

Nucleus-highlighting (terminal/lethal) staining of adherent live U2-OS cells by Erythrosine B

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Erythrosine B (tetraiodofluorescein; ErB) is a negatively charged viability stain commonly used to assess cell viability in a hemocytometer or in adherent mammalian cell cultures. Being considered membrane-impermeable, it preferentially stains dead cells that have lost their membrane integrity (pink color within 1 min of treatment). It is often recommended over Trypan Blue staining for providing a faster and more reliable dead cell staining and for being a generally non-toxic reagent. While exposure to Trypan Blue is toxic to mammalian cells, ErB is often stated to be relatively harmless and suitable for non-terminal cell staining. However, Erythrosine B actually enters live cells and eventually stains them lethally but the speed of this process varies considerably between cell lines and can take from several minutes (U2-OS) to more than 30 minutes (HEK293T).

In the presence of 0.06% w/v (or higher) ErB in serum-free media the adherent U2-OS cells get efficiently stained after 10 min resulting in a pronounced nuclear and a faint cytoplasmic staining while retaining the general cell morphology and adherence. This can be used for a fast terminal staining and counting of adherent U2-OS cells or other cell lines that interact with ErB in the same way.

Lower ErB concentrations in serum-free media often lead to a fast cell disintegration and loss of nuclei in U2-OS cells without staining. The discussed possible destructive and toxic effects of Erythrosine B on mammalian cells should be taken into account when using it as a vital dye. The presence of serum in the media temporarily protects live U2-OS cells from the toxic action of ErB (at concentrations below 0.1% w/v), so it can be recommended when staining dead cells to avoid affecting the live ones.

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INTRODUCTION

Erythrosine B (ErB) is a pink dye used as a food colorant (Acid Red 51; Red No. 3) and is generally considered non-toxic. It is a tetraiodinated derivative of fluorescein and (unlike fluorescein) it non-specifically binds to proteins and can be used as a protein stain (Soedjak, 1994). It is negatively charged at neutral pH which prevents crossing lipid bilayers, so it is usually considered cell-membrane impermeant. These two properties are similar to Trypan Blue (TB; see Fig. 1) stain (Avelar-Freitas et al, 2014), and both dyes are used to selectively stain "dead" or damaged cells in adherent cell cultures and in flow cytometry: the dyes preferentially enter cells that have lost their membrane integrity and stain the cytoplasmic proteins making such cells intense blue (TB) or pink (ErB).

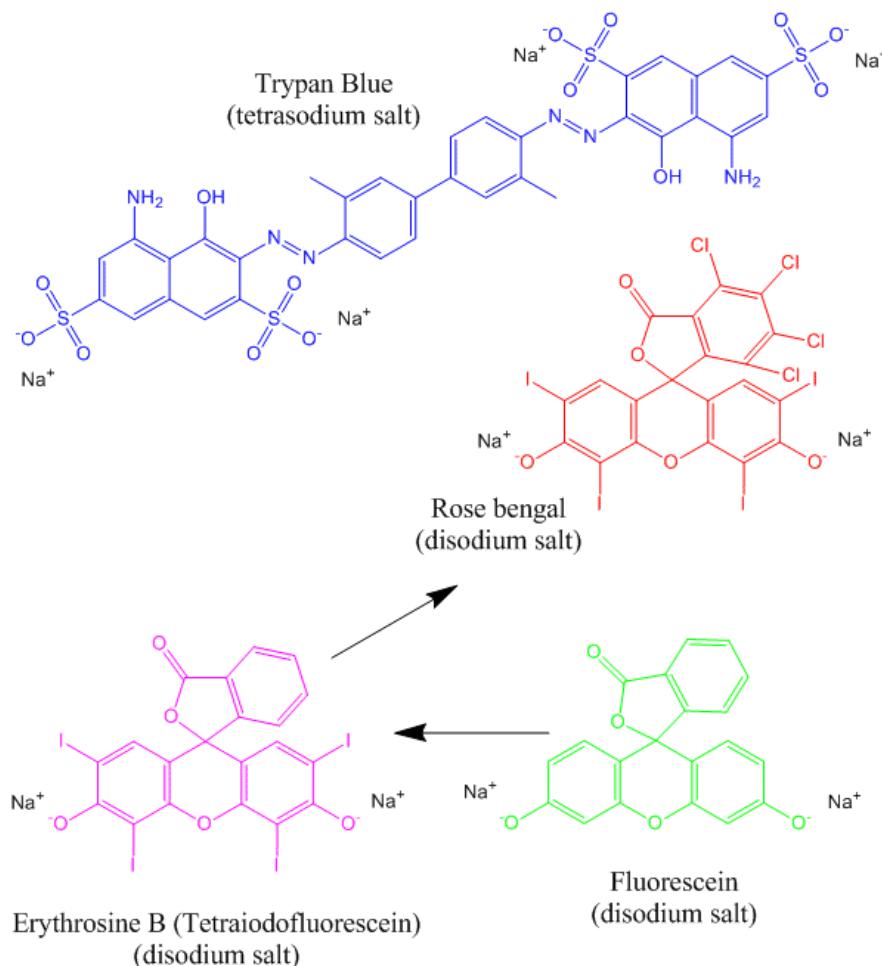


Fig. 1. Chemical structure of relevant cell and tissue dyes: Trypan Blue, Fluorescein, Erythrosine B and Rose Bengal.

