

## Chapter

# Trypan Blue Exclusion Assay, Neutral Red, Acridine Orange and Propidium Iodide

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## Abstract

Cytotoxicity and cell viability assessments are very important parameters that are widely used in fundamental research and drug development to determine the safety profile of toxic compounds. These assays measure the degree to which a substance can cause toxic damage to cells or cell death. There are different assays that have been employed to determine the cytotoxicity of substances. These assays either determine enzymatic function, cell viability, mitochondrial activity, lipid metabolism, cell proliferation and/or cell death. These assays entail use of different kinds of dyes such as trypan blue exclusion dye, neutral red, acridine orange and propidium iodide to stain the cells. Trypan blue dye permeates compromised cell membrane to stain necrotic cells. However, this can lead to false positive and false negative results as it does not provide information on sub-lethal injury. As a result, neutral red and acridine orange can be used as counterstains for trypan blue to stain the lysosome of live cells. Acridine orange can also be used to stain nucleic acids in living cells and is usually co-stained with propidium iodide or ethidium bromide. This is because propidium iodide and ethidium bromide permeate only compromised plasma membrane thus co-staining cells with these dyes can provide vital information that can be used to differentiate between live and dead cells.

**Keywords:** cytotoxicity, trypan, acridine, iodide, neutral, blue, red, orange, propidium

## 1. Introduction

Today, humans consume food and vegetables rich in phenolic compounds and are continuously exposed to increased volume of xenobiotics. These chemicals such as phenolic compounds as well as pharmaceutical agents induce cellular toxicity and/or genomic instability most especially in the liver [1]. These chemicals are absorbed and biotransformed by the liver to their metabolites that may be less or more toxic to cells. Consequently, the evaluation of hepatocytotoxicity and erythropoietic cytotoxicity are key components of safety assessment during drug development [2]. Cytotoxicity is the ability of chemicals to destroy the functioning of living cells [3, 4]. Cytotoxic

chemicals cause damages to the components of DNA and thus induce mutations that may increase the risk of cancer development. Cytotoxicity and cell viability assessments are based on targeting of functions of different cells, including the liver and bone marrow [5, 6].

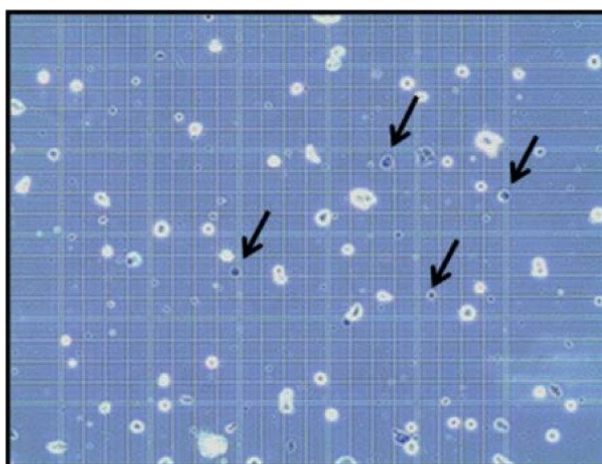
Cytotoxicity assays are experimental methods used in pharmacology and *in vitro* toxicology studies to measure loss of cellular functions [5–7]. These assays generally screen xenobiotics and predict human toxicity. These assays predict the risk of these xenobiotics towards human health by relating the cytotoxic effects between the *in vitro* and *in vivo* systems. There are different *in vitro* cytotoxic assays that have been employed in this stead with different results [5–7]. In these *in vitro* systems commonly used, cells are treated or exposed to a chemical or test compound and incubated for some period. Afterwards, a marker is measured to reflect the number of viable cells present compared to the positive (toxin) and negative (vehicle) control treatments. In addition, dead cells are also evaluated to distinguish between cytotoxicity and growth arrest. Knowledge of the number of live and dead cells present during or after the end of the experiment enhances the statistical robustness of these assays [5–8]. These assays commonly estimate dead cells via two common ways: (a) penetration of an otherwise non-permeable dye into the cells due to loss of cell membrane integrity; (b) the ability of indicator molecules to partition into a compartment not achievable if the cell membrane is intact. Therefore, these cytotoxicity assays directly or indirectly assess cytotoxicity by providing information on cell membrane integrity, cell metabolism, cell machinery, cell mortality and cell proliferation [5–8]. Some of these sensitive and reproducible assays are used to obtain colorimetric, luminometric and fluorometric measurements hence entails the use of different dyes such as trypan blue exclusion dye, neutral red, acridine orange and propidium iodide [1, 9–12]. These dyes offer different advantages and disadvantages hence a combination of two or more dyes is usually employed in *in vitro* studies to avoid overestimation or underestimation of the toxicity of a chemical substance thereby increasing the reliability of the results obtained.

