampling, and dim response properties (10% change over 100 mV change) are likely not HCS compatible, unless a device is optimized for their rapid response times and sensitivity. Yet some of the other fast-response (>40 ms) dyes have been optimized for high-throughput applications. The voltage sensor probes (VSPs) provide a FRET-based voltage-sensing technology for live cells ***(33,34)***. The FRET donor-acceptor pair in each VSP consists of a fluorescent, membrane-bound, coumarin-labeled phospholipid donor (CC2-DMPE) and one of two highly fluorescent, mobile, voltage-sensitive oxonol acceptors: $DiSBAC_2(3)$ (bis-[1,3-diethylthiobarbituric acid] trimethine oxonol) or $DiSBAC_6(3)$ (bis-[1,3-dibutylbarbituric acid] trimethine oxonol). In resting cells, the oxonol acceptors: orient on the extracellular side of the plasma membrane, away from the net negatively charged cell interior and near its FRET partner. With depolarization or net positive ion buildup internally, the oxonols reorient to the inner leaflet, separating the FRET pair and disrupting the FRET signal. When optimized there is 40% change in the FRET emission ratio per 100 mV change. Again, as with the fast styrl dyes, a specialized machine is required. The voltage ion sensing device from Aurora Biosciences, (San Diego, CA) is one such instrument in which more than 32,000 wells a day can be screened using this technology ***(35)***.

$DiBAC_2(3)$, $DiBAC_4(3)$, and other slow response oxonol dyes, can also be used alone ***(36)***, or with carbocyanine-based $DiOC_2(3)$ and $DiOC_5(3)$ reagents. These indicators require that the depolarization event to be maintained for minutes before a clear change in fluorescence is detectable (increased light emitted with depolarization for DiBACs or decreased light for DiOCs). The oxonol based or DiBAC dyes can be used in any microplate reader, including the popular fluorometric imaging plate reader ***(37,38)*** from Molecular Devices, Cell-Lux from PerkinElmer and the Hamamatsu FDSS imaging based plate reader for kinetic cellular assays. With kinetic updates of 0.5–1 s a more complete picture of voltage responses can be obtained. The long-term nature of their signal production can potentially make them highly suitable for live cell analysis under HCS conditions as well in which individual cell-to-cell variations in response are anticipated. For example, as primary cells are brought into the screening environment, such variability will likely arise.

Table 2
Cellular Probes From Invitrogen

Nucleoli	
SYTO®RNASelect™ green fluorescent cell stain	***Lv***-FX
Nucleus	
SYTO® Red fluorescent nucleic acid sampler kit for live cells	***Lv*** and ***Lv***-FX
Hoechst 33342	***Lv***
TO-PRO®-3	P-FX?
CellMask™ Red	***Lv***-FX
Vybrant® DyeCycle™ violet, green, or orange	***Lv***-FX
SelectFX® Nuclear Labeling Kit[a] DAPI, SYTOX® Green, 7-AAD, TO-PRO®-3	P-FX
DAPI	P-FX
Mitochondria	
MitoTracker Red CMXRos, Green FM or Orange CMTMRos	***Lv***-FX
MitoSOX™ Red Mitochondrial Superoxide Indicator	***Lv***
JC-1	***Lv***
SelectFX Alexa Fluor® 488 Cytochrome *c* Labeling Kit	P-FX
Anti-cytochrome oxidase subunit 1	P-FX
Endoplasmic reticulum	
ER-Tracker™ Blue-white DPX	***Lv***-FX[a]
ER-Tracker Red (BODIPY® TR glibenclamide)	***Lv***-FX[a]
ER-Tracker Green (BODIPY® FL glibenclamide)	***Lv***-FX[a]
SelectFX Alexa Fluor® 488 Endoplasmic reticulum labeling kit[a] for fixed cells[a]	P-FX
Golgi	
BODIPY® TR C_5-ceramide complexed to BSA	***Lv***
BODIPY® FL C_5-lactosylceramide complexed to BSA	***Lv***
Anti-golgin-97 (human), mouse IgG_1, monoclonal CDF4 (anti-Golgi)	P-FX
Lysosome	
LysoTracker® Green DND-26	***Lv***
LysoTracker® Red DND-99	***Lv***
LysoSensor™ Yellow/Blue DND-160	***Lv***
Cytoskeleton/actin microtubules	
TubulinTracker™ Green (Oregon Green® 488 Taxol, bis-acetate)	***Lv***
Alexa Fluor 488, 568, 594 phalloidin	P-FX
TRITC and FITC phalloidin	P-FX
Anti-α-tubulin (bovine), mouse IgG_1, monoclonal 236-10501	P-FX
Cytosol	
Calcein AM	***Lv***
CellTracker™ Green CMFDA (5-chloromethylfluorescein diacetate)	***Lv***-FX
CellTracker™ Red CMTPX	***Lv***-FX
CellMask™ Deep Red	P-FX
CellMask™ Blue	P-FX
CellMask™ Red	***Lv***-FX
Plasma membrane, lipid rafts	
FM® 4-64 FX[a]fixable analog of FM® 4-64 membrane stain	***Lv***-FX
FM® 1-43FX[a] fixable analog of FM® 1-43 membrane stain	***Lv***-FX
Alexa Fluor 350, 488, 555, 594, 647, 680 wheat germ agglutinin conjugates	***Lv***-FX[a]
Vybrant multicolor cell-labeling kit[a] DiO, DiI, DiD solutions, 1 mL each	