Historically, TB is the most widely used and became a traditional vital dye for viable cell counting. However, TB is considered a hazardous material and as more toxic, so the use of ErB instead is commonly recommended (ATCC Cell culture guide, 2020; Scott & Merrett, 1995; Kim et al, 2016). In addition, in case of ErB the staining of all dead cells occurs immediately upon the cell exposure to the dye (Krause et al, 1984; Kim et al, 2016) while not all cells are optimally stained by TB (Krause et al, 1984) and some dead cells can lose integrity after staining affecting the readout (Chan et al, 2020). Thus, ErB staining of dead cells is often reported as more reliable. Apart from eucaryotic cells, ErB was reported to be a reliable vital dye for bacteria (Franke et al, 2020)

Apart from colored staining, binding of ErB to non-denatured proteins is often accompanied by an increase of ErB fluorescence and a blue shift of its excitation/emission (Ganesan & Buchwald, 2013; Mathavan et al, 2009; Sabilin et al, 2016). This fluorescence can be used as another efficient cell staining readout for both ErB (Krause et al, 1984; Franke et al, 2020) and TB (Avelar-Freitas et al, 2014). Fluorescence detection is more sensitive than the colorimetric one (Krause et al, 1984).

Despite being used as vital dye to exclusively stain dead cells, TB is considered toxic, can slowly enter alive cells as well (Nguyen et al, 2020) and can leave detrimental effects on live cells after several minutes of exposure (Tsaousis et al, 2013). In this regard, ErB is often considered a better vital dye with much lower toxicity on mammalian cells (e.g. see Fig. 3 in (Kim et al, 2016)). But as shown below, ErB actually also enters the interior of live mammalian cells and eventually stains them as well. This is particularly pronounced in serum-free media and buffers because otherwise serum proteins bind the bulk of ErB present in solution alleviating or delaying the toxic effects of ErB. This staining of live cells is lethal due to extensive binding to cytoplasmic proteins and accumulation of ErB in the nucleus. However, the speed of this process varies considerably between cell lines: it can take only a few minutes for U2-OS cells but for HEK293T cells a considerable staining occurs after 30-60 min.

In the presence of 0.06% w/v (680 µM) ErB in serum-free media the adherent U2-OS cells get efficiently stained within 5-10 min resulting in a pronounced nuclear and a faint cytoplasmic staining while retaining the general cell morphology and adherence. This can be used for a fast terminal staining and counting of adherent U2-OS cells or other cell lines that interact with ErB in a similar way. In case of U2-OS cells, lower ErB concentrations in serum-free media often lead to a fast cell disintegration and loss of nuclei without staining.

OBSERVATIONS: INTERACTION OF MAMMALIAN CELL LINES WITH ERYTHROSIN B

Typically, a quick treatment of adherent mammalian cells with ErB (followed by replacement of the medium with a clean, ErB-free one) can serve as a fast and convenient way to detect dead cells (Fig. 2).

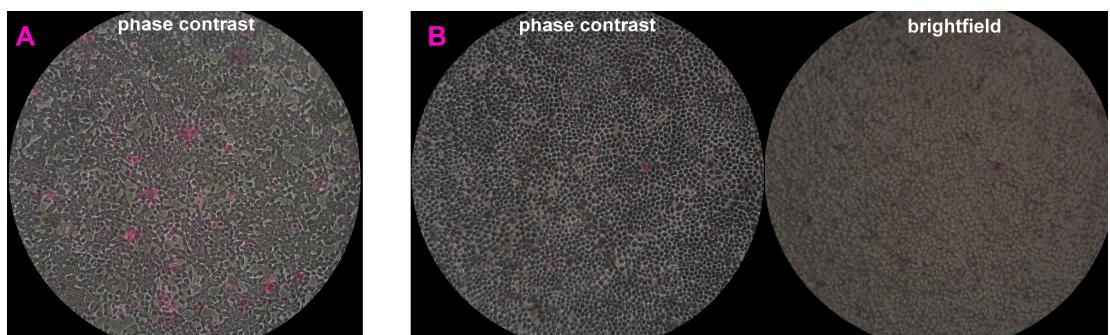


Fig. 2. Example of staining dead cells among adherent cell culture.
A well with adherent HEK293T cells (left after various experiments) were washed by 500 µl 0.06% w/v ErB in PBS (with Ca/Mg) buffer (pH 7.4) and then washed by PBS (with Ca/Mg) buffer.
A) HEK293T cells close to full confluence. A number of stained dead cells can be noted.
B) Confluent HEK293T cells (from another experiment). A few stained dead cells can be noted.

