Cell analysis

Fluorescent cell viability assays on Countess Automated Cell Counters

Assessing cell viability is a key step in daily cell manipulation and is required for accurate and efficient downstream processing. The most common method by brightfield microscopy is trypan blue staining; however, many fluorescent options also exist and are frequently used in microscopy and flow cytometry applications. Fluorescent staining techniques offer benefits such as reduced counting of noncellular objects, selective staining of nucleated cells, and the ability to multiplex assays. Here we discuss four commonly used fluorescent viability kits, and the flexibility offered by Invitrogen™ EVOS™ 2.0 light cubes, which allow samples to be analyzed using a variety of proven reagents, depending upon workflow requirements.

Cell viability assay using the ReadyCount Green/Red Viability Stain

Introduction

The Invitrogen™ ReadyCount™ staining reagent is benchtop stable and provide the same ease of use for cell counting as trypan blue staining. ReadyCount Green/Red Viability Stain (Cat. No. A49905) contains a combination of acridine orange and propidium iodide to enable counting of all live and dead cells in green and all dead cells in red.

Materials

- Invitrogen™ Countess™ 3 FL Automated Cell Counter (Cat. No. AMQAF2000)
- Invitrogen™ Countess™ Cell Counting Chamber Slides (Cat. No. C10228) or Countess™ Reusable Slide (Cat. No. A25750)
- ReadyCount Green/Red Viability Stain (Cat. No. A49905): stains all nucleated cells; detected using EVOS GFP and EVOS RFP or Texas Red 2.0 light cubes

Methods

Instrument setup

1. Turn on the Countess 3 FL Automated Cell Counter and install the appropriate EVOS light cubes for the stains being used. Most commonly, the ReadyCount viability stains require the GFP and Texas Red 2.0 light cubes.
2. Install the appropriate slide holder for either the disposable or reusable slide.
3. Obtain a Countess disposable or reusable slide.

Protocol

1. Acquire the cell suspension and cell viability reagent. PBMCs or other primary sample isolates work well for this method, too.

1. Mix the ReadyCount Green/Red Viability Stain 1:1 (v/v) with the sample—10 µL of stain with 10 µL of your cell suspension. Mix well by pipetting up and down a few times.
2. Incubate up to 5 minutes at room temperature.
3. Gently pipet 10 µL of the sample into the sample loading area of the slide. The sample is loaded into the chamber through capillary action.
4. Optional: The default counts with fluorescent light cubes do not factor in a dilution factor.
   - Press “Protocols” and enter the names of the desired light cubes. Then enter the 1:1 dilution factor.
5. Let the sample mixture settle in the chamber for 30 seconds, and then insert the slide into the slide adapter to initiate autofocus. Your will hear a soft click once the slide is pushed in correctly.
6. Adjust light intensities to minimize fluorescent background. (The Countess II FL instrument requires manual fluorescent intensity adjustment, while the Countess 3 FL instrument has auto-lighting in fluorescence mode.)
7. Press “Count”.

Results

The ReadyCount Green/Red Viability Stain enables counting of all live and dead cells in green and all dead cells in red (Figure 1).

Summary

When used with cells stained with the ReadyCount Green/Red Viability Stain, the Countess 3 FL instrument displays total concentration, concentration and percentage of the counted objects that have green fluorescence (all cells), concentration and percentage of the counted objects that have red fluorescence (dead cells), and concentration and percentage of the counted objects that have both green and red fluorescence (dead cells).

Cell viability assay using the LIVE/DEAD Viability/Cytotoxicity Kit

Introduction

Ubiquitous intracellular esterase activity and an intact plasma membrane are distinguishing characteristics of live cells. The Invitrogen™ LIVE/DEAD™ Viability/Cytotoxicity Kit (Cat. No. L3224) quickly discriminates live from dead cells by simultaneously staining with green-fluorescent calcein AM to indicate intracellular esterase activity, and red-fluorescent ethidium homodimer-1 (EthD-1) to indicate loss of plasma membrane integrity. It is adaptable to most eukaryotic cells where cytotoxic conditions produce these cellular effects. This fluorescence-based method of assessing cell viability can be used in place of trypan blue exclusion, the ^{51}Cr release assay, and similar methods for determining cell viability and cytotoxicity.