[a]Fixation okay but not permeabilization. For example, detergent will remove labeling and for some dyes, they are not aldehyde fixed but rather show high retention with Fixation.

Live only, *Lv*; Live and retained with fixation, *Lv*-FX; Postfixation only, P-FX.

4.4. K^+ and Na^+ Probes

In resting cells, internal Na^+ concentrations are held low, whereas K^+ concentrations are high. Externally the reverse is true. Consequently, a large Na^+ influx into a cell during an action potential generates a higher percentage concentration change in a restricted volume and is more amenable to detection. K^+ efflux has been more difficult to address, despite the critical need for its detection. For example, the hERG K^+ channel safety assay required of all candidate drug compounds, would benefit greatly from an improved fluorescence based approach. The similarity between K^+ and Na^+ further confounds the production of Ca^{2+} equivalent selective sensing reagents. Moreover, nature uses these ions for the most part in their salt forms, thereby influencing brief physiological properties, but not long lasting structural properties through chelation.

Compounds utilizing a crown ether moiety have been developed that have advanced analysis of these ions *(39)*. For sodium flux changes, Molecular Probes, offers a Sodium Green™ Na^+ indicator CoroNa™ Green Na^+ and red shifted, CoroNa Red Na^+. All of these dyes function nonratiometrically and work in the visible wavelengths producing brighter signals on Na^+ binding. The two fluorogenic CoroNa dyes, have been shown to label approx 20% of test cells, whereas Sodium Green™ Na^+ loaded less than 5%. This is most likely a result of the large size of the crown ether moieties. In microplate or single-cell assays these dyes represent the current "best-in-class" offerings. HCS would be an ideal way to amplify the apparent efficacy of these dyes—by ignoring nonloaded cells, differences will be markedly higher than a per-well integrated read typical of HTS readers.

4.5. "The Dyes That Bind": Low Cost Alternatives to Antibodies

A number of other reagents are worth noting for their utility in any image-driven physiological-based screens. Many existing HCS assays incorporate antibodies or expression reporters to measure specific targets. Costs per well, critical in scale up applications, typically run quite high for any antibody-dependent approach. Along with reagent expense, these approaches require more steps and are often only feasible on fixed and permeabilized preparations. Expression tags require cumbersome cloning projects, isolation of uniformly expressing cells, and are difficult with any primary line. Whenever a small organic molecule can be substituted, savings in time, effort, and expense are realized. A number of small molecule reagents are available with high-selectivity to subcellular compartments and proteins that are ideal for these applications.