However, in case of U2-OS cells it becomes apparent that ErB enters live cells as well after a few minutes (Fig. 2).

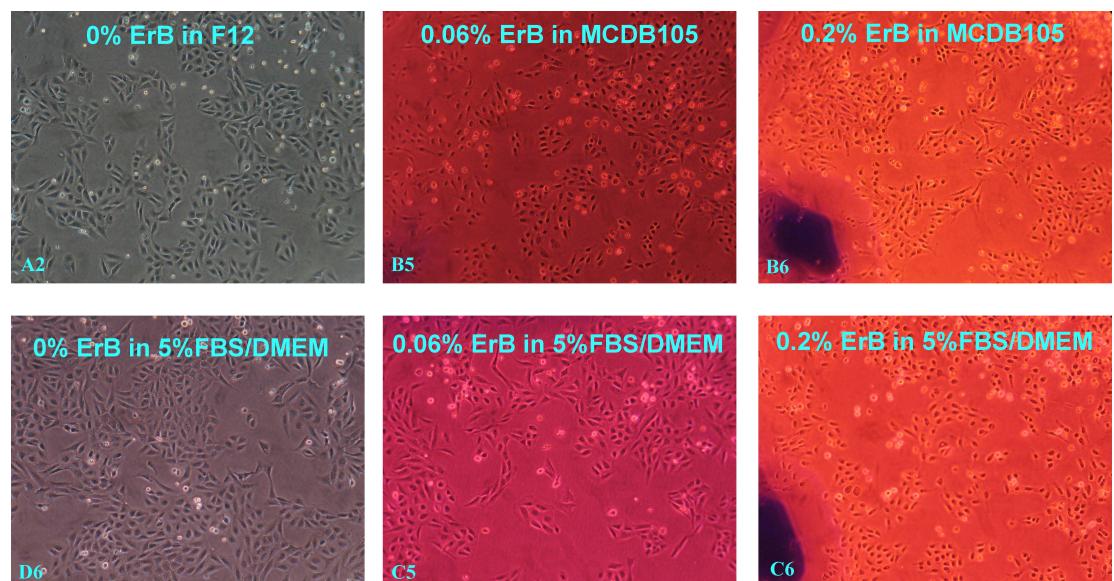


Fig. 2: Staining of adherent U2OS cells after 10 minutes with Erythrosin B. Effect of 0% (control), 0.06% and 0.2% w/v ErB in the absence (MCDB105 or F12 medium) or presence (5% FBS / DMEM) of serum.

The presence of serum in the media protects U2-OS cells from staining at 0.06% ErB and lower while 0.2% ErB still stains the cells (Fig. 2). The protective effect of serum at 0.06% ErB is temporary and incubation for several hours still stains the cells (Table 1).

0.06% w/v ErB in serum-free media can be used to efficiently stain U2-OS cells within 10-15 min leading to a pronounced nuclear and a weak cytosolic pink coloration (Fig. 3).

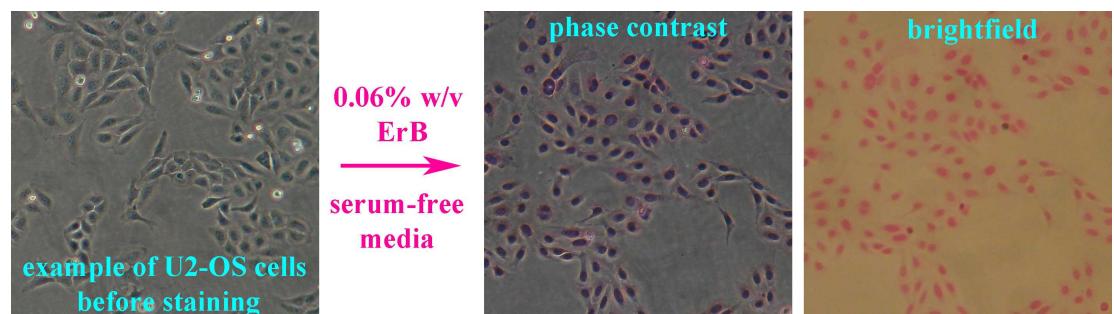


Fig. 3. Staining of adherent U2-OS cells by 0.06% ErB in a serum-free media. A distinct staining is achieved after 10-15 min of incubation.