A

B

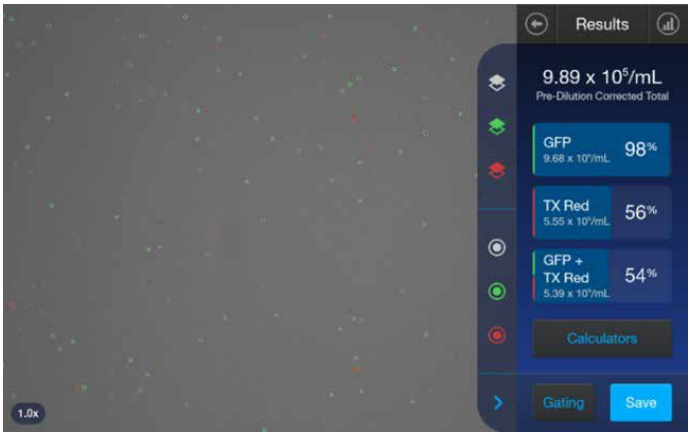

Figure 1. Viability assay via cell staining using the ReadyCount Green/Red Viability Stain. (A) Live and heat-killed U2OS cells were mixed and then stained with ReadyCount Green/Red Viability Stain at a 1:1 ratio, which is optimal for use with Countess instruments. The resulting suspension was counted on the Countess 3 FL instrument with EVOS GFP and Texas Red 2.0 light cubes, with a protocol factoring in the 1:1 dilution. **(B)** Sample of isolated PBMCs stained as described in (A), visualized with brightfield and fluorescent image overlay.

Materials

- Countess 3 FL Automated Cell Counter (Cat. No. AMQAF2000)
- Countess Cell Counting Chamber Slides (Cat. No. C10228) or Countess Reusable Slide (Cat. No. A25750)
- Calcein AM (included with the kit; Ex/Em: 494/517 nm): a cell-permeant dye that is converted to fluorescent calcein by live-cell esterase activity to produce intense, uniform green fluorescence. Calcein is detected with standard FITC/GFP filter sets or the EVOS GFP 2.0 light cube.
- EthD-1 (included with the kit; Ex/Em: 528/617 nm): a high-affinity stain that enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, producing bright red fluorescence in dead cells. EthD-1 is excluded by the intact plasma membrane of live cells. EthD-1 can be detected with EVOS RFP and Texas Red 2.0 light cubes; less spectral overlap and thus less gating is required with the Texas Red 2.0 light cube. Background fluorescence levels are low with this assay because the dyes are virtually nonfluorescent before interacting with cells.

Methods

Instrument setup

1. Turn on the Countess 3 FL Automated Cell Counter and install the appropriate EVOS light cubes for the stains being used. Most commonly, the LIVE/DEAD Viability/Cytotoxicity Kit requires the GFP and Texas Red 2.0 light cubes.
2. Install the appropriate slide holder for either the disposable or reusable slide.
3. Obtain a Countess disposable or reusable slide.

Protocol

1. Allow the calcein AM and EthD-1 to thaw and fully equilibrate to room temperature.
2. Make a 2 mL staining solution containing 2 µM calcein AM and 4 µM EthD-1 by adding 20 µL of each to 1.96 mL of PBS. Mix thoroughly.
3. Remove the culture medium from the cells after centrifugation for 5 min at 400 x *g*.
4. Add 100–200 µL of the staining solution directly to the cells.
5. Incubate for up to 30 minutes at room temperature.
6. Gently pipet 10 µL of the sample into the sample loading area of the slide. The sample is loaded into the chamber through capillary action.
7. Let the sample mixture settle in the chamber for 30 seconds, and then insert the slide into the slide adapter to initiate autofocus. Your will hear a soft click once the slide is pushed in correctly.
8. Adjust light intensities to minimize fluorescent background.
9. Press “Count”.

Results

The LIVE/DEAD Viability/Cytotoxicity Kit enables counting of all live cells in green and all dead cells in red (Figure 2).

