



## Traditional and emerging methods for analyzing cell activity in cell culture

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**Abstract.** The selection of appropriate techniques to assay for markers of cell activity is important for obtaining optimal results in cell culture-based research. This paper is intended as a guide to many of the assays currently available and new techniques that have been recently introduced in the literature. This paper addresses both manual assay techniques, including the use of hemocytometers, phase contrast microscopy, cell staining, and the immunofluorescent antibody assay (IFA), and automated assays for cell activity, including stained optical density, prolif-

erating cell nuclear antigen, creatine kinase assay, DNA quantification, electronic cell counting, flow cytometry, magnetic cell sorting, image analysis, chemiluminescence, radioisotope labeling, precursor incorporation, *in-situ* hybridization/ligand binding, and enzyme-linked immuno-culture assay (ELICA). Advantages/disadvantages and applicability of these assays to different areas of cell culture research are discussed, and guidelines for selecting an appropriate assay are suggested.

**Key words:** Assays for activity, Cell culture, ELICA, IFA, Molecular markers

**Abbreviations:** ABTS = 2,2'-azino-di-(3ethylbenzthiazidine sulfonate); BrdU = 5-bromo-2-deoxyuridine; DMEM = Dulbecco's modified Eagle's medium; DNA = deoxyribonucleic acid; ELICA = enzyme-linked immuno-culture assay; FBS = fetal bovine serum; HRP = horse radish peroxidase; IFA = immuno-fluorescent assay; SDS/PAGE = sodium dodecyl sulfate/polyacrylamide gel electrophoresis; TMB = 3,3',5,5'-tetramethylbenzidine

### 1. Introduction

Development of cell culture systems has increased our understanding of the physiology of a large number of cell types. Examples of this growth in knowledge range from observations that some tumor-derived cells require specific nutrients, such as glutamine, to support cancerous cell growth [19], to definition of specific media formulations that support the growth and/or trigger differentiation of skeletal muscle satellite cells [23].

Analysis of cell activity *in vitro* may involve various assays, from simply counting absolute cell numbers over time, to identifying multiple markers on the single cell level. If cultures contain more than one cell type, counting alone may not provide useful information, requiring that more cell-specific assays be used. Markers commonly assayed include receptors, enzymes, hormones, structural proteins, and RNA coding for specific proteins as an early indicator of expression of a specific developmental program.

Cellular activity is influenced by a variety of

factors including soluble hormones/growth factors, environmental conditions, nutrient availability, culture substrate, and time in culture. Determining the effects of exogenous factors on the activation, proliferation, and differentiation of cells in culture, or when certain proteins are expressed in cellular growth and differentiation/maturation, requires assays for markers of these processes that are both sensitive and specific [22, 84]. Markers based on the expression of specific genes that regulate cellular growth and development have been developed for many cell types.

Different techniques lend themselves to certain situations, such as the use of automated assays for determining the effects of large numbers of different treatments or microscopic assays for mapping colocalization of specific markers to areas on single cells. The selection of a technique should be based on knowledge of what assays are available and determination of which assay is most applicable to the area of interest. This paper is intended as a guide to the most prevalent techniques for analyzing the activity of cells in culture, as well as emerging

techniques that are currently being developed. Examples from muscle satellite cell culture have been included, where appropriate, based on our experience using these cells with a variety of assay protocols.

## 2. Procedures

### *Manual assays*

These are methods for quantifying the number of cells of a specific type in a culture of interest via direct observation through a microscope. A hemocytometer may be used to count cells that are cultured in suspension or adherent cells that have been released from the culture substratum [17]. The hemocytometer consists of a glass slide with a chamber which contains a specific volume when a coverslip is in place. Grids engraved on the surface of the glass allow a consistent area to be counted each time it is used. Adherent cells may also be counted while growing in culture through the use of phase contrast microscopy, which allows the cells to be visualized based on the diffraction of light using specialized filters and objectives [43, 50]. A number of 10× fields are counted and averaged to approximate the actual density, which is then multiplied times the area of the plate.

Phase contrast microscopy does not provide great detail of intracellular structure, but it does allow cell counting based on the outline of individual cell membranes. Greater detail of the morphology of adherent cells in culture may be achieved by staining with different dye solutions for viewing under light microscopy. Examples include stains such as Giemsa [23], methylene blue [15], Wright's stain, MTT tetrazolium [56], gentian violet, and neutral red [42], all of which stain viable cells, and trypan blue [64], which stains non-viable cells. There are also stains which are specific for lipids, such as oil red O [75]. Fluorescent chromatin stains such as Hoechst 33258, 33342, or DAPI [63, 77] may also be used for manual cell counting procedures on a fluorescent microscope, which allows assessment of nuclear condensation as an indicator of cellular apoptosis [43].