Complete information about our live-dyes for tubulin, lysosomes, Golgi, endoplasmic reticulum, nucleoli, nucleus, mitochondria, plasma membrane, cytosol, and lipid rafts can be found in our website or handbook and overviewed in **Table 2**. Of these the ER tracker, nucleoli, nuclear, mitochondrial, plasma membrane (our FX versions of the FM dyes), and cytosolic dyes can be used with live cells then fixed or retained with most standard formaldehyde-based fixatives. These fixable live-dyes or *Lv*-FX dyes present a very utilitarian option for physiology studies in which live responses are sought, but the convenience and stability of a fixed sample are desired as well.

Organelle markers are not just suitable for landmarking subcellular regions, but make convenient fiduciaries in phenotype hunts, in which perturbations in normal cell structure or appearance is under examination. Although never specifically prompted for HCS, our cell friendly and plate friendly assays for viability, proliferation, and apoptosis are available that are compatible with live cells (**Table 3**) and many that work on fixed cells.

Criteria for accurately determining if a cell is undergoing apoptosis are nearly as vast as the current 100,000 and counting papers devoted to this critical area *(40–42)*. The TUNEL assay based on immunodetection of BrdU incorporation at fragmented DNA ends, and others that detect DNA laddering *(43,19)* remain the most reliable in terms of indicating irreversible entry into apoptosis. However other assays, including our recent offering of a mitochondrial permeability

Table 3
Vybrant Assays in Kit Form for Live Cell Study

Assays for live cell study
Vybrant Phagocytosis Assay Kit
Vybrant CFDA SE Cell Tracer Kit
Vybrant MTT Cell Proliferation Assay Kit
Vybrant Multidrug Resistance Assay Kit
Vybrant Cell Adhesion Assay Kit (calcein AM and SYTOX® green.)
Vybrant Cell Lineage Tracing Kit
Vybrant Cell Metabolic Assay Kit with C_{12}-resazurin
Vybrant Cytotoxicity Assay Kit G6PD Release Assay
Vybrant Alexa Fluor 488, 555, or 594, Lipid Raft Labeling Kit
Vybrant FAM Poly Caspases Assay Kit for Flow Cytometry
Vybrant Tubulin Staining Kit[a] for Live Cells 100 Assays
Apoptosis assays
APO-BrdU™ TUNEL Assay Kit with Alexa Fluor 488 anti-BrdU
Vybrant Apoptosis Assay Kit 1 Alexa Fluor 488 annexin V/SYTOX® green
Vybrant Apoptosis Assay Kit 2 Alexa Fluor 488 annexin V/propidium iodide (PI)
Vybrant Apoptosis Assay Kit 3 FITC annexin V/propidium iodide
Vybrant Apoptosis Assay Kit 4 YO-PRO®-1/propidium iodide
Vybrant Apoptosis Assay Kit 5 Hoechst 33342/propidium iodide
Vybrant Apoptosis Assay Kit 6 biotin-X annexin V/Alexa Fluor 350 streptavidin/PI
Vybrant Apoptosis Assay Kit 7 Hoechst 33342/YO-PRO®-1/propidium iodide
Vybrant Apoptosis Assay Kit 8 R-phycoerythrin annexin V/SYTOX green
Vybrant Apoptosis Assay Kit 9 allophycocyanin annexin V/SYTOX green
Vybrant Apoptosis Assay Kit 10 allophycocyanin annexin V/C_{12}-resazurin/SYTOX green
Vybrant Apoptosis Assay Kit 11 Alexa Fluor 488 annexin V/MitoTracker® Red CMXRos

transition pore assay, are useful in indicating the earliest emergence of apoptotic characteristics. In the MPTP assay, dye leaking out of mitochondria is quenched by cobalt in the cytoplasm: dimmer mitos are in the very earliest stages of apoptosis *(44)*.