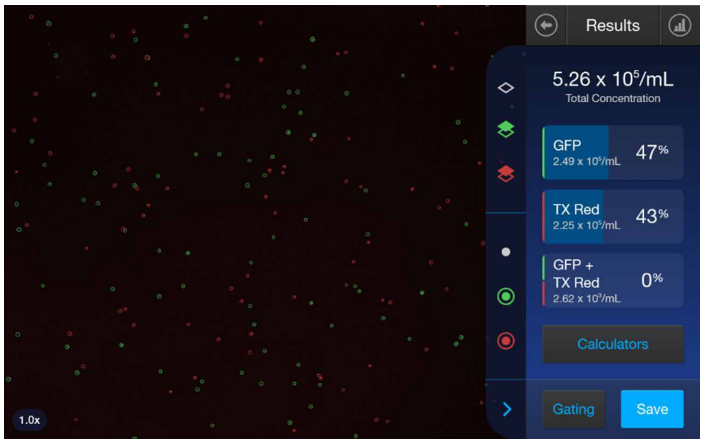

Figure 2. Viability assay via cell staining using the LIVE/DEAD Viability/Cytotoxicity Kit. Live and heat-killed human PBMCs were mixed and then stained with calcein AM and EthD-1 supplied with the kit. The resulting suspension was counted on the Countess 3 FL instrument with EVOS GFP and Texas Red 2.0 light cubes. The Countess 3 FL instrument is easier to use than the Countess II instrument because of the auto-lighting and histogram features in fluorescence mode.

Summary

When used with cells stained with the LIVE/DEAD Viability/Cytotoxicity Kit, the Countess 3 FL instrument displays total concentration, concentration and percentage of the counted objects that have green fluorescence (live cells), and concentration and percentage of the counted objects that have red fluorescence (dead cells).

Cell viability assay using the ReadyProbes Cell Viability Imaging Kit, Blue/Green

Introduction

The Invitrogen™ ReadyProbes™ Cell Viability Imaging Kit, Blue/Green (Cat. No. R37609), can be used to quickly and easily determine cell viability using an alternative blue and green dye combination, which is extremely useful in samples expressing red fluorophores such as RFP or mCherry. Similar assays cannot be done using the standard acridine orange/propidium iodide mixtures due to spectral overlap. Just add 2 drops each of room temperature–stable Invitrogen™ NucBlue™ Live reagent and NucGreen™ Dead reagent to 1 mL of cell growth medium, and then determine viability by counting total vs. dead cells.

Materials

- Countess 3 FL Automated Cell Counter (Cat. No. AMQAF2000)
- Countess Cell Counting Chamber Slides (Cat. No. C10228) or Countess Reusable Slide (Cat. No. A25750)
- NucBlue Live reagent (included with the kit; Ex/Em: 360/460 nm): stains the nuclei of all cells; detected with a standard DAPI filter.
- NucGreen Dead reagent (included with the kit; Ex/Em: 504/523 nm): stains only the nuclei of dead cells with compromised plasma membranes; detected with standard FITC/GFP (green) filter sets.

Methods

Suggestions for use

- NucBlue Live and NucGreen Dead reagents can be added directly to cells in a complete growth medium or a compatible buffer solution.
- In most cases, 2 drops per milliliter of suspension and an incubation time of 5–30 minutes will give bright nuclear staining; however, optimization may be needed for some cell types, conditions, and applications. In such cases, simply add more or fewer drops to obtain optimal staining intensity.

Instrument setup

1. Turn on the Countess 3 FL Automated Cell Counter and install the appropriate EVOS light cubes for the stains being used. Most commonly, the NucBlue Live and NucGreen Dead reagents require the DAPI and GFP 2.0 light cubes.
2. Install the appropriate slide holder for either the disposable or reusable slide.
3. Obtain a Countess disposable or reusable slide.

Protocol

1. Culture cells in an appropriate medium.
2. Add 2 drops each of NucBlue Live and NucGreen Dead reagents per milliliter of cell suspension.
3. Incubate 5–30 minutes.
4. Gently pipet 10 µL of the sample into the sample loading area of the slide. The sample is loaded into the chamber through capillary action.
5. Let the sample mixture settle in the chamber for 30 seconds, and then insert the slide into the slide adapter to initiate autofocus. Your will hear a soft click once the slide is pushed in correctly.
6. Adjust light intensities to minimize fluorescent background.
7. Press “Count”.