## 2. Experimental dyes used in measuring cytotoxicity

### 2.1 Trypan blue dye exclusion assay

Trypan blue dye exclusion assay is one of the most frequently used routine methods to determine cell viability [9, 13]. It involves the selective staining of dead cells with trypan blue and microscopic examination on haemocytometer [9]. It was developed in 1975 to measure viable cells and provide information on cell mortality [2]. Trypan blue, a non-permeable cell membrane dye, is an azo dye derived from toluidine. This is a vital stain used in bioscience to exclusively stain necrotic (dead) cells (**Figure 1**). Therefore, this assay is based on the principle that viable cells have intact cell membranes, which trypan blue cannot penetrate thus, trypan blue is excluded from viable cells [5].

In contrast, trypan blue dye penetrates cell membrane of necrotic (dead) cells due to loss of cell membrane integrity and enters the cytoplasm. Under light microscope, only necrotic (dead) cells absorb this blue colour [15]. The trypan blue staining technique is usually performed on a single sample or relatively small number of samples from simple experiments [6].



**Figure 1.**  
*Cells stained with trypan blue. Neubauer chamber counter (Adapted from Hong et al. [14]).*

However, there have been some questions raised about the integrity of the trypan blue technique. These include:

1. There is the possibility of false-positive or false-negative results. Trypan blue exclusion assay can only demonstrate either viable or dead cells without detecting cells with sub-lethal injury [6]. Compromised viable cells may be counted as dead cells whilst the cell is still alive or cells with intact membranes may be counted as live cells even though they are no longer functioning as viable cells. This is because sometimes a cell may have an intact membrane and at the same time, the cell will be unable to grow or function as a viable cell [6, 16]. On the contrary, a cell may lose its cell membrane integrity and still be able to repair itself to become viable again [16]. Longer incubations with trypan blue dye may also result in faint staining of viable cells possibly due to slow uptake of the dye [16].
2. False-positive and/or false-negative results may also be due to subjective judgement of the user to determine what is a dead cell or stained debris [6]. Cellular uptake of trypan blue dye is determined by the human eye, and it is possible to overlook any small amount of the dye uptake following cellular damage due to stress or injury [9, 13]. Cells are counted manually using a haemocytometer hence a false result may arise due to human errors especially when processing many cell counts. Also, it takes a lot of time and manual labour to count and measure multiple samples [6]. Thus, if different operators count the same sample, there may be a lot of inconsistencies.
3. Trypan blue dye can also function as a mutagenic and carcinogenetic agent [6, 17]. Therefore, high concentration and prolonged exposure of the dye to cells may lead to toxic effects on the cells thus cause gradual cell damage and thence leading to false cell counts [18]. Due to its toxicity, trypan blue can also cause changes in intracellular protein expression, which may require some time to be observed/reflected in the cell count [18]. The amount of trypan blue dye exposed

to a cell with time may influence the expression of *P53* and *P21* genes, which are crucial in cell cycle. *P53* helps in making intracellular protein located in the nucleus and plays a major role in cell cycle; controlling cell division and cell death whereas *P21* is a cell cycle inhibitor including cell cycle arrest, apoptosis, and DNA repair [19–21]. Therefore, the impact of prolonged exposure of trypan blue dye to a cell on *P53* and *P21* genes expression imposes a challenge that contradicts the use of this assay [18].

In conclusion, there is need for optimisation of concentration and incubation time depending on the type of cell involved to eliminate the possibility of this dye causing a gradual damage on the cells. Also, trypan blue dye may likely underestimate cellular damage when performed immediately after treating cells with a cytotoxic agent. As a result, many researchers combine this technique with another dye to ensure the cytotoxic results are robust. Benchtop instruments have also been designed to automate imaging and improve the biased analysis steps of this assay.