Protocols for detecting specific markers may be used for manual counting when the cells are stained using specific antibodies, coupled with an enzyme/insoluble substrate (Table 1) detection system (such as HRP and 4-chloro-1-naphthol substrate [84]). This technique allows identification of cells that are expressing a specific marker of interest, which may be correlated with morphological characteristics, if present, for identification of a specific cell subtype.

With manual counting techniques, it is possible to look for single or multiple markers at the indi-

**Table 1.** Enzymes and substrates with insoluble precipitates<sup>a</sup>

Enzyme	Substrate	Color of precipitated product
Alkaline phosphatase	5-bromo-4-chloro-3-indolylphosphate p-toluidine salt ( BCIP)	Blue/purple
	Nitroblue tetrazolium chloride (NBT)	Blue/purple
	Naphthol-AS-GR-phosphate and fast blue BN	Green
	Naphthol-AS-phosphate and fast blue BN	Blue
	Naphthol-AS-phosphate	Reddish purple
	Naphthol-AS-BI-phosphate	Reddish purple
	Naphthol-AS-BI-phosphate and fast red TR <sup>b</sup>	Red
	Naphthol-AS-BI-phosphate and new fuchsin	Crimson red
	Naphthol-AS-MX-phosphate and fast red TR	Red
	Pyronin Y	Reddish purple
Horseradish peroxidase	3-amino-9-ethylcarbazole (AEC)	Reddish brown
	4-chloro-1-naphthol (4CN)	Blue
	3,3'-diaminobenzidine tetrahydrochloride dihydrate (DAB)	Brown
	DAB with nickel enhancement	Blue/black
	Hanker-Yates Reagent	Blue/black
	Naphthol	Pinkish red
β-galactosidase	3,3', 5,5'-tetramethylbenzidine (TMB) <sup>c</sup>	Greenish brown
	5-bromo-4-chloro-3-indolyl-8-D-galactopyranoside (BCIG)	Blue
	5-bromo-4-chloro-3-indolyl-8-D-galactoside (X-gal)	Blue
	Halogenated indolyl-β-D-galactoside (Bluo-gal)	Blue
	Naphthol AS-BI-β-D-galactoside	Red

<sup>a</sup> Adapted from [38] – product names are used for example only, and implies no approval of products to the exclusion of other suitable products.

<sup>b</sup> Not suitable for Immunohistochemistry because the product is soluble in alcohol.

<sup>c</sup> Forms soluble or precipitable product depending on reaction conditions.

vidual cell level and to count only those cells that express specific markers or combinations of markers. Examples of this include identifying those cells that are undergoing a transition from quiescence to proliferation, or from proliferation to differentiation, based on both cell morphology and staining characteristics. Although manual counting is labor intensive, it has the advantage of not requiring specialized or elaborate equipment beyond a laboratory microscope (light, phase contrast, or fluorescent) with at least 4×, 10×, and 40× objectives and a hemocytometer, if desired. Counting cells for determination of cell number to approximate the level of proliferation is relatively accurate. However, when quantifying differentiation in cells that show morphological changes (such as skeletal muscle satellite cells), manual counting is more subjective. This is due to the difficulty of defining criteria to identify cells that have undergone differentiation, since events such as differentiation and morphological fusion of satellite cells may be, for a time at least, disassociated from each other [83].

Because the relationship between morphological changes and physiological stages is not absolute, it is possible to have results that are not representative of the actual transitions based on morphology alone. If performed in a consistent manner, cell counting may yield reasonable results, but different personnel and equipment may cause variation. There are always qualitative judgments that affect accuracy by introducing uncontrolled variables, and this occurs even when standards for determining differentiation (for example, myotubes defined as having three nuclei) have been implemented.

*A. Immunofluorescent assay.* The immunofluorescent assay (IFA) is a procedure using primary antibodies

against markers of interest present in cultured cells or tissue sections, followed by secondary fluorescent-labeled antibodies against the appropriate antibody class and species in which the primary antibody was produced. IFA has utility in many instances where precise visualization is important and provides the capability of localizing a specific marker to a specific population of cells, or even a specific location on an individual cell [7, 28, 41, 70].

Through the use of multiple antibodies and distinct fluorescent labels, multiple markers of interest can be simultaneously probed, which enables co-localization of specific proteins to be observed and documented using fluorescent microscopy [46, 70, 74]. This does, however, require that the primary antibodies used be produced in different species or be of a different IgG subtype or antibody class (i.e. IgM), and that the secondary antibodies have sufficient specificity for the species or subtype against which they were produced [31, 41]. Background fluorescence may be a problem if antibody specificity is not high enough or if there is non-specific binding of the antibodies due to inadequate blocking procedures.

