Functional Assays and Toxicity Screening

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Contents

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What You will Learn in This Chapter and Associated Exercises

Students will be taught how to evaluate viability of isolated cells using several widely accepted methods. Cell type–specific functional assays will be then briefly discussed. Concept of the standard curve, blank, and positive and negative controls will be then introduced, followed by practical exercises to test toxicity of different compounds using these concepts.

5.1 Assessment of Cell Viability

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Viability assays are used to determine how many viable cells are present after their isolation from the tissue, and how these cells survive in culture under different culturing conditions. They are also used to assess the success of routine steps such as cell passaging, cryopreservation, or thawing (more in ▶ Chap. 6). During the 3D printing process, evaluation of cell viability is important during multiple time points, that is, *before* cells are mixed with bioink material, *after* extrusion and crosslinking, and *during* subsequent tissue formation. Lastly, viability assays are widely used to evaluate the toxicity of different drugs or treatments. Most viability assays can be modified to work for cells in suspension, cultured cells, as well as for cells residing with scaffolds of engineered tissue.

Based on the specific end goal, viability assays rely on different aspects of cell metabolism, being it mitochondrial activity, protein turnover, ATP production, DNA replication rate, and others [1]. Regardless of the chosen assay, there are many indirect factors that can influence the assay's outcome. These factors include possible effects of media components, cell surface-to-reagent volume ratio, fluid evaporation, and many others. Before choosing an assay, students have to understand exactly what information has to be quantified, being it the number of live/dead cells (viability/cytotoxicity assay), the total number of cells (proliferation assay), or the exact mechanism of the cell death (e.g., necrosis, apoptosis). The simplest method to examine viability is to stain cells with Trypan blue as described in ▶ Chap. 4. Trypan blue is a synthetic dye that selectively stains cells with compromised membranes. Upon entry into the cell, Trypan blue binds to intracellular proteins, thereby rendering cells dark. Cell viability can be then calculated as the percent of live cells versus total cell numbers as shown in ◘ Fig. 4.4.

5.2 The Most Commonly Used Assays to Evaluate Overall Cell Viability: DAPI, Ethidium Bromide

Another method of distinguishing dead or dying cells is the use of DNA binding fluorescent dyes. These dyes are impermeable through the cell membrane when cells are live; therefore, they can be used as fluorescent indicators of dead cells. There are many fluorescent dyes such as propidium iodide (PI), ethidium bromide (EB), 7-aminoactinomycin D (7-AAD), 4′,6-diamidine-2-phenylindole (DAPI), and acridine orange (AO) that can be used on live cells without fixing. Upon interaction with nucleotides, these dyes exhibit intense fluorescence. DAPI molecules attach at the minor groove of the DNA double helix. EB intercalates inside the DNA double helix.

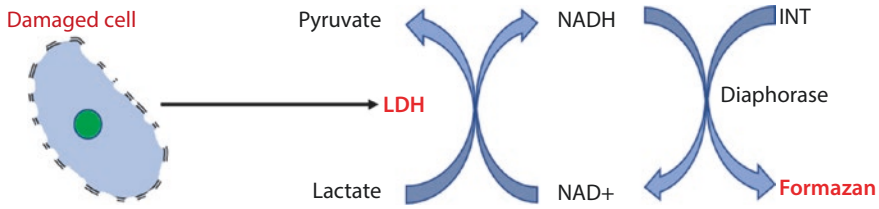

Fig. 5.1 Chemistry behind the LDH cytotoxicity assay

LDH assay Lactate dehydrogenase (LDH) is a cytosolic enzyme found in all living cells. LDH cytotoxicity assay measures the release of LDH into the media, which occurs when the cell plasma membrane is damaged. To quantify the amount of released LDH, the following enzymatic reaction is used. First, LDH catalyzes the conversion of lactate to pyruvate acid, as it converts NAD^+ to NADH. Second, diaphorase uses NADH to reduce a tetrazolium salt to a red formazan product (Fig. 5.1). Therefore, the level of formazan formation is directly proportional to the amount of released LDH in the medium.

Resazurin reduction assay Resazurin sodium salt is a cell-permeable non-fluorescent dye that can be used to monitor the number of viable cells. After adding blue colored resazurin in the media, viable cells reduce it into the pink and fluorescent resorufin. The produced resorufin quantity is proportional to the number of viable cells and can be quantified either by measuring a red fluorescence using a fluorometer (Excitation/Emission 560/590 nm) or by measuring a change in absorbance at 570 nm (Fig. 5.2).