In contrast to a fast penetration of ErB into U2-OS cells, HEK293T cells get stained much slower, so initially the staining of live cells is unnoticeable. A partial staining occurs after about 30 min and usually more than 1 hour is needed to fully and deeply stain an adherent HEK293T cell layer (Fig. 4). The staining leads to cell rounding and nuclear coloration is much less distinct than in U2-OS cells (Fig. 4 & Fig. 5).

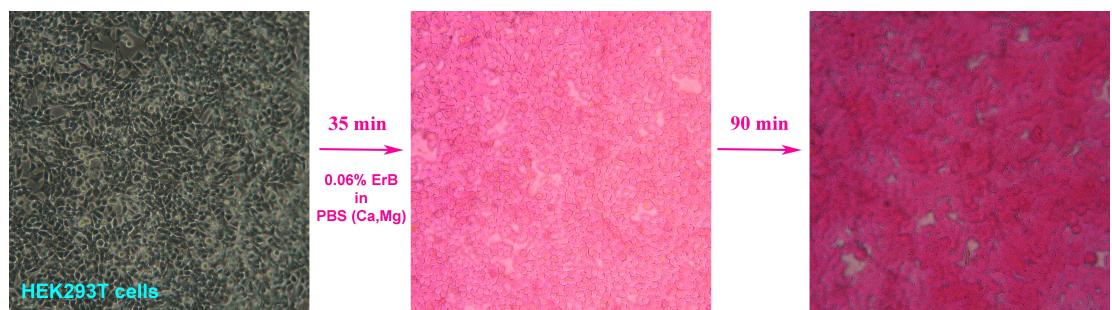


Fig. 4. Staining of adherent live HEK293T cells by ErB.

While the nucleus is more intensely colored, the ErB fluorescence is much stronger in the cytosol in HEK293T (Fig. 5) or U2-OS cells (not shown). This is likely as a result of self-quenching of ErB in the nucleus due to the high concentration of ErB in this compartment - similar to the described for the Rose Bengal dye (Feenstra & Tseng, 1992). Interestingly, a pronounced ErB fluorescence occurs only for non-denatured cellular proteins: cells fixed with ethanol or methanol are quickly stained by ErB but do not show ErB fluorescence using YFP filters (not shown).

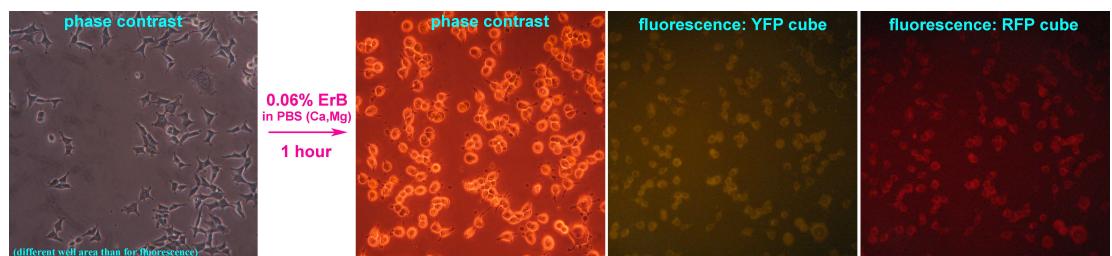


Fig. 5. Staining of adherent live HEK293T cells by ErB and imaging of ErB fluorescence of stained cells.

While 0.06% w/v stains U2-OS cells in serum-free media and preserves their morphology essentially fixing them, lower levels of ErB tend to cause cell damage without visible staining (Fig. 6). Typically, the nucleus becomes more distinct (likely due to accumulation of ErB) which is followed by its separation from the rest of the cell and/or cell disintegration. The use of media buffered only by 5% CO₂ (e.g. F12) exacerbates this effect, so that the damage occurs faster and is more pronounced (likely due to the alkaline pH of the media which is attained quickly when handling cells outside the CO₂ incubator). A brief (3 min) treatment of U2-OS cells with 0.06% ErB followed by its removal also leads to cell damage (Fig. 6).