The use of confocal microscopy allows additional information to be obtained, including co-localization of three or more separate fluorescent markers on separate channels and the combination of ultra high detail and three dimensional constructs for intracellular localization. [20, 41, 70]. The labeling compounds available range in emission wavelength from the blue area of the spectrum at 420 nm with 4',6-diamidino-2-phenylindole (DAPI) [20], to the green areas of the spectrum at 520 nm with fluorescein isothiocyanate (FITC) [41], and then into the red wavelengths with rhodamine (TRITC) at 570 nm [20, 29] and Texas Red at 620 nm [46, 74]. There are also numerous other markers (Table 2) between these

**Table 2.** Compounds for immunofluorescent assays<sup>a</sup>

Fluorescent compound	Excitation wavelength <sup>b</sup> (nm)	Emission wavelength <sup>b</sup> (nm)
Allophycocyanin	610–640	660
4', 6-diamidino-2-phenylindole (DAPI)	365	> 420
Fluorescein isothiocyanate (FITC)	450–495	520
Hoechst 33258	360	470
Lissamine rhodamine	570	590
R-phycoerythrin	555–620	634
B-phycoerythrin	545–565	575
R-phycoerythrin	480–565	578
RED613 <sup>TM</sup>	488	613
RED670 <sup>TM</sup>	488	670
Rhodamine	552	570
Spectrum Green <sup>TM</sup> (fluorescein derivative)	497	518
Spectrum Red <sup>TM</sup> (rhodamine derivative)	559	588
TEXAS RED <sup>®</sup>	596	620

<sup>a</sup> Adapted from [38] – product names are used for example only, and implies no approval of products to the exclusion of other suitable products.

<sup>b</sup> The wavelength may vary depending on the pH.

wavelengths [77]. These options allow markers to be selected so that the signals have adequate separation, providing clear images of individual markers on separate channels. The cells of interest need to be grown on glass cover slips or other surfaces that may be inverted onto a glass cover slip to allow the transmission of the laser light to the cells and clear imaging of the fluorescence. Growing cells on these surfaces be challenging, since they are often sub-optimal for attachment and growth.

Orientation with regard to cellular morphology may be ascertained based on the use of a confocal microscope to visualize intracellular structures such as the nucleus, using propidium iodide or ethidium bromide [37, 84], or a cytoskeletal component, such as actin, which is detected by utilizing a separate fluorescent marker [8, 47]. The resolution that can be obtained gives exceptional detail and illustrates colocalization by providing lucid visualization of the position of each of the markers. The digital images obtained with confocal microscopy may also be utilized for image analysis, which could include quantification of the amount of signal from a specific fluorescent marker and correlation with cell numbers or levels of other markers. This could allow automation of cell counts and comparisons between populations expressing different cellular markers and could also increase the amount of information that is obtained from confocal IFA.

#### *Automated assays*

Automated cell activity quantification may be accomplished in a variety of ways and has the advantage of allowing large numbers of cultures to be assayed with high rapidity, consistency and repeatability. The methods addressed range from those that simply count all cells to those that count only cells that meet specific criteria, which may include staining characteristics, cell size or morphology, and molecular markers expressed either on the cell surface or inside the cell. In some cases, the cells are counted indirectly by quantifying a marker which correlates with cell numbers, but these techniques still provide reliable results.

**A. Cell culture staining.** Estimates of cell numbers may be obtained by staining cells in the culture vessel with crystal violet [48], Coomassie blue, sulforhodamine B [13, 64], or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), which stains only viable cells [56], and then taking a measurement of the optical density of the well. These densitometer measurements can be correlated to cell number in a linear relationship, facilitating their use as an automated cell counting technique. Methylene blue has been used to stain adherent cells, cultured in 96 well plates, as a method for determining cell counts in proliferating cultures of pure equine

satellite cells or fibroblasts [15, 52]. Methylene blue is taken up by viable cells, and after any excess stain is removed, the stain contained in the cells is released into solution by lowering the pH, allowing the absorbance to be read on an automated spectrophotometric plate reader. This technique improves the accuracy over densitometer readings, since the stain in solution is homogenous and absorbance can be read at precisely 620 nm, reducing uncontrolled variables. Assays based on proliferating cell nuclear antigen (PCNA) can specifically identify cells undergoing active proliferation, utilizing one of several antibodies that have been developed against this antigen [44, 51, 53, 79]. This assay compares favorably to other methods for detecting proliferation, such as BrdU [72].

**B. Creatine kinase activity.** Creatine kinase, a late marker of differentiation in myogenic cultures, may be used to determine the level of satellite cell differentiation based on enzymatic activity [26]. This method utilizes an automated 96 well plate reader set at 405 nm to determine the activity of creatine kinase on thio-NAD substrate and allows large numbers of cultures to be assayed for the number of cells in the later stages of differentiation [57, 85].

**C. DNA quantification.** The chromatin stain diaminobenzoic acid (DABA) [34, 38, 84] allows automated cell counts to be performed based on the fluorescence of DABA bound to cellular DNA, which is elicited using a 420 nm excitation wavelength, resulting in emission at 510 nm. The results are correlated to known DNA quantities using a standard curve [84] or manual cell counts to relate the results to cell number. Other stains which can also be used for DNA-based assays include the Hoechst dyes 33258 and 33342 [27, 58, 63, 71] and DAPI [20, 63, 77].