MTT Tetrazolium reduction assay The methyl-thiazolyl-tetrazolium assay is a standard colorimetric assay used to assess the metabolic activity of cells. It measures the reduction of yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

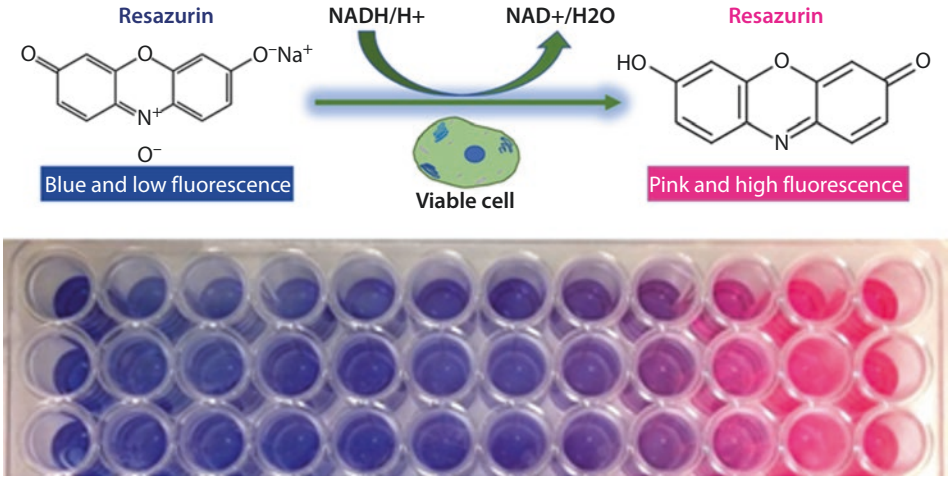

Fig. 5.2 Resazurin assay. Top: intracellular conversion of non-fluorescent blue resazurin to pink fluorescent resorufin. Bottom: an image of a cell plate showing the conversion of blue resazurin to pink resorufin with triplicates for each condition with cells progressively losing their viability from right to left

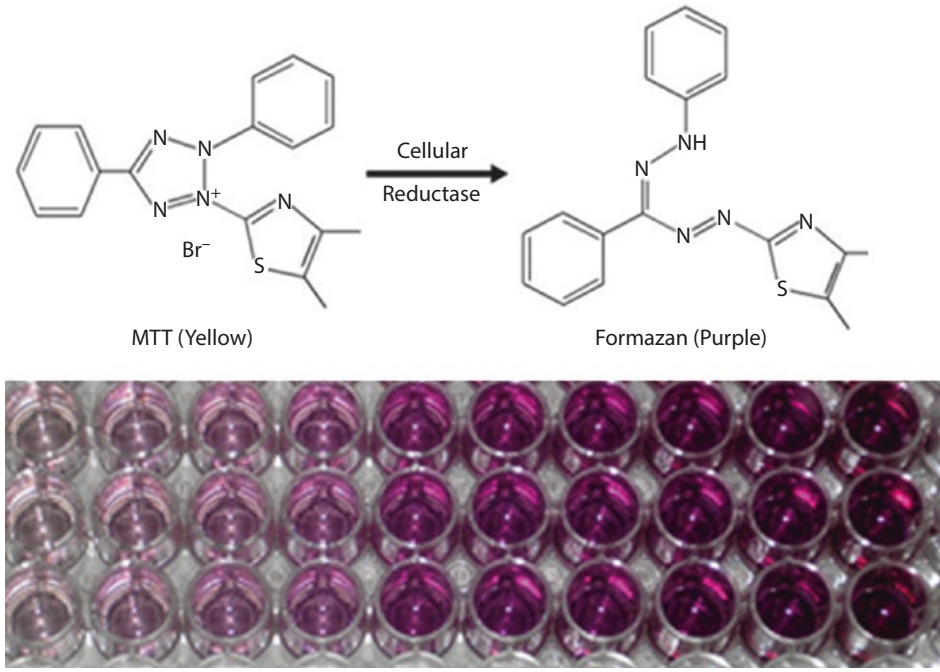

Fig. 5.3 MTT assay. Top: formula showing the conversion of MTT to formazan upon the action of intracellular reductases. Bottom: image of a cell plate after MTT assay showing the conversion of a clear solution to purple with three samples for each condition with cells progressively losing their viability from right to left

by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, colored formazan (dark purple) product (Fig. 5.3). To dissolve precipitated formazan crystals, MTT solvent should be added. The quantity of formazan is measured by reading absorbance at 570 nm using microplate spectrophotometer.