We have also reformulated our very accurate CyQUANT® plate based cell counting assay. Now called CyQUANT NF (new formulation or no-freeze, [Molecular Probes/Invitrogen]), Assay for Cell Proliferation. This version is compatible with automation, imaging and now even postcount analysis with antibody probes. The existing version of CyQUANT requires –80°C freeze then thaw cycle. Although the freeze step provides a convenient storage mode, it remains cumbersome for robotics and severely compromises cell structure for later analysis. The flexibility, accuracy, multiplexing-capability, sensitivity, dynamic range from less than 100 cells to more than 20 K cells, compatibility with 96- and 384-plates and robust protocol of CyQUANT NF makes this new assay a perfect choice if (1) a wide range of cell numbers is being determined or (2) robotics are being utilized or (3) structural integrity, is critical. Like fluo-4 NW, following media removal, it is add and read.

Many competing cell count assays require a stable or constant metabolic activity for their determination—not always achievable or predictable in screens. As if to emphasize that point, BioLog (Hayward, CA) has built a cell-based phenotypic screening device, which measures the immediate alterations in normal cell metabolism with exposure to test compounds. Specifically, reductions in tetrazolium-based reagent turnover, a measure of cell redox or respiratory activity, is interpreted as a measure of a compound's cytotoxicity. With the DNA based cell counting tools, CyQUANT and Molecular Probes' new CyQUANT NF assay (Eugene, OR), fluctuations in cell number determinations produced by variations in metabolic activity can be avoided. Although it is somewhat tenable that compounds and treatments can affect DNA content, this is easier to control for. However, in critical applications, parallel determinations combining metabolic indicators, like

MTT (3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltertrazolium bromide) or the orange and more water-soluble version XTT (2,3-bis-[2-methoxy-4-nitro-5 sulfophenyl]-2H-tetrazolium-5-carboxyanilide) and CyQUANT NF would be the best safe guard.

Knowing cell number and shape, especially in dense cultures, is a challenge for any software system. Reagents that hasten cell segmentation or boundary determination are critical in most every assay, physiology-based or otherwise. One common trick is to use nucleic acid stained nuclei to infer cell number, whereas cell segmentation or cell masks are created by cytosolic markers like cytoskeleton or plasma membrane stains. However, a single dye for both nuclei and generalized cell masks could achieve both ends. The red emitting Draq-5 (BioStatus, Leicestershire, UK) is a popular reagent for staining DNA in live cells, and can be detected in multiple emission channels from green to red ***(45)***. But at most concentrations, faint cytoplasmic staining is observed and can serve this dual role as cell mask and nuclear stain.

In one of those ironic cases of not knowing when a flaw is indeed a feature, we have traditionally failed nucleic acid stains that showed this type of faint cytoplasmic staining and broad excitation maxima. But indeed compounds exist that have exactly these features and improve on Draq-5 by being fixable, even working in fixation solutions and are stable, working well in cells and tissue preparations. Our new dye CellMask Red will help in assays in which, nuclear and cytoplasmic dimensions are sought in one step on fixed cells and tissues as well as live cells. By comparison Draq 5 is poorly retained in fixed cells or tissue. In our studies it has been quite useful in cell counts and cell spreading, segmentation and similar cell masking protocols.

There are additional physiological indicators that space here does not permit covering. For our part, we continue to qualify reagents based on the uniformity of their response, fluorogenic always preferred, cell permeability and fidelity to a given response a requirement. The mitochondrial superoxide sensor, MitoSOX™ is a prime example. Overburden a cell with oxidative stress and this dye will glow—likely indicative of cell stress or toxicity. This has not been shown yet to be HCS friendly—nor is it fixable but it reads out a key new therapeutic target in cancer, aging, fertility, ADME-Tox, and more ***(46)***. Additional reagents for lipid metabolism accumulation, glucose, nitric oxide, and others await testing under the demanding auspices of HCS paradigms.

4.6. Standards and Optimizing Photon Output

Translocation based assays built on expression tags are emerging as critical tool in this field, despite requiring cell transfections and often heavy licensing fees ***(8,6,47)***. The biggest contribution to this set of assays are the collection of FPs and expression tags, discussed in great detail in Chapters 14 and 15.

Due in large part to the abundance of GFP reagents, dyes that fall outside of this excitation/emission range can facilitate multiplexing. Expression reporter compatible dyes are blue- and red-shift and when partnered with a FP indicator can make valued tools for unraveling complex physiological pathways.