**D. Electronic cell counters.** These cell counting systems (such as the Coulter counter) function by drawing cells in suspension through a small orifice. As the cells pass through, a current flowing through the orifice is altered, resulting in pulses that are proportional to cell volume. This enables both automated cell counting and cell size determination to be performed, which is important for ascertaining the level of cell maturity or proportions of different cells in mixed cultures. This technique is commonly used on cells that are cultured in suspension and may also be used on adherent cell types after an enzymatic step to release them from the culture substrate [32, 61].

**E. Flow cytometry.** This technique allows identification and separation of subpopulations of cells based on cell size (flow cytophotometry) or expression of a given cellular marker through the use of a variety of stains or specific fluorescent antibodies (flow

cytofluorometry) [4, 11, 12, 16, 33, 68, 76]. Cells pass through a laser beam, and based on the amount of light scatter or fluorescence detected by a photomultiplier tube, cells are diverted by applying a charge as they pass between oppositely charged plates [76]. Automated cell sorting using flow cytometry allows cells to be segregated into subpopulations with specific characteristics for further culture, alleviating the confounding possibility of other cell types being present. There are several different instruments that are capable of flow cytophotometry/cytofluorometry, including the fluorescent activated cell sorter (FACS) (Becton Dickinson), the Cytofluorograph (Ortho), and the Coulter cell sorter. These are all quite expensive and require substantial technical expertise to operate, but they provide a high level of accuracy when counting and separating cells based on a variety of size and cell membrane characteristics.

*F. Magnetic cell sorting.* This technique allows separation of certain populations of cells based on cell surface antigens [24, 55, 73]. The cell suspension is mixed with a specific antibody that has been conjugated to iron-containing beads. The antibody-bead complex then binds to the cell marker, allowing cells to be sorted by running the cell suspension/antibody conjugate past an electromagnet. This process attracts all the complex-bound cells to the magnet, while other cells are removed. The electromagnet is then switched off, releasing the complex-bound cells as a relatively pure population. The bead-antibody complex is then disassociated from the cells by enzymatic digestion (using trypsin or DNase depending upon the type of link) or trituration (vigorous pipeting). Cell counting can then be performed, and the sorted cells cultured for experimental use as above under flow cytometry. The advantage of this technique is that it can be performed without the expense associated with the flow cytometry apparatus, while providing similar, if not as accurate, results.

*G. Image analysis.* Image analysis involves the capture of digitized images and the subsequent manipulation these images to yield a clear contrast between the cell (or subcellular component) of interest and other cell types or background signals. This technology may be used to identify cell types based on morphology or staining characteristics that are not evident in the raw image or to automate cell counting procedures based on enhanced digital images. Acquiring a digital image allows flexibility to perform numerous manipulations, such as increasing contrast, decreasing background signal through filtering, and sharpening edges. This may be done for improved visualization of cell characteristics or in preparation for automated cell counting. Once the cells of interest can be distinguished by a

unique signal, the computer may then quantitate the area occupied by the cells labeled with a specific marker, which may then be correlated to cell size and number. This may be accomplished in many different ways, based on the type of information needed and the equipment available.

Ide et al. [36] developed an image-based cell counting method that needs no specialized image capture equipment but still requires the operator to identify and mark individual cells before the system may classify and count the cells of interest. Other image-based cell counting procedures are more fully automated [18, 69] and are even capable of assaying individual cell characteristics, such as the volume of secretory granules contained in cells of interest [25] or cell size and shape [59]. However, these methods require additional hardware and/or software to provide the extra features. Some of this equipment is becoming more readily obtainable and can add extensive capabilities, helping to justify its purchase.

Charge-coupled device (CCD) cameras that are compatible with microscope camera mounts may capture images directly from the microscope in real time, which allows the field to be viewed and corrected during the capture process and opens the possibility of capturing real time or time lapse video of cells which display locomotive or morphologic changes [1]. Ultra high sensitivity, photon-counting video image acquisition may be used in combination with the luminescent marker luciferase to identify cells that have been infected with a recombinant virus which carries the luciferase gene, while allowing the cells to continue living in culture [49].

The confocal microscope is another type of extremely sensitive device which obtains digital images and allows the use of specific channels to capture targeted wavelengths of fluorescence, facilitating the identification of cells with a specific marker without the interference from other wavelengths. Confocal microscopy also provides the capability of producing three dimensional images with precise colocalization of multiple markers (see section on Immuno-Fluorescent Antibody Assays). Image analysis equipment often has proprietary software that has been designed to complement the capabilities of the device, but additional features frequently can be added through supplemental software packages. Some of the packages currently in use include Mocha Software [59], NIH Image [36], and Image Pro Plus [54], among others. Software for image analysis is in a state of rapid evolution, which mandates that the latest software packages be evaluated when designing a system for a specific purpose.