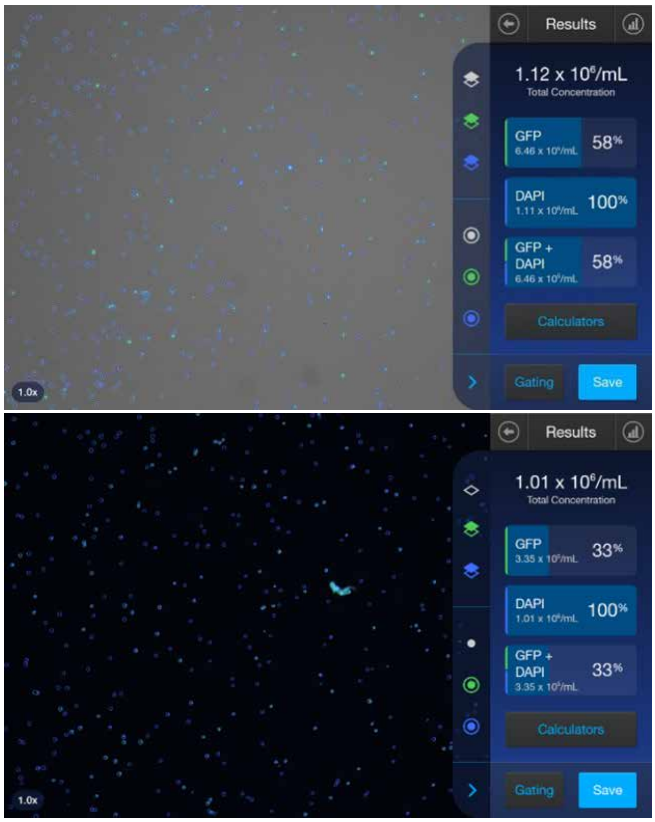

Figure 3. Viability assay via cell staining using the ReadyProbes Cell Viability Imaging Kit, Blue/Green. (A) Live and heat-killed U937 cells were mixed and then stained with the NucBlue Live reagent and NucGreen Dead reagent supplied with the kit. The resulting sample was counted on the Countess 3 FL Automated Cell Counter with EVOS DAPI and GFP light cubes installed. The Countess 3 FL instrument is easier to use than the Countess II instrument because of the auto-lighting and histogram features in fluorescence mode. **(B)** Human PBMCs stained as described in (A), visualized using only fluorescent channel overlay.

Results

Reagents in the ReadyProbes Cell Viability Imaging Kit, Blue/Green, stain all cells in blue and all dead cells in green (Figure 3).

Summary

When used with cells stained with the ReadyProbes Cell Viability Imaging Kit, Blue/Green, the Countess 3 FL instrument displays total concentration, concentration and percentage of the counted objects that have green fluorescence (dead cells), concentration and percentage of the counted objects that have blue fluorescence (all cells), and concentration and percentage of the counted objects that have both green and blue fluorescence (dead cells).

Cell viability assay using the ReadyProbes Cell Viability Imaging Kit, Blue/Red

Introduction

The Invitrogen™ ReadyProbes™ Cell Viability Imaging Kit, Blue/Red (Cat. No. R37610), can be used to quickly and easily determine cell viability. The combination of dyes provided in the kit allows for fluorescent evaluation of viability with samples expressing GFP, unlike the standard acridine orange/propidium iodide method. Just add 2 drops each of room temperature–stable Invitrogen™ NucBlue™ Live reagent and propidium iodide to 1 mL of cell growth medium, and then determine viability by counting total vs. dead cells.

Materials

- Countess 3 FL Automated Cell Counter (Cat. No. AMQAF2000)
- Countess Cell Counting Chamber Slides (Cat. No. C10228) or Countess Reusable Slide (Cat. No. A25750)
- NucBlue Live reagent (included with the kit; Ex/Em: 360/460 nm): stains the nuclei of all cells; detected with a standard DAPI filter.
- Propidium iodide (included with the kit; Ex/Em: 535/617 nm): stains only the nuclei of dead cells with compromised plasma membranes; detected with standard TRITC/RFP (orange) filter sets.