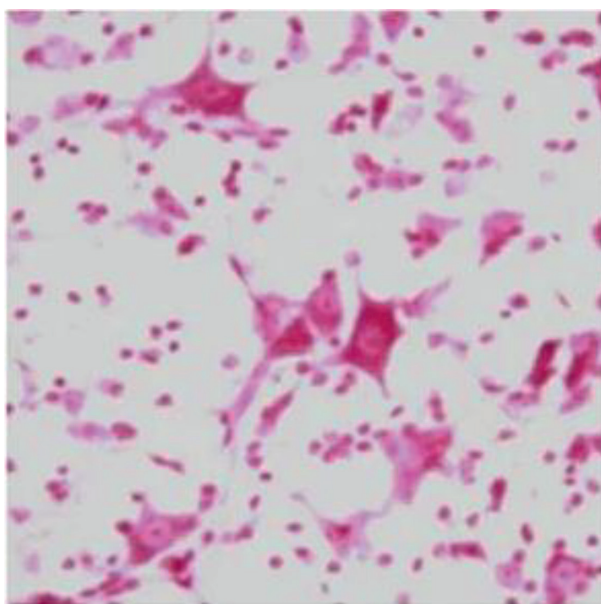
## 2.2 Neutral red

Neutral red assay was first described by Finter in 1969 as a chemosensitivity assay [22, 23]. This assay provides information on cell machinery and quantifies cell viability and measures cell replication. In addition to hepatocytes and erythrocytes, this assay can also be done in non-adherent cells such as fibroblasts. Cells with biotransformation capacity are recommended to be used when neutral red is used to assess the cytotoxicity of chemicals requiring metabolic activation to toxic metabolites [1, 24].

Its principle is based on the ability of viable cells to absorb neutral red, a weak cationic dye, which penetrates the cell membrane and concentrates in the lysosomes of the cell [24]. The mechanism by which the dye penetrates the cell membrane and accumulates in the lysosomes is not well understood. Micropinocytosis with subsequent fusion of vesicles with secondary lysosome was first suggested however entry by non-ionic passive diffusion has always been postulated. Once inside the lysosome, it binds to the anionic and/or phosphate groups of the lysosomal matrix by electrostatic hydrophobic bonds [24–26]. The dye is then extracted from viable cells using an acidified ethanol solution, and the absorbance of the solubilised dye is quantified using a spectrophotometer at 540 nm wavelength.

The uptake of neutral red by viable cells depends on their capacity to maintain pH gradients through the production of ATP [1, 8]. The dye presents a net charge close to zero at physiological pH that enables it to penetrate the cell membrane. Once inside the lysosome, the dye becomes charged and is retained inside the lysosome due to a proton gradient in the lysosome that maintains a pH lower than that of the cytoplasm [1, 8]. As a result, the amount of retained dye is proportional to the number of viable cells (**Figure 2**).

However, alterations in cell surface or lysosomal membranes can modify the uptake of neutral red by viable cells. For example, a variety of chemicals or pharmaceutical agents induce damage to cell surface or lysosomal membrane that may alter or decrease dye uptake and subsequent retention [27]. Due to specific lysosomal capacities in different cells for taking up the dye, neutral red can be used to differentiate between viable, damaged, or dead cells. Viable cells have intact lysosome and tend to absorb more neutral red dye more than dead cells or cells undergoing apoptosis.



**Figure 2.**  
*Cells stained with neutral red (Adapted from Repetto et al. [24]).*

Therefore, uptake of the dye as well as lysosomal integrity are highly sensitive indicators of cell viability. In addition, the results of neutral red assay are dependent on (a) the degree of acute toxicity (b) number of viable cells in the culture which determine the timing of the assay [25, 26, 28].

Neutral red dye is non-specific and non-toxic and is often used as a counterstain for dyes such as trypan blue dye. Therefore, neutral red assay poses some advantages over other cytotoxicity assays as it is very sensitive, simple, cheap, readily quantifiable, presents less interference and does not require equipment or unstable reagents such as tetrazolium salts that measure lactate dehydrogenase enzyme activity by the chemical reduction of the salts to formazans [22, 29, 30]. The neutral red assay also has advantages, which include speed and reproducibility of data. Also, neutral red estimates can be done on the same cell culture alongside protein determination.