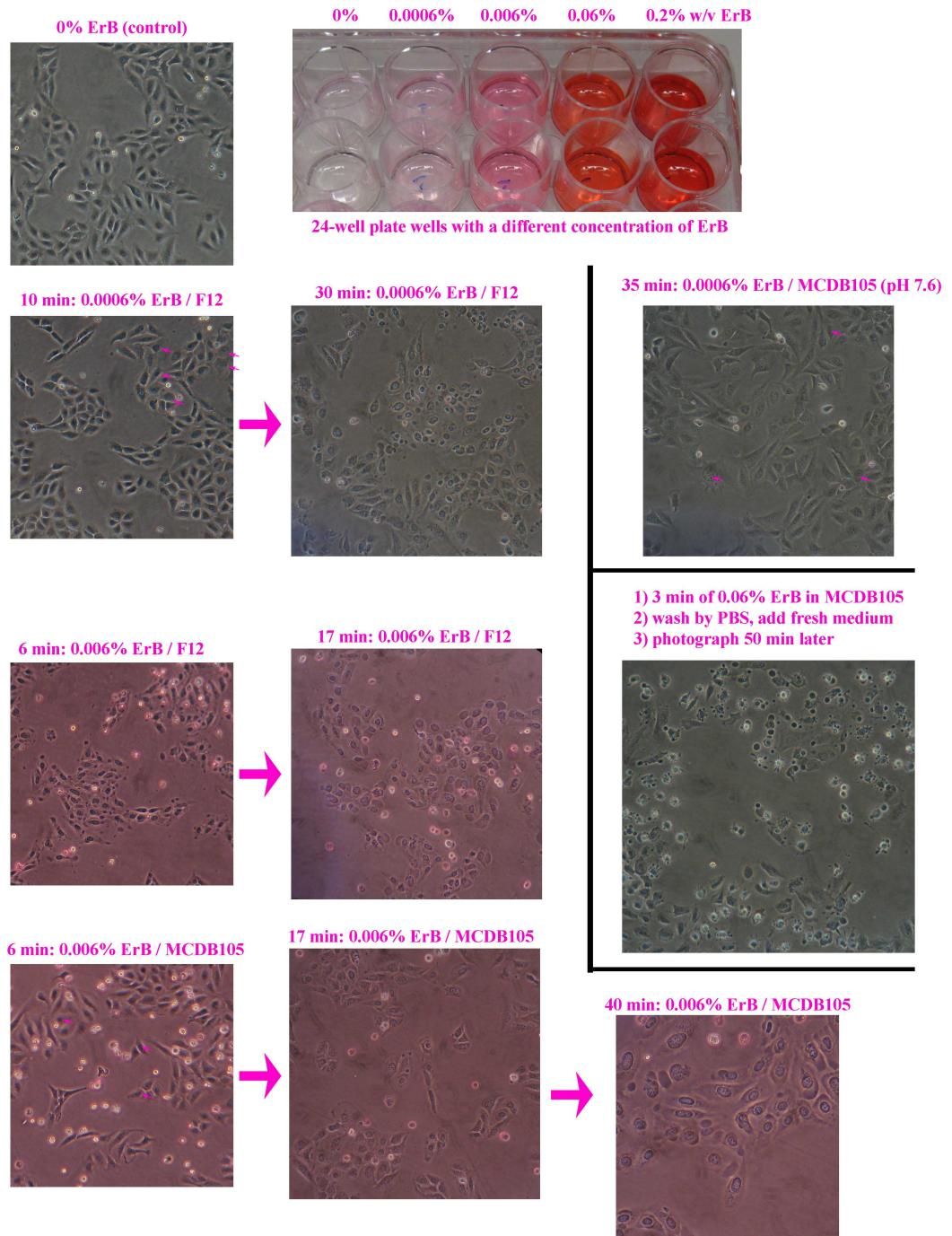


Fig. 6. Effects of treating adherent U2-OS cells with 0.0006% and 0.06% w/v ErB in serum-free media.

Cells were placed either under F12 or MCDB105 media (Ham & McKeehan, 1979). F12 is buffered by 5% CO₂ and gets alkaline outside the incubator while MCDB105 is buffered by HEPES for pH 7.6 at ambient conditions. Cells were incubated in a cell culture incubator (37°C, 5% CO₂) but handled and photographed under ambient room conditions. Small arrows in some of the images point out changes in cell nucleus as an initial effect to the presence of ErB.

Some of the visual effects caused by ErB on U2-OS cells in the absence and presence of serum are summarized in the Table 1 below.

Erythrosine B concentration	B	C	D
% w/v (microM)	after 10-20 min with ErB	after 10-20 min with ErB	4 hours with ErB: cell fluorescence (YFP filter)
	FBS-free media: F12 or MCDB105 (pH 7.6)	5% FBS / DMEM	5% FBS / DMEM
0.2% (2270 uM)	cells are stained	cells are stained	adherent cells are stained, cell fluorescence is less pronounced than at 0.06% ErB due to a media (FBS) background
0.06% (680 uM)	cells are stained	cells look normal	adherent cells are stained, ErB is depleted from the medium (accumulates in cells => low background absorbtion/fluorescence)
0.006% (68 uM)	separation of the nucleus and other cell damage, no visual staining; cell damage occurs faster in a non-buffered (F12) medium	cells look normal	adherent cells are not killed or stained; floating (dead) cells are stained
0.0006% (6.8 uM)	cell damage in a non-buffered (F12) medium; almost no changes in a buffered (MCDB105, pH 7.6) medium	cells look normal	adherent cells are not killed or stained; floating (dead) cells are not stained

Table 1. Effects of different ErB concentrations on adherent U2-OS cells (grown in a 24-well plate, 500 ul media is added per well) in the absence and presence of 5% serum (FBS). See also Fig. 2 and Fig. 6.