Methods

Suggestions for use

- NucBlue Live reagent and propidium iodide can be added directly to cells in a complete growth medium or a compatible buffer solution.
- In most cases, 2 drops per milliliter of suspension and an incubation time of 5–30 minutes will give bright nuclear staining; however, optimization may be needed for some cell types, conditions, and applications. In such cases, simply add more or fewer drops to obtain optimal staining intensity.

Instrument setup

1. Turn on the Countess 3 FL Automated Cell Counter and install the appropriate EVOS light cubes for the stains being used. Most commonly, the ReadyProbes Blue/Red kit requires the DAPI and RFP 2.0 light cubes.
2. Install the appropriate slide holder for either the disposable or reusable slide.
3. Obtain a Countess disposable or reusable slide.

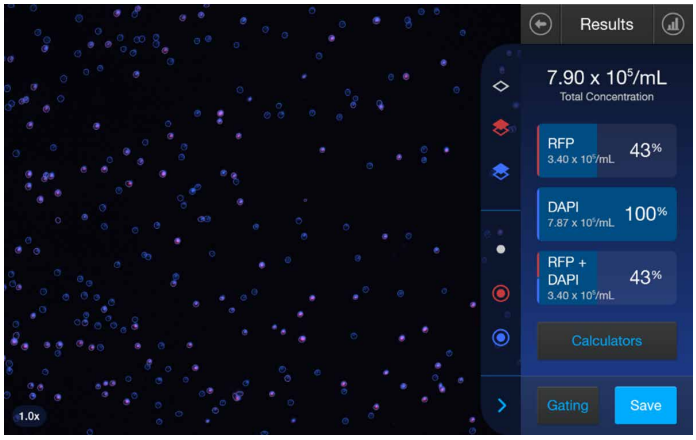

Figure 4. Viability assay via cell staining using the ReadyProbes Cell Viability Imaging Kit, Blue/Red. Live and heat-killed U2OS cells were mixed and then stained with the NucBlue Live reagent and propidium iodide supplied with the kit. The resulting sample was counted on the Countess 3 FL Automated Cell Counter with EVOS DAPI and RFP light cubes installed. The Countess 3 FL instrument is easier to use than the Countess II instrument because of the auto-lighting and histogram features in fluorescence mode.

Protocol

1. Culture cells in an appropriate medium.
2. Add 2 drops each of NucBlue Live reagent and propidium iodide per milliliter of cell suspension.
3. Incubate 5–30 minutes.
4. Gently pipet 10 µL of the sample into the sample loading area of the slide. The sample is loaded into the chamber through capillary action.
5. Let the sample mixture settle in the chamber for 30 seconds, and then insert the slide into the slide adapter to initiate autofocus. Your will hear a soft click once the slide is pushed in correctly.
6. Adjust light intensities to minimize fluorescent background.
7. Press “Count”.

Results

Reagents in the ReadyProbes Cell Viability Imaging Kit, Blue/Red, stain all cells in blue and all dead cells in red (Figure 4).

Summary

When used with cells stained with the ReadyProbes Cell Viability Imaging Kit, Blue/Red, the Countess 3 FL instrument displays total concentration, concentration and percentage of the counted objects that have red fluorescence (dead cells), concentration and percentage of the counted objects that have blue fluorescence (all cells), and concentration and percentage of the counted objects that have both red and blue fluorescence (dead cells).

Counting standards and fluorescent gating

Introduction

When counting cell samples over time or between users, having a stable standard is quite useful as it enables users to monitor the performance of instruments and the impact of instrument settings. This is especially true when counting fluorescent samples, since there are more variables present than in a brightfield-only count. Two standards that are commonly used with Countess automated cell counters are Invitrogen™ Countess™ Test Beads and the Countess™ 3 Standard Slide.

The Countess Test Beads were developed for the first-generation Countess instrument but will work in all Countess instrument versions as a simple counting standard. Countess Test Beads perform very well as a brightfield counting standard only and do not offer any viability or fluorescence properties. However, the Countess Test Beads are particularly useful if the user wishes to evaluate sample-to-sample variability while pipetting, as the beads are offered in suspension and must be pipetted into a chamber slide.

The Countess 3 Standard Slide was developed directly in response to customer demand for a standard that is highly stable over time, useful in brightfield and fluorescence applications, and closely represents the morphology of cells stained with trypan blue for brightfield viability assays. Since the Countess 3 Standard Slide is preloaded with an immobilized sample, it does not offer insight into user-to-user variation in sample preparation like the Countess Test Beads do.