LIVE/DEAD assay A number of commercially available viability assay kits use two-color fluorescent reagents to discriminate the population of live cells from the dead cell population. The most commonly used LIVE/DEAD cell assay measures intracellular esterase activity using properties of *calcein-AM* and plasma membrane integrity using *ethidium bromide*. Esterase activity removes esters from the non-fluorescent and cell-permeable form of calcein-AM dye converting it to a highly fluorescent non-ester form. The latter has multiple charges and as such is well retained within live cells. It yields an intense cytosolic fluorescence with excitation/emission maxima of 495 and 515 nm, respectively. Ethidium bromide enters cells with a damaged membrane. Once inside the cell nuclei, it undergoes a ~40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence in dead cells with excitation/emission maxima of 495 and 635 nm, respectively (Fig. 5.4).

Knowing the mechanism of each assay is critical for the correct interpretation of its results. For example, use of calcium-AM staining to evaluate the viability of cells upon treating them with a compound that affects intracellular esterase activity, while not necessarily killing these cells, can yield an erroneous conclusion. This is because

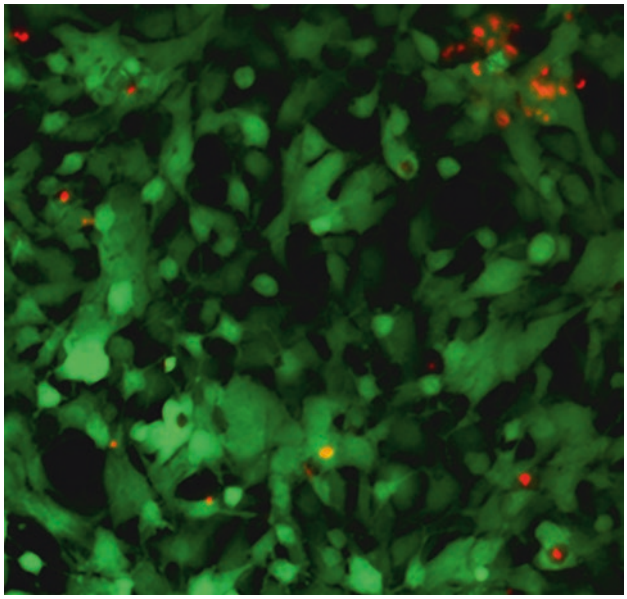

Fig. 5.4 Culture of mouse fibroblasts stained with LIVE/DEAD Cytotoxicity Assay Kit. Live cells exhibit bright green color due to the presence of calcein in the cytosol, whereas dead cells with compromised membranes have red-stained nuclei

intracellular esterases are involved in removing ester moieties of calcium-AM dye, which leads to trapping dye within the cell. One has to also keep in mind that cell viability is a rather general term that can include cytotoxic, cytostatic, and antiproliferative effects.

5.3 Controls and Linear Range

Another important issue to consider is the design of appropriate controls. Many factors can affect measurement endpoints, so one needs to make sure that outcomes of viability assays are not mistakenly attributed to other factors. There are three types of controls that can be used in cell viability assays. Those include positive control, negative control, and blank. *The positive control* is a sample that for sure will be affected by the treatment. For example, high concentration hydrogen peroxide can serve as a positive control. *The negative control* is a sample w/o treatment. Lastly, *the blank* means sample without any cells but with the media. The latter is needed to subtract the values of background fluorescence or absorbance for all the samples.

It is very important to establish the linearity of the assay curve as per Fig. 5.5. For each assay it is advised to set up a standard curve, which will allow to later transform data into a concentration-response curve. In the case of LDH assay, it can be a concentrated LDH solution; in case of resazurin, it can be a resorufin standard. The measurement data obtained from the treated samples should be compared to a standard curve of serially diluted standards specific for each assay.

To ensure the reproducibility of the measurements, it is recommended to perform all types of viability assays using at least three samples, that is, *triplicates*. The average of the three can then be used as a measure of viability for each sample in that particular experiment. The experiment itself then needs to be performed at least three times (see ▶ Chap. 1).