Nevertheless, there are limitations of the neutral red assay such as underestimation of the toxicity of chemicals, which require metabolic activation to a toxic product and of substances, which bind to serum proteins [22, 29, 30]. On the contrary, some chemicals may induce irreversible precipitation of the neutral red dye into fine, needle-like crystals, which may result in an overestimation of the toxic effects [27]. Some chemicals have a localised effect on lysosomes, and this may result in low or high uptake thus leading to overestimation or underestimation of cellular toxicity. Therefore, this assay is suitable for detecting chemicals such as chloroquine that selectively target lysosome and alter its pH thus inducing a greater effect of neutral red uptake than most chemicals [24]. This causes an overestimation of the toxic effects induced by these chemicals. Changes in cell proliferation may also interfere with the estimation of lysosomal function [26, 28, 29]. To prevent this, the assay can be performed in conjunction with other cytotoxicity assays. However, performing neutral red estimates followed by determination of enzymatic function such as lactate



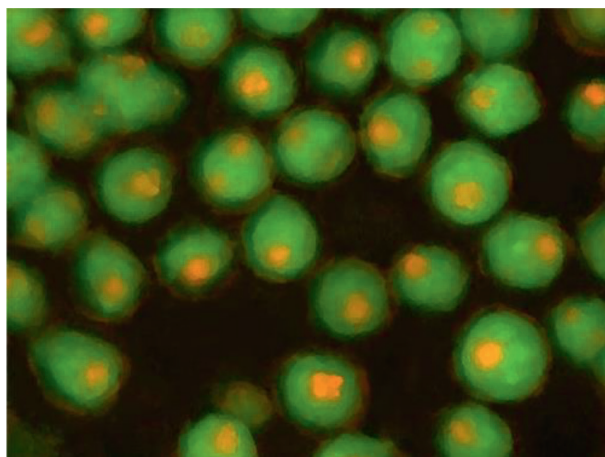
dehydrogenase and glucose-6-phosphate dehydrogenase or total protein determination in the same cell culture may lead to a reduction in the amount of protein estimated to be present in the cell culture.

### 2.3 Acridine orange

Acridine orange is a heterocyclic organic compound that was first extracted from coal tar. It acts as a weak basic nucleic acid dye, which can permeate the cell membrane and accumulate in these acidic organelles such as lysosome in a pH dependent manner [31]. Acridine orange is hydrophobic, which enables it to permeate the cell membrane quickly, enter the cytoplasm and accumulate in the lysosome [31–33]. Therefore, acridine orange can be used to stain lysosome, vacuoles, and nucleus where it specifically binds to double stranded DNA and RNA in living cells by intercalation or electrostatic attractions (**Figure 3**). Acridine orange stabilises the pigment-DNA complexes via charge neutralisation of DNA backbone phosphate group [33, 35].

Under acridine orange staining, lysosome fluoresce bright-red or orange red at a wavelength of 590 nm whereas the nucleus and cytoplasm emit green fluorescence at a wavelength of 525 nm. Due to its low molecular weight (256 g/mol), it rapidly diffuses into the cytoplasm of living cells to bind to DNA and RNA [36, 37]. Once bound to single stranded DNA or RNA, red fluorescence is emitted whereas green fluorescence is emitted when bound to double stranded DNA. As a result, acridine orange can be used in apoptotic studies to stain apoptotic cells orange or red depending on the degree of loss of membrane integrity. This dye also has the capacity to label dead cells thus differentiating the apoptotic cells into early apoptosis (green) and late apoptosis (orange red) hence it offers superior accuracy than the older methods [10, 11, 38].

However, fluorescence response of acridine orange is dependent upon the concentration of acridine orange used, the solvent used in dissolving the dye, fixation, time of staining, ions present, ionic strength of the medium, pH, temperature, and complexing substrate [37, 39, 40]. Lowering the concentration of acridine orange causes a decrease in fluorescence whilst an increase in acridine orange concentration will cause a metachromatic shift. In addition, acridine orange is usually co-stained with other



**Figure 3.** Stromal cell line, HS-5 cells, stained with acridine orange, which stained the nucleus of the cells (Adapted from Okeke [34]).

dyes such as ethidium bromide and propidium iodide [10, 37–41]. The stock solution of acridine orange (100 mg/ml) and ethidium bromide/propidium iodide (100 mg/ml) is usually made in phosphate buffered saline (PBS) or distilled water and stored in a foil-wrapped bottle at 4°C. This is because acridine orange is light-sensitive and can degrade upon exposure to light.