For calibrating your imaging instrumentation Molecular Probes offers excellent bead-based dye standards, for intensity, size, spectral accuracy, and even spectral unmixing. Flow Cytometrists have long appreciated the critical need of a calibrated instrument, understanding the value of both accuracy and precision of their device, especially in any longitudinal studies. For that reason microspheres are routinely used in many flow labs every day to calibrate their instruments. The case for equivalent vigilance in imaging has been made as well ***(48,49)***. At present these microspheres are available on slides or in solution and provide useful means to establish signal-intensity level evaluate spectral accuracy, and alignment, and assist in size determination, Z-resolution, and more. It would be nice to know that an ever dimming signal is in fact a machine drifting out of specs, a bulb or lamp source failing, a thicker dish impairing light delivery or a score of other maladies, and not the assay itself.

The majority of existing HCS applications are fixed end-point reads, that incorporate one or two cell markers and an analyte selective reagent, usually an antibody or expression probe. Picking the right secondary antibody detection reagent is critical, with cost and brightness being the most important criteria to consider when scaling up for HCS, especially considering the comparatively light-starved dry objectives used in most instruments. To expand the dynamic range of antisera visualization, bright, photostable dyes are essential. Brightness is achieved through many mechanisms, initial brightness, maintained brightness, and amount of fluor per antibody. Although often expressed in terms of quantum yield and extinction coefficients, real-life sample-based determinations are more reasonable expressed in the simple values of how bright and for how long. The Alexa Fluor dyes (Molecular Probes/ Invitrogen) start out bright and are more photostable than equivalent organic dyes. Of additional virtue is the ability to load more dyes per IgG molecule than can be achieved with other dyes. Dye overloading leading to intramolecular quenching is a common failing of other dyes, whereas multiple molecules of Alexa Fluor dyes can be incorporated. Labeled secondaries are offered in nearly every variety. For the researcher interested in avoiding the extensive wash steps incumbent on secondary detection protocols, directly labeled primaries with high degree of labeling with these proprietary Alexa Fluor dyes might prove convenient. New microscale antibody kits optimized to label antibodies from 20 µg to 1 µg are available to expedite such labeling (Molecular Probes/Invitrogen).

In October of 2005, driven by activities on the Eugene Campus, Invitrogen purchased two companies making semiconductor nanocrystals, BioPixel and Quantum Dot Corp Inc. (Hayward, CA). Many features of these nanoparticles have intriguing possibilities in automated imaging. First, their initial brightness is equivalent to if not better than standard organic dyes. Second, they can all be excited at a single wavelength, removing much of the instrument costs and variability in excitation light. Third, their narrower spectral widths allow more analytes in the visible and near infrared region. Finally, their nearly concrete photostability allows for more constant signal strength for accurate intensity determinations, longer exposure times for dim signals, and storage convenience for later testing and retesting if needed.

In the near term we will continue to add parameters in characterizing our dyes that more accurately predict their utility in this promising area of automated, scaled up imaging. Wash steps, a trivial inconvenience in small samples can present an enormous barrier to automation and cost containment. Moreover, wash steps confound any discovery assays, in which perturbants of cell adhesion are sought. Serum in media confounds analysis by binding up dye and blocking their uptake. We are seeking ways to reduce this effect—and can recommend the Advanced D-MEM offered by our affiliate, GIBCO (Invitrogen). This DMEM substitute is designed to reduce serum formulations down to 1% thereby avoiding some of the confounding effects and costs of serum. In addition we are considering the strain on budgets and workflow that our current packaging and pricing directed at small throughput users is creating. Molecular Probes and Invitrogen as a whole is hoping to better enable all aspects of image-based discovery—from lead discovery to optimization to ADME-Tox to animals.

For more complete information on many of the Molecular Probes/Invitrogen Detection Technology products mentioned, please view the website (www.probes.invitrogen.com), or request a free copy of the most recent Molecular Probes/Invitrogen Handbook, volume 10.

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