After adding ErB, the 24-well plate was periodically removed from a cell culture incubator (5% CO₂, 37°C) to observe and photograph cells in different wells.

The MCDB105 medium is buffered (pH 7.6) at the ambient atmosphere while F12 and DMEM require 5% CO₂ to maintain neutral pH and quickly become alkaline during periods outside of a cell culture incubator.

DISCUSSION

As shown above, despite being a vital dye, ErB in cell culture has a propensity to enter and stain mammalian cells leading to cell death and other toxic effects.

In 2011 Ganesan et al have reported that the promiscuous protein binding ability of ErB readily inhibits many protein-protein complexes at 5-10 µM including cell surface receptor-ligand interactions (Ganesan et al, 2011). They have shown that ErB exhibits

a clear toxicity in cell culture above 100 μ M which was attributed to these inhibitory effects. As shown in the current work, ErB also has a more general cell staining propensity and concurrent toxic effects on cell morphology. ErB was quite unique among other food colorants to show promiscuous protein binding and protein inhibition in the μ M concentration range (Ganesan et al, 2011). Among structural analogs of ErB, a closely related dye Rose Bengal (Fig. 1) showed even somewhat stronger inhibitory and toxic effects than ErB (Ganesan et al, 2011).

In 1992 Feenstra & Tseng have reported very similar effects of Rose Bengal in cell culture as the ones described here for ErB. It stained cultured cells leading to a pronounced nuclear coloration, higher fluorescence in the cytosol than in the nucleus, cell toxicity and various detrimental effects on cell morphology (Feenstra & Tseng, 1992; Lee et al, 1996). At the same time Rose Bengal was used as a generally safe dye to stain eye damage in ophthalmology. This discrepancy was explained by the ability of proteins in a tear liquid to bind Rose Bengal and reduce its toxic effects (Feenstra & Tseng, 1992) - similar to what was observed here for serum and ErB.

- How do negatively charged vital dyes enter cells?

ErB, Rose Bengal and TB are negatively charged and do not cross lipid bilayers per se. Their entry into mammalian cells is likely mediated by some small molecule importers located in the plasma membrane. Even in the presence of cross-membrane transport, negatively charged small molecules tend to be preferentially excluded from the cytoplasm due to the negative electric potential of the plasma membrane. However, protein-binding dyes are still expected to accumulate inside cells due to their binding to the intracellular proteins those cytosolic concentration (>200 mg/ml) (Milo et al, 2013) is much higher than in the extracellular space.

The differences in the speed of dye transport between cell lines are not unexpected since they can have different types and levels of relevant membrane small molecule transporters (importers and exporters). For example, U2-OS cells also show a faster intracellular/extracellular equilibration (several minutes) for another negatively charged small molecule - D-luciferin substrate during live cell bioluminescent assays - while HEK293T cells require about 20 min (unpublished observations).

- Why do ErB, Rose Bengal and TB accumulate in the nucleus?

The preferential accumulation of ErB, Rose Bengal and TB (for the latter, see Fig. 4 in (Nguyen et al, 2020)) in the nuclei of live cells rather than in the cytosol is puzzling. These are all promiscuous protein- but not DNA-binding dyes. Cell nucleus was reported to have a negative membrane potential (Loewenstein & Kanno, 1963; Matzke & Matzke, 1985; Julian & Diacumakos, 1977), which again should preferentially exclude negatively charged dyes from it. This phenomenon may warrant further investigation.

TAKE-HOME MESSAGES

1. Despite being a vital dye for dead cells, Erythrosine B (ErB) gradually enters live cells as well, stains the cytosolic proteins and prominently accumulates in the nucleus. This process is lethal.
2. The speed of this process varies between cell lines (and likely cell types): it occurs within a few minutes in U2-OS cells but >30 minutes is needed to noticeably affect HEK293T cells.
3. 0.06% w/v ErB can be used to [terminally/lethally] stain adherent live U2-OS cells within 10 min resulting in relatively fixed cells with pink nuclei.
4. This can be useful for their counting in brightfield or phase contrast microscopy images.
5. Lower concentrations (<0.01%) or a transient treatment with ErB in serum-free media lead to damage and loss of integrity of live U2-OS cells without their visible staining.
6. Serum (5% FBS) temporarily protects U2-OS cells from these effects up to 0.06% ErB. Therefore, the presence of serum can be recommended when using ErB as a vital dye - particularly, with cell lines that can show adverse reactions to ErB within a few minutes like U2-OS. This will prevent damage of live cells during the assay which could affect their counting relative to dead cells.
7. The described effects of ErB on live mammalian cells lines should be taken into account when using ErB with them as a vital dye, especially in serum- and protein-free media.
8. ErB accumulation in the nucleus, its effects on the nucleus in live cells and its protein inhibitory activity can be potentially used in some other applications.
9. Judging from the literature, a related Rose Bnsengal dye (Fig. 1) is expected to show similar (and probably stronger) effects as ErB.