*H. Chemiluminescence.* This technique utilizes luminescent substrates that give off light in proportion to the enzymatic activity of the bound enzyme, resulting in a highly sensitive amplified assay. The main advantage of chemiluminescent techniques is that

they allow detection of proteins down to sub-picogram levels [30]. Development of chemiluminescent substrates for HRP [78], alkaline phosphatase [14], and  $\beta$ -galactosidase [60] allows these sensitive enzymatic systems to be further amplified, resulting in a 10–100 fold increase in sensitivity. These can be used in conjunction with the ELICA protocol (see following ELICA section) directly on cells, or as an ELISA on cell culture supernatants.

There are also chemiluminescent techniques that involve removing a cell from culture and isolating the protein for Western blotting. This technology allows the detection of protein products isolated from a single cell, which is accomplished by labeling the proteins of the cell of interest with biotin esters of *N*-hydroxysuccinimides, which form covalent links to  $\alpha$ - and  $\epsilon$ -amino groups [30]. These biotinylated proteins are then separated using SDS/PAGE to allow classification based on molecular weight and then transferred to charged nylon membranes (electroblotting) to allow them to be labeled with a streptavidin-conjugated alkaline phosphatase enzyme. This enzyme then acts on the enzymatic substrate which decomposes at a low rate following phosphorylation and gives off photons that may be detected with standard radiographic films [14].

To maintain a sufficiently low background, a blocking agent should be selected which is effective and has been purified to remove any biotin or biotinylated proteins by running it through an avidin-agarose gel [30]. The use of pre-transfer biotinylation causes a shift in the molecular mass that corresponds to the number of biotin molecules that attached to the proteins, which must be compensated for in the results. This technique also requires that the proteins from the single cell go through several treatments, including isolation, biotinylation, SDS/PAGE, immunoblotting, and the chemiluminescence protocol before identification can be accomplished. Knowledge of the molecular weight of the protein, the level of biotinylation, and the charge characteristics of the protein must be obtained prior to using this technique.

**I. Radioisotope labeling.** Radioisotope labeling involves choosing a protein or a metabolite of interest and either incorporating a radioactive marker during synthesis (such as [ $^{125}$ I]) to the protein of interest [82] or using a simple compound that already contains a radioactive marker, such as [ $^{14}$ C] labeled  $\text{CO}_2$  [19], which functions in cellular metabolism. Radioisotope-labeled proteins will interact with the binding sites, cell receptors, and other cellular processing mechanisms of the cell in culture and allow localization of the receptor, binding protein, or site of action based on detection of radioactive decay. The labeled [ $^{14}$ C] $\text{O}_2$  may be used to evaluate cellular oxidation rates, and with new methods that have been developed, these assays may now be performed in 24-well

cell culture plate formats without needing to dislodge the adherent cells [19]. This provides valuable information about the cellular oxidative characteristics of the cells with respect to specific substrates. Other techniques that rely on radiolabeling include precursor incorporation and *in situ* hybridization/*in situ* ligand binding, but these are addressed in separate sections. Radioisotopes that are also commonly used in research include [ $^3$ H], [ $^{32}$ P], [ $^{33}$ P], [ $^{35}$ S], [ $^{45}$ Ca], [ $^{51}$ Cr], [ $^{59}$ Fe] and [ $^{131}$ I], of which [ $^{131}$ I] has the shortest half-life at 8.07 days, and [ $^{14}$ C] has the longest at 5568 years [9]. The use of specific radioisotopes is based on the detection requirements of the research, but the radioisotope with the shortest half-life and lowest risk to personnel should be chosen.

All techniques that require the use of radioactive materials have several drawbacks, including requiring long incubation times for detection and special handling procedures, licenses, and protocols for disposal of waste. This adds to the cost and technical difficulties of using the assay, unless other radioactive applications are already in use.

**J. Precursor incorporation.** The use of precursors of macromolecules as markers for cell activity is of value when assaying cells for activation or proliferation (cells that have entered the cell cycle), since this is the time that cells will incorporate labeled precursors in the process of DNA synthesis. A common metabolite for this purpose is BrdU, which can be detected after uptake and incorporation into the DNA, through the use of anti-BrdU antibodies [5, 65, 70]. BrdU is also used for inhibiting differentiation in cell cultures, such as muscle satellite cells, when added at  $1.6\text{--}8 \times 10^{-5}$  M concentration to the culture media [2, 3, 10]. Another precursor that is commonly used is [ $^3$ H]-thymidine, which is detected by autoradiography of the cultures or liquid scintillation counting after they have been exposed to the compound for a period of hours (pulse labeling) [3, 21, 81]. This pulse labeling marks those cells that were actively dividing at the time when the [ $^3$ H]-thymidine was added to the cultures, identifying the percentage of the population that had engaged in S phase at a given time point.