Co-staining of cells with acridine orange and ethidium bromide or propidium iodide provides information on nuclear morphology (perinuclear chromatin condensation, nuclear collapse, and eventual fragmentation) [10, 37, 40, 41]. Ethidium bromide and propidium iodide can bind with core histones of DNA nucleosome structure but lack the metachromatic property of acridine orange and only stain dead cells when combined with acridine orange [10, 37, 40, 41]. This enables earlier identification of damaged cells by suppressing the DNA-specific green fluorescence induced by acridine orange. Ethidium bromide and propidium iodide are impermeable to intact cell membranes and intercalate the DNA and emits red or orange fluorescence when cells lose their membrane integrity [10, 12, 37–40]. In the presence of two dyes, the cells stain red or orange because the molar concentration of propidium iodide/ethidium bromide is more than 100× greater than that of acridine orange thus propidium iodide/acridine orange has greater affinity and specificity for nucleic acids than acridine orange. In addition, the active accumulation of acridine orange in the cells stops upon death, which reduces the concentration of acridine orange in the cells thereby making the red fluorescence of ethidium bromide/propidium iodide is more apparent [12, 38, 40].

Therefore, dual staining of cells with acridine orange and propidium iodide/ethidium bromide aids the detection of four main types of cells: (a) Viable cells with uniform green nuclei with organised chromatin structure (b) Necrotic cells with uniform orange red or red nuclei with organised structure due to loss of membrane integrity (c) Early apoptotic cells with irregular structured green nucleic but chromatin is condensed as apoptotic bodies or green patches/fragments (d) late apoptotic cells, with orange or red nuclei with extremely condensed or fragmented chromatin [10, 11, 38, 40–42]. Thus, since the cellular detail and nuclear outline are distinct, co-staining cells with acridine orange and ethidium bromide/propidium iodide offers a rapid, stable, sensitive, and easy-to-perform way to simultaneously visualise and identify all the possible nuclear stages with increased accuracy and ease of interpretation. In addition, light microscopy of cells co-stained with these dyes also provide visualisation of a complete morphological profile of an apoptotic cell [12, 37, 41–43]. Therefore, this assay also permits the staining and scoring of multiple specimens in batches thus it is cost-effective and labour saving. It also works well at room temperature and not subject to interference by extracellular enzymes.

However, fluorescence microscope is required to perform this dual staining thus expertise in this field is paramount. In addition, propidium iodide and ethidium bromide are carcinogenic and can cause debilitating effects to the DNA [11, 12, 38]. Interestingly, acridine orange is also carcinogenic and has been used as an anti-tumour agent in targeting different cancer cells via photosensitization as it selectively binds to malignant cells compared to normal cells [40–42]. Accumulation of acridine orange in the lysosome at low pH is also crucial for photosensitization thus resulting in the release of oxygen radicals, especially in malignant cells.

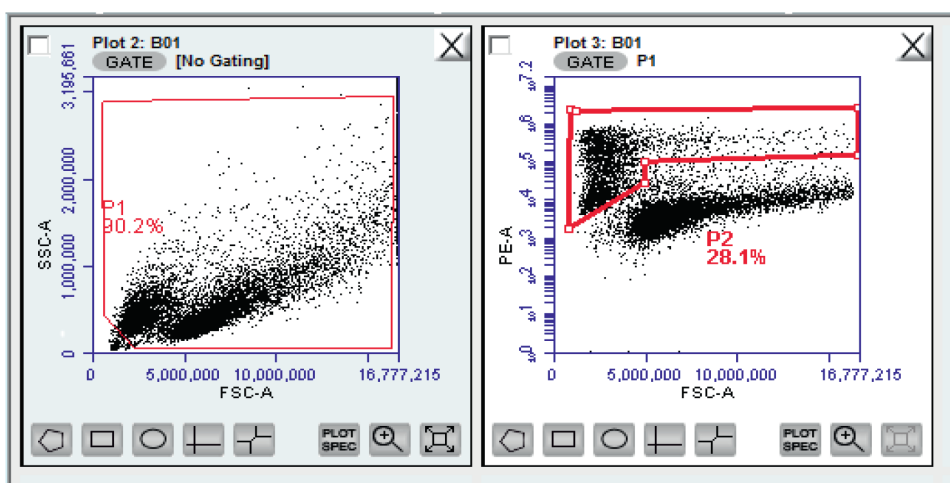
## **2.4 Propidium iodide**

Propidium iodide is an analogue of ethidium bromide. It is an exclusion dye that binds to short non-specific double stranded RNA or DNA at excitation wavelength of