Reagents

 Erythrosine B (disodium salt) TCI

 Chemicals Catalog #T0557

 U-2 OS cell

 line ATCC Catalog #HTB-96

STOCK SOLUTIONS

6% w/v Erythrosine B stock in water.

- dissolve ErB (disodium salt) to 6% w/v in water (68 mM ErB)
- can be filtered to sterilize and to remove any undissolved impurities (if present).
- store at room temperature.

(ErB can also be dissolved to 6% w/v in propylene glycol which can be useful in other applications. In this case the dissolution will require a long time and some heating at 42-50°C)

PHYSIOLOGICAL BUFFERS

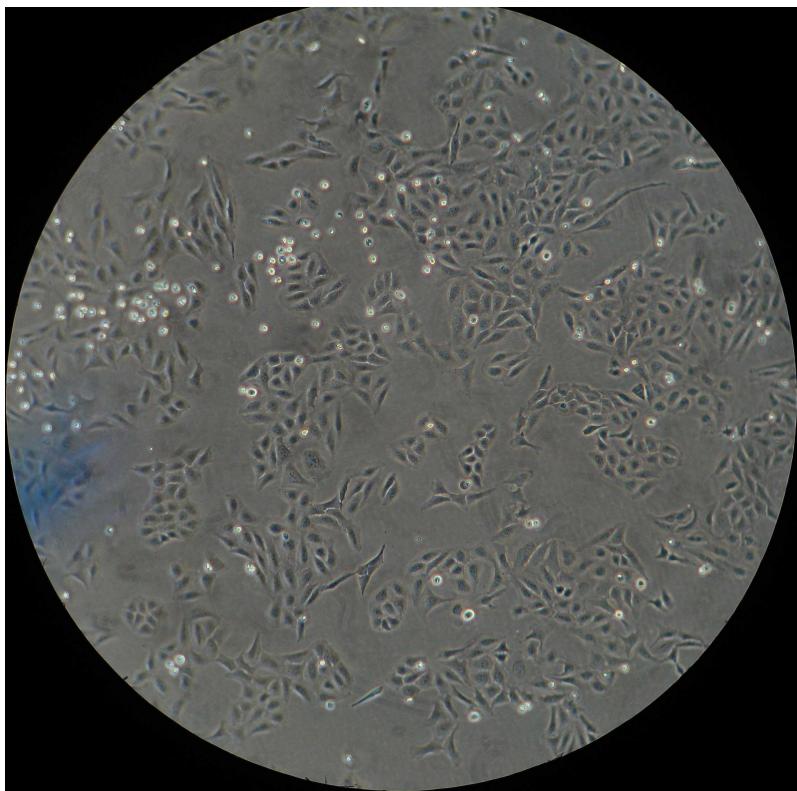
A	B
PBS (with Ca/Mg), pH 7.4 (Phosphate buffered saline with calcium and magnesium)	8.2 mM Na2HPO4 1.8 mM KH2PO4 148 mM NaCl pH 7.40 (adjust with NaOH) 0.5 mM MgCl2 0.9 mM CaCl2
PB1 (Physiological buffer, pH 7.3)	25 mM MOPS 120 mM NaCl 2.8 mM KCl 1.2 mM KH2PO4 1.8 mM CaCl2 0.6 mM MgCl2 0.3 mM MgSO4 10 mM glucose pH 7.30 (adjust with NaOH)

Possible buffers to wash or stain live cells.

Prepare the required stock solution and media. Read the Guidelines & Warnings section describing experimental examples and the literature background of the method.

Cell staining protocol steps

- 1 Grow U2-OS cells in a desired vessel.



Adherent U2-OS cells grown on a surface of 24-well plate (TC-treated transparent polystyrene plate).

U2-OS mammalian cell line demonstrates a strong adherence to the growth substrate and can grow well in a variety of media of different nutritional composition (DMEM, McCoy's 5A, F10, F12, MCDB105, etc) in the presence of 1-10 % serum. Most commonly it is grown in DMEM or McCoy's 5A medium in the presence of 5-10% FBS.

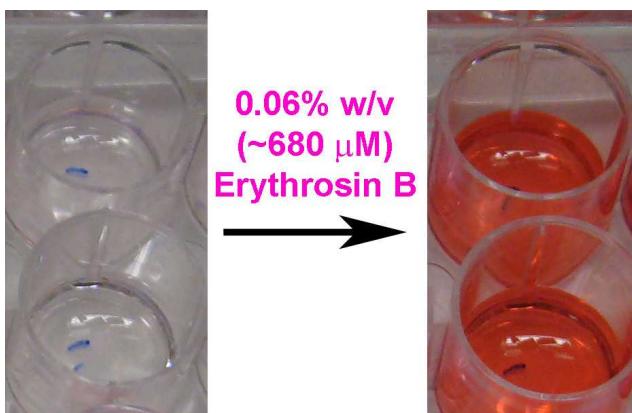
- 2 Remove the culture media from the vessel (cell culture plate or well) with adherent U2-OS cells.