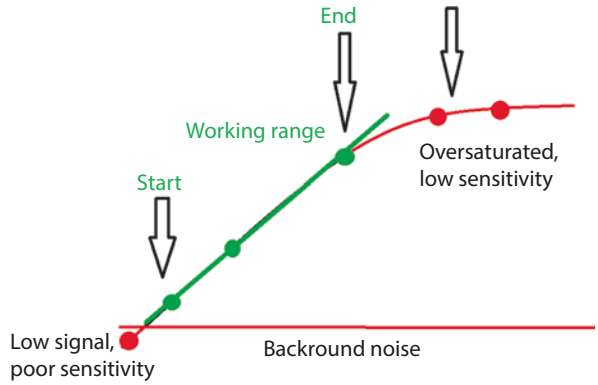

Fig. 5.5 Optimal results are obtained when sample values are found within the linear range shown in green

5.4 Functional Assays

The above viability assays are applicable to almost all cell types. The second set of assays is more specific as is used to evaluate a particular function of specific cell types (Fig. 5.6, *top panel*).

For example, the dynamic monitoring of intracellular calcium is a standard way to monitor the functional performance of muscle cells. This is done by loading cells with live calcium indicators, such as Fluo-3, Fluo-4, Fura, and many others. Upon binding of calcium ions to these dyes, their fluorescence dramatically changes. Calcium transient is a momentary >10-fold increase in the concentration of cytosolic calcium (Ca^{2+}). It occurs due to the rapid release and re-uptake of calcium ions from the sarcoplasmic reticulum. This transient increase in Ca^{2+} triggers the contraction of both cardiac and skeletal muscle cells. Quantitative indices extracted from recordings of calcium transients, such as transient amplitude, half times of upstroke and decay, and synchronicity, provide a great deal of information regarding the expression of specific channels in these cells. Voltage-sensitive dyes are used to record the activity of electrically active cells. These cells also called excitable cells and include cardiac, skeletal, and smooth muscle cells together with neurons.

Other types of functional assays are aimed at labeling the expression of particular genes or the production of different proteins coupled to a specific cell phenotype. For example, mature hepatocytes are expected to produce significant amounts of albumin; functionally active chondrocytes should deposit g*lycosaminoglycan*s (GAGs), fibroblasts, collagen, etc. Production of specific proteins or expression of specific genes can be monitored on both cell and tissue constructs levels. But there are also functional assays that only work for engineered tissues exceeding mm scale (Fig. 5.6, *bottom panel*). In the latter case, assessment relies on properties of engineered tissue that consists of many interconnected cells in close interaction with surrounding ECM or artificial scaffold material. Such macroscopic tissues can be stretched, compressed, bent, and electrically stimulated to produce measurable active force or tested for their barrier function (in case of tissue constructs emulating the skin).

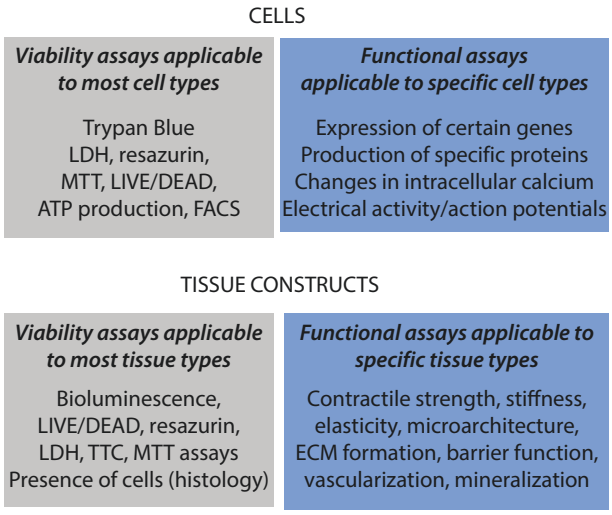

Fig. 5.6 Summary of different types of assays that can be used to evaluate survival and specific functions of cells and tissue constructs

Session I

Demonstration

The instructor exposes plated cells to different concentrations of hydrogen peroxide followed by either resazurin, LDH, or MTT assay. Recommended doses of H_2O_2 for 10 min exposure include 0.1 mM, 1 mM, 10 mM, and 100 mM peroxide. Assessment of cell contractions and other tissue-specific functional assays for toxicity assessment is also demonstrated and discussed. For example, the use of Fluo-4 to monitor calcium transients using plated rat neonatal cardiomyocytes from the previous week can be shown.