**K. *In situ* hybridization/*in situ* ligand binding.** These procedures are sensitive methods for detecting a specific mRNA of interest prior to protein production using radiolabeled antisense RNA probes and protein ligands [80]. These two techniques may be applied to cells in culture and to tissue sections, allowing study of both *in vitro* and *in vivo* systems for identification of the markers of interest. The use of radiolabeled probes allows localization of the sites of interaction through the detection of the radioactive decay. This provides information about the presence of mRNA prior to protein synthesis [44] or the affinity and ability of a receptor to bind a ligand.

These methods may be used to determine the effects of growth factors and hormones on the expression of mRNA and the production of receptors and proteins in either cell culture or whole tissue systems. The complexity and number of factors that influence the success of an *in situ* hybridization/*in situ* ligand binding protocol, including prevention of mRNA contamination, probe production, etc., can make this a challenging technique to implement.

*L. Enzyme linked immuno-culture assay (ELICA).* This assay is similar to the ELISA technique, but in place of antigen or antibody bound to the plate, the ELICA uses the cell culture directly as the antigen source, leaving the cells bound to the plate, and transferring the reacted substrate to a separate plate for analysis [84]. The ELICA utilizes a specific primary antibody, which is followed by a biotinylated secondary antibody against the antibody class and the species of the primary antibody. An amplification system utilizing an avidin-conjugated enzyme, which binds to the biotinylated antibody complex, is then detected with one of several soluble enzyme substrates (Table 3), including fluorogenic [39, 62] or

chemiluminescent [15, 60] amplified substrates (for a review of immunoenzymatic techniques, see [6]). The reacted substrate is then transferred to a 96-well plate, which is read in a spectrophotometric plate reader set at the appropriate wavelength for the specific substrate (Table 3). The ELICA method was first described by Young et al. [84] as a highly efficient protocol for detection of cellular markers, allowing automation of cell culture activity assays (Figure 1). In this protocol, horseradish peroxidase (HRP) was the enzyme used, and ABTS was used as the HRP substrate. ABTS is, however, less sensitive and more hazardous than other substrates, such as TMB, which is also more stable once the reaction is stopped [45]. This allows for greater accuracy and flexibility when reading the absorbance of the sample, since the reaction is not markedly light sensitive and does not proceed at a significant rate after addition of the stop solution.

A protocol using the TMB substrate has been developed [42, 66], with applications in detecting markers of differentiation associated with skeletal muscle satellite cells in culture. Used in combination with specific antibodies against markers of differen-

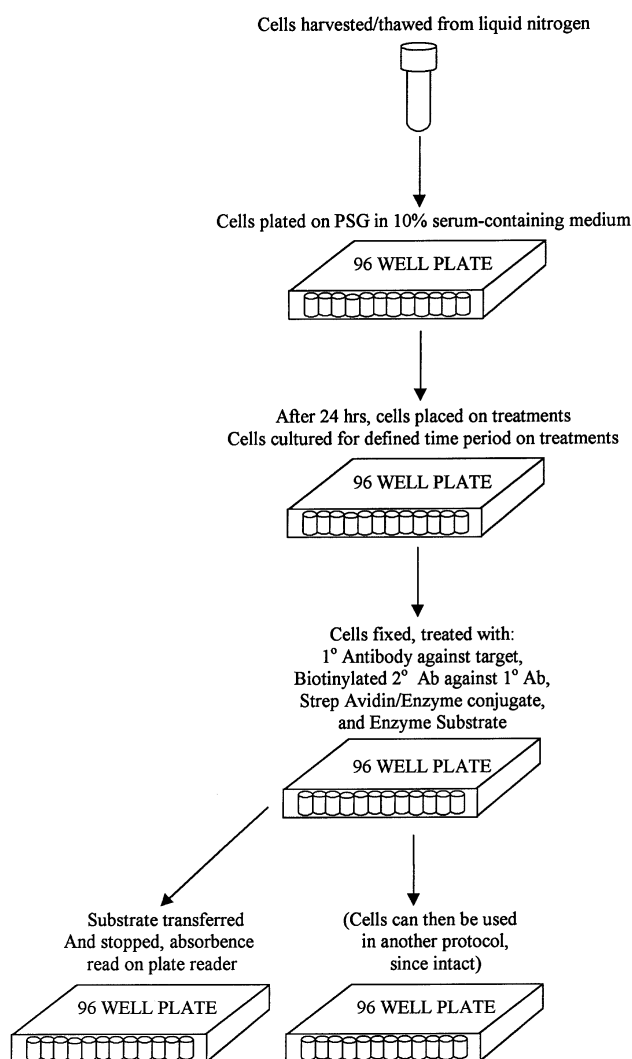
**Table 3.** Enzymes and soluble substrates used for ELICA/ELISA<sup>a</sup>