Materials

- Countess 3 FL Automated Cell Counter (Cat. No. AMQAF2000)
- Countess Cell Counting Chamber Slides (Cat. No. C10228) or Countess Reusable Slide (Cat. No. A25750)
- Countess Test Beads (Cat. No. C10284)
- Countess 3 Standard Slide (Cat. No. A51876)

Methods

Protocol

1. Insert the Countess 3 Standard Slide or disposable slide with Countess Test Beads into the Countess instrument.
2. Confirm that the autofocus has yielded a well-focused image.
3. Press “Count”.
4. Adjust gates for size, brightness (brightfield and fluorescence), and circularity as you see fit; the Countess instrument will update in real time.

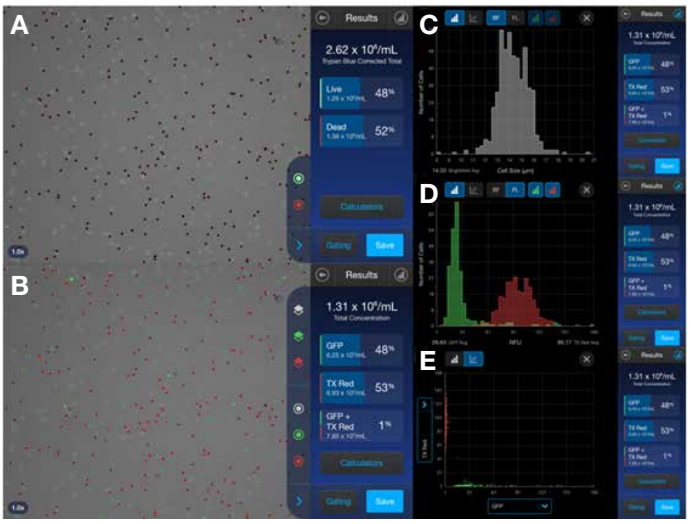

Figure 5. Countess 3 Standard Slide counted on the Countess 3 FL Automated Cell Counter. Images and counts for **(A)** brightfield and **(B)** fluorescence mode. Measurements are displayed as a traditional histogram for **(C)** brightfield number of cells vs. cell size (µm), **(D)** fluorescence number of cells vs. intensity (RFU), and **(E)** a scatterplot of intensity measures of each fluorescent event (GFP and Texas Red light cubes).

5. Compare results between users, profiles, or over time, keeping in mind that different gating parameters will likely yield different results.

Results

The Countess Standard Slide is highly stable over time, useful in brightfield and fluorescent applications, and closely represents the morphology of cells in both brightfield and fluorecence. The concentration in brightfield is double the concentration in fluorescence (Figure 5A and 5B), because the brightfield concentration shows an example of the trypan blue–corrected total, factoring in a 1:1 dilution.

Summary

The Countess Test Beads and Countess 3 Standard Slide are useful tools for monitoring instrument performance and sample preparation consistency. Their use is straightforward and amenable to the user’s requirements for test frequency and protocol. However, these standards are also good instrument training tools. If we consider the Countess 3 Standard Slide, users can apply gates and observe the impact on cell counting, in real time. Some of the most useful strategies are gating by size and fluorescence brightness while observing the impact of gating via the histogram and scatter plot views of the Countess 3 FL instrument.

Ordering information

Description	Cat. No.
Countess 3 Automated Cell Counter	AMQAX2000
Countess 3 FL Automated Cell Counter	AMQAF2000
Countess 3 Automated Cell Counter Starter Package	A49865
Countess 3 FL Automated Cell Counter Starter Package	A49866
Countess Reusable Slide	A25750
Countess Cell Counting Chamber Slides	C10228
ReadyCount Green/Red Viability Stain	A49905
LIVE/DEAD Viability/Cytotoxicity Kit	L3224
ReadyProbes Cell Viability Imaging Kit, Blue/Green	R37609
ReadyProbes Cell Viability Imaging Kit, Blue/Red	R37610
Countess Test Beads	C10284
Countess 3 Standard Slide	A51876

Learn more at **thermofisher.com/countess**