It is also possible to use the starting serum-containing growth media without a change but the final concentration of Erythrosin B will need to be increased to 0.2% w/v or more to achieve the same results. Otherwise, serum proteins bind ErB blocking or reducing most of the described effects on live cells.

3 Gently add a serum-free cell culture media or buffer (preferably maintaining pH 7.2-7.6 in the absence of the CO₂ atmosphere) to cover the cells.

- In principle, any buffer or media not affecting live cells in a short time scale is suitable, since the staining is terminal and short.
- A physiological buffer with calcium and magnesium or a cell culture medium similar to the one used for cell growth can be a good choice.
- It is convenient to have a transparent media without phenol red
- If using a cell culture medium, it is useful if it's buffered to have pH 7-7.6 in the absence of CO₂ atmosphere (like MCDB media or regular cell culture media formulations without sodium bicarbonate) if cells are to be handled outside cell culture incubator. However, the described terminal staining works similar regardless of this point.

4 Add a small volume of 6% w/v (~6.8 mM) Erythrosin B stock (in water) to the media above cells to achieve the final 0.06% concentration (1:100 dilution). Mix by gently shaking the plate.



- In serum-containing media the concentration of ErB should be increased to 0.2% (with 5% FBS) or more to achieve the same results.
- 0.006% ErB is not sufficient to stain live U2-OS cells but leads to cell damage (see the Guidelines section); this concentration still stains dead cells.

5 Incubate the plate for 10-15 min in a cell culture incubator (37°C).

During this time, living U2OS cells will uptake ErB from the media leading to a faint pink coloration of the cytosol with a pronounced pink staining of the nucleus.

- 6 The medium can be exchanged to a fresh transparent one or removed. Cells can now be imaged in phase contrast or brightfield: nuclei become intensely pink while cytosol remains quite faint. If placed under ErB-free medium, cells will lose some of the accumulated ErB.

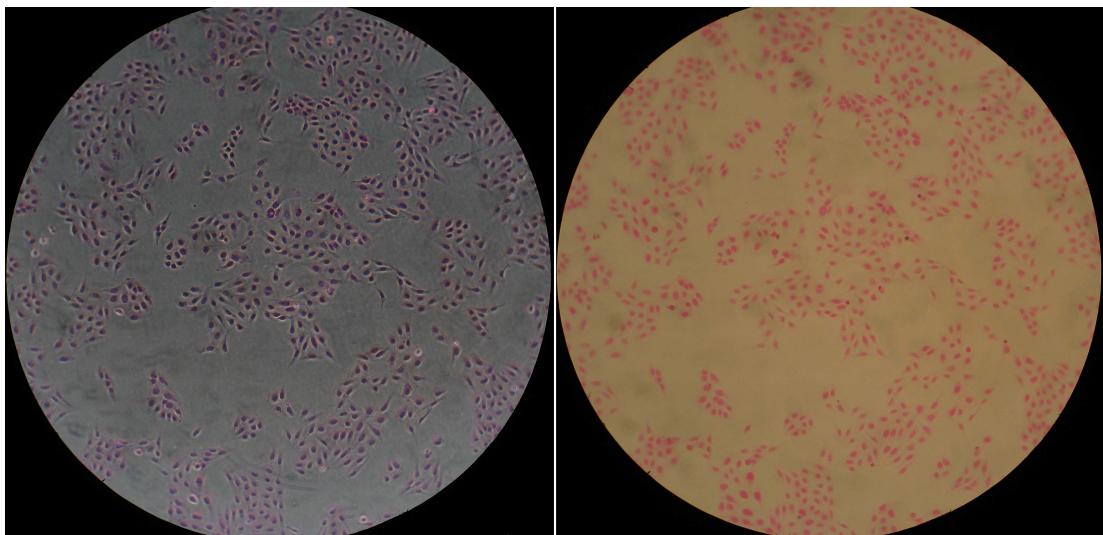


Image of (initially) live U2OS cells stained with ErB. Left: phase contrast image; Right: brightfield image. Cells were grown and stained in wells of a 24-well TC-treated polystyrene plate.

Post-protocol notes

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- A flat shape and large size of U2OS cells generally results in a good visual separation of pink nuclei between neighboring cells.
- Stained cells loose their viability but remain fixed.
- The use of ErB concentrations below 0.01% leads to the separation of nuclei from cells and disintegration of many U2-OS cells rather than staining and fixation.

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