Enzyme	Substrate	Wavelength for monitoring reactions (nm)	
		Reacted	Stopped
Alkaline phosphatase	3-nitrophenyl phosphate (pNPP)	405	
	4-methylumbelliferyl-phosphate <sup>b</sup>	(Excitation 365)	(Emission 455)
	Disodium 4-chloro-3-(methoxyspiro [1,2-diox-etane-3,2'-(5'-chloro)tricyclo [3.3.1.1 <sup>3,7</sup> ] decan}-4-yl)phenyl phosphate	(Chemiluminescent) (CDP-Star <sup>TM</sup> )	(Visible light)
	Disodium 3-(4-methoxyspiro[1,2-diox-etane-3,2'-(5'-chloro)tricyclo[3.3.1.1 <sup>3,7</sup> ] decan}-4-yl)phenyl phosphate	(Chemiluminescent) (CSPD)	(Visible light)
Horseradish peroxidase	5-aminosalicylic acid (5AS)	450	
	2,2'azino-di-(3-ethylbenzidine sulfonate) (ABTS)	410	410
	o-dianisidine	403	
	o-phenylenediamine dihydrochloride (OPD)	450	492
	3,3', 5,5'-tetramethylbenzidine (TMB) <sup>c</sup>	650	450
	N-(4-amino-5-methoxy-2-methylphenyl) benzamide(AMMB) <sup>b</sup>	(Excitation 302)	(Emission ~450)
	N-acetyl-3,7-dihydroxyphenoxazine (Amplex Red) <sup>b</sup>	(Excitation 563)	(Emission 587)
	Luminol (diacylhydrazide)/4-iodophenol	(Chemiluminescent)	(Visible light)
$\beta$ -galactosidase	o-nitrophenyl- $\beta$ -D-galactopyranoside (oNPG)	410	
	p-nitrophenyl- $\beta$ -D-galactopyranoside (pNPG)	405	410
	chorophenolred- $\beta$ -D-galactopyranoside (CNPG)	578	
	4-methylumbelliferyl- $\beta$ -D-galactoside <sup>b</sup>	(Excitation 365)	(Emission 455)
	Galacton Plus <sup>TM</sup> with Emerald II <sup>TM</sup>	(Chemiluminescent)	(Visible light)

<sup>a</sup> Adapted from [38] – product names are used for example only, and implies no approval of products to the exclusion of other suitable products.

<sup>b</sup> Fluorogenic substrates require an excitation light source at the first wavelength, and then emit light at the second wavelength.

<sup>c</sup> Forms soluble or precipitable product depending on reaction conditions.



**Figure 1.** Flow diagram of the ELICA protocol. (a) Cells are harvested for primary cultures, or thawed from liquid nitrogen for clonal or cell line cultures, plated into a 96 well cell culture plate coated with a PSG substratum, and supplied with a 10% serum-containing medium for the first 24 hours. (b) Cultures are then placed on treatments, and grown for the length of time that has been set in the experimental design. (c) Cultures are next fixed in methanol and treated with: the primary antibody to the marker of interest, the biotinylated secondary antibody specific for the species or subtype of the primary antibody, and the avidin conjugated enzyme. Blocking agents are used to prevent non-specific binding, and extensive washing steps remove any unbound reagents. The enzyme substrate is then incubated in the wells which contain the labeled cells. (d) Next, the enzyme substrate is transferred to a second 96-well plate which contains the pre-measured stop solution to stop the reaction. The absorbance of the stopped substrate in this plate is read using the spectrophotometric plate reader set at the correct wavelength for the reaction. The original cell culture plate still contains the adherent cells, which can be further analyzed using other protocols, including morphological and marker localization, since the cellular structure remains intact [74].

tiation and protein products of differentiation, the ELICA has a high level of sensitivity (down to ~80 pg) and can be adapted and used efficiently for multiple markers of interest at different stages of differentiation [57, 66, 84]. Other cell types and cellular markers at any stage of cell growth or development could also be assayed using this system, since the attributes mentioned above would be equally advantageous to other areas of interest, and the adaptations should only increase the number of markers that can be assayed from the 33 which were tested by Young et al. [84].

Depending on the location of the marker of interest, the ELICA may be used to detect both intracellular and cell surface markers by adjusting the solubilization process to remove or retain the cellular membrane [84]. The assay does not disturb cells, allowing further analysis using other assay protocols, such as a second precipitating chromogenic substrate (4-chlor-1-naphthol) for direct visualization and localization of the marker of interest or cellular DNA quantification to correlate the results of the ELICA to a standard cell quantification assay [84]. The combination of the high sensitivity, the rapid and non-destructive technique of the assay, and the 96-well plate format make this a useful assay for a wide range of applications.

### 3. Discussion

The techniques presented in this paper are suited to many different cell culture situations and to the detection of various markers of cell activity in cell culture. A method may be selected that will be most applicable to the area of research, depending on the type of cell activity that is being assayed, the number of cells or the amount of marker that may be present in the sample of interest, the needed sensitivity level, what is known about the expression and characteristics of the marker of interest, and the number of cultures that need to be assayed (Table 4). Deciding whether protein expression will allow detection of the product, or if the early presence of mRNA would make it serve as a superior marker, will determine if *in situ* hybridization is necessary [35]. The level of sensitivity required should be considered, and if ultra high sensitivity is needed, an amplified assay, such as the biotin/avidin/enzyme ELICA or chemiluminescent assay, may be optimal.