525 nm and an emission wavelength of 491–495 nm to produce red or orange fluorescence [44]. Propidium iodide is impermeable to the plasma membrane in living cells however it easily permeates compromised plasma membrane [45–47]. Once inside the cell, the dye binds to DNA, which leads to a 30-fold increase in fluorescence shifting the excitation maximum by 30–40 nm up to 525 nm to the red and emission maximum by 15 nm to the blue [44–46]. Thus, propidium iodide provides an objective number of dead or necrotic cells in a cytotoxicity assay when observed with a fluorescent microscope or flow cytometer (**Figure 4**). Therefore, assessing cytotoxicity with propidium iodide is easy, cost-effective and aids in measuring a large set of samples using automated flow cytometer.

In addition to acridine orange, propidium iodide is often co-stained with annexin V [12, 38, 41]. Annexin V binds to phosphatidylserine (PS) exposed on the outer membrane of necrotic cell surface. PS is usually situated in the inner membrane leaflet in viable cells and only translocate to the outer membrane leaflet in these cells upon induction of apoptosis [12, 48]. Co-staining propidium iodide with annexin V offers the ability to simultaneously detect and measure viable and non-viable cells. The cells are grouped into four groups: (a) viable cells (annexin V-negative/propidium iodide-negative), (b) necrotic cells (annexin V-negative/propidium iodide-positive), (c) early apoptotic (annexin V-positive/propidium iodide-negative), (d) late apoptotic (annexin V-positive/propidium iodide-negative) [3, 10, 12, 49]. Therefore, propidium iodide co-staining with annexin V or acridine orange provides reliable, reproducible results and distinguishes subpopulation of apoptotic cells with accuracy.

However, in some cases, late apoptosis can be characterised by some loss of membrane integrity therefore flow cytometric analysis of cytotoxicity using propidium iodide and annexin V cannot differentiate between late apoptosis and necrosis, which can both be annexin V-positive and propidium iodide-positive. In addition, apoptotic cells stained with propidium iodide present different hypodiploid peaks and sizes on the red fluorescent channels, which could be representative of the sub G1 (debris) in the sample [46, 49]. Therefore, despite propidium iodide being a universal cell death indicator, results can be skewed by nuclear material in solution although this



**Figure 4.** Cells treated with 20 µg/mL of PI/FACS buffer for 10 min on Ice and analysed on the flow cytometer. Exclude debris using (P1 left panel) then isolate non-viable cells on the PE-A vs. FSC-A using P2 (right panel).



can be avoided by excluding the debris by size in the forward and side scatter plots or using RNase A to digest RNA. Propidium binding to the DNA can be affected by the chromosome structure and this can be exploited to study the effects of drugs and xenobiotics on cell cycle [46, 49]. This is because propidium shares similar properties to some chemotherapy agents such as mitoxantrone, which can mask its reading and lead to underestimation of toxic effects [50, 51].

### 3. Conclusion

Cytotoxicity assessment is an important assessment in pharmaceutical and environmental industry to produce novel drugs and identify potentially harmful substances. In recent years, experimental methods to evaluate cytotoxicity have improved due to the progress of modern biology. However, there is no uniform cytotoxicity assay and the most popular methods currently used still entail tracking changes in cell morphology, cell proliferation and differentiation by labelling cells with colorimetric or fluorescent dyes that target compromised membranes of dead cells or specific organelles in live cells. Trypan blue dye is the most common dye used in cytotoxicity assays however due to its lack of specificity and sensitivity, it should be made mandatory to counterstain cells with dyes like neutral red, acridine orange and propidium iodide to provide a robust result. This would help eliminate potential drastic patient outcomes that may result from pushing a toxic compound into the market.

### Author details

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