Homework

Based on a literature search, each team designs an experiment in which passaged cells from the previous week can be exposed to chemical or physical stress followed by cell viability assessment.

Session II

Team Exercises

Teams test chosen stress conditions and methods to determine cell viability afterward. Assay manufacturers provide a detailed description of each assay. Students are advised to carefully read these manuals before experiments. Their goal should be to create a viability curve that should have at least three points control (i.e., no damage), mid-point (some damage), and max damage (i.e., all cells dead) points. Cultured cells from the previous week or commercial cell lines can be used to conduct these experiments.

Homework

Teams use their experimental data to create a graph illustrating the effect of their chosen treatment on cell viability. Positive, negative, and blank samples must be included.

Sample Protocols

DAPI staining protocol

1. Remove culture media and rinse the sample with PBS.
2. Dilute dye stock solutions with PBS to 300 nM final concentration.
3. Add the appropriate volume of the diluted staining solution to the prepared coverslip. Make sure that the cells are completely covered.
4. Incubate for 5 min.
5. Wash the sample three times with PBS. Drain excess buffer and mount with Mowiol mounting medium.
6. Use a fluorescence microscope with appropriate filters to view the sample.

Protocol for LDH assay

1. Transfer supernatant from cell culture plates or treated cells into a new multiwell plate.
2. Add LDH reaction mixture according to manufacturer suggestions.
3. Incubate at room temperature for 30 min.
4. Add stop solution to stop the enzymatic reaction.
5. Measure absorbance with a spectrophotometer at 490 nm.

Protocol for resazurin assay

1. Add the specified amount of resazurin solution to the samples (in case of 96 wells this can be 10 μL of 0.15 mg/mL resazurin to the well containing 90 μL of cell culture media).
2. Incubate for 2–4 h at 37 °C in the dark.
3. Record fluorescence using a 560/590 nm (excitation/emission) filter set, or absorbance at 570 nm.

Procedure for MTT assay

1. Add a specified amount of MTT solution to the samples (in case of 96 wells this can be 10 μL of 5 mg/mL MTT solution to the well containing 90 μL of cell culture media).
2. Incubate for 2–4 h at 37 °C in the dark until purple precipitate becomes visible.
3. After incubation, remove the culture medium with excess MTT salt.
4. Add 100 μL of MTT solvent (10 mM HCl, 10% Triton-X100 in isopropanol).
5. Shake the plate for 20 min at room temperature to ensure complete dissolving of formazan crystals.
6. Measure the absorbance at 570 nm using a microplate spectrophotometer, or transfer the content of each well into an individual cuvette for separate measurements.

Take-Home Message/Lessons Learned

After reading this chapter and performing the requested assignments and exercises, students should be able to:

- Understand the difference between viability assays and functional assessment of cells and tissues
- Familiarize with most common ways to evaluate cell viability and differences between them
- Design cell viability experiment, which includes blank and positive and negative controls
- Make sample dilutions so measurements are taken within linear part of the standard curve

Self-Check Questions

Q.5.1. Which of the following assays is considered to be a tissue-specific *functional* assay?

A. Release of lactate dehydrogenase
B. Permeability to Trypan blue
C. Chondroitin production
D. Reduction of resazurin

Q.5.2. Which of the following assays is often used to noninvasively evaluate the survival of implanted tissue constructs?

A. Tensile strength
B. Load-bearing
C. Bioluminescence
D. LIVE/DEAD assay

Q.5.3. What type of control is NOT included when testing response to a drug or a treatment?

A. Blank
B. Neutral control
C. Positive control
D. Negative control

Q.5.4. Which assay recommends the transfer of accumulated media to a new well for viability testing?

A. Resazurin
B. LDH
C. MTT
D. Ethidium bromide

Q.5.5. Viability assays can rely on different aspects of cell metabolism, EXCEPT

A. Mitochondrial activity
B. Protein turnover
C. ATP production
D. DNA mutation rate

References and Further Reading

1. T.L. Riss, R.A Moravec, A.L. Niles et al. Cell Viability Assays. 2016. In: Sittampalam GS, Grossman A, Brimacombe K, et al., editors. Assay Guidance Manual [Internet]. Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences, https://www.ncbi.nlm.nih.gov/books/NBK144065; Präbst K, Engelhardt H, Ringgeler S, Hübner H. Basic Colorimetric Proliferation Assays: MTT, WST, and Resazurin. Methods Mol Biol. (1601), 1–17 (2017)