Another consideration is whether the protocol chosen leaves the cells attached and intact, allowing further analysis with other protocols, as can be done with the ELICA system [84]. Choosing methods that do not require toxic or radioactive substances, prolonged incubation times, or difficult manipulations of the cultures can facilitate productivity and improve safety. The systems that are currently available offer many capabilities, and further development of assays

**Table 4.** Summary of techniques presented

Category	Name of technique	Equipment required	Functional limits	Advantages/drawbacks
Manual assays	Hemocytometer [17] (cell suspensions)	Microscope, hemocytometer	$< 10^4$ , $> 10^7$ cells/ml	Inexpensive/labor intensive, cells must be in suspension
	Phase Contrast Microscopy [43, 50] (living adherent cells)	Phase contrast microscope	$> 200$ cells/HPF	Visualize living cell structure/labor intensive, inaccurate
	Cell Staining [15, 23, 42, 56, 64, 75, 77] (fixed adherent cells)	Light microscope	$> 200$ cells/HPF	Enhanced visualization/ labor intensive
	Ab/Enzyme/Precipitating Substrate [84] (fixed adherent cells)	Light microscope	$> 200$ cells/HPF	Visualization of marker/ labor intensive
	Immunofluorescent Assay (IFA) [46, 70, 74] (fixed adherent cells)	Fluorescent/ Confocal microscope	$> 300$ cells/HPF (3-D = increased #)	Colocalization, high detail, 3-D/expensive instrument
Automated assays	Stained Optical Density [13, 44, 48, 56, 79] (fixed adherent cells)	Densitometric 96 well plate reader	$< 10^3$ , $> 10^4$ cells/well	Rapid, simple/lower accuracy, reliability
	New Methylene Blue [15, 52] (fixed adherent cells)	Spectrophotometric 96 well plate reader	$< 10^3$ , $> 10^4$ cells/well	Rapid, reliable/only for cell proliferation
	Creatine Kinase [26, 57, 85] (fixed adherent cells)	Spectrophotometric 96 well plate reader	$< 10^3$ , $> 10^4$ cells/well	Rapid, reliable/only for myogenic cell differentiation
	DNA Quantification [20, 27, 34] (fixed adherent cells)	Fluorometric 96 well plate reader	$< 10^3$ , $> 10^4$ cells/well	Rapid, reliable/only for DNA, dividing cells alter results
	Electronic Cell Counting [32, 61] (living cells in suspension)	Electronic cell counter (Coulter counter)	$< 10^3$ , $> 10^7$ total cells	Rapid, reliable, counting and sizing/cells must be in suspension
	Flow Cytometry [4, 11, 12, 16, 33, 68, 76] (living cells in suspension)	Flow cytometer (FACS)	$< 10^3$ , $> 10^7$ total cells	Rapid, reliable, counting, precision separation of marked cells/cells must be in suspension, expensive instrument
	Magnetic Cell Sorting [24, 55, 73] (living cells in suspension)	Electromagnetic cell sorting chamber	$< 10^2$ , $> 10^8$ total cells	Reliable counting and separation of marked cells/ Cells must be in suspension
	Image Analysis [1, 18, 36, 59, 69] (fixed adherent cells)	CCD camera or scanner	$< 1$ , $> 50$ cells/mm <sup>2</sup>	Improved image quality, analysis capabilities/ expensive equipment
	Chemiluminescence [14, 30, 60, 78] (fixed adherent cells)	+/- SDS/PAGE/ Western Blot Apparatus	$\sim 1$ , $> 10^4$ cells/well	High sensitivity/ complex procedure
	Radioisotope Labeling [19, 82] (fixed adherent cells)	Radioactivity shields, dedicated hood, Geiger counter	$< 10^3$ , $> 10^4$ cells/well	High sensitivity/ dangerous reagents
	Precursor incorporation [2, 3, 5, 10, 65, 70] (living/fixed adherent cells)	Radioactivity shields, dedicated hood, Geiger counter	$< 10^3$ , $> 10^4$ cells/well	High sensitivity/ dangerous reagents
	<i>In Situ</i> Hybridization/ Ligand Binding [44, 80] (fixed adherent cells)	Radioactivity shields, dedicated hood, Geiger counter	$< 10^2$ , $> 10^4$ cells/well	High sensitivity, ability to detect mRNA, specific markers/dangerous reagents
	Enzyme-Linked Immuno- Culture Assay [15, 39, 60, 62, 66, 84] (fixed adherent cells)	Spectrophotometric 96 well plate reader	$< 10^2$ , $> 10^4$ cells/well	High sensitivity, rapidity, reliability, cells intact/ new technique

for cell activity will open other possibilities for additional advanced procedures in the future.

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