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# A rapid and inexpensive viability assay for zoospores and zoosporangia of *Batrachochytrium dendrobatidis*

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## Abstract

The fungus *Batrachochytrium dendrobatidis* is causing global amphibian declines. Here we describe a simple, rapid and inexpensive methylene blue staining protocol to determine *B. dendrobatidis* viability, regardless of life-stage. The viability of cells in suspension or adherent monolayers can be determined using either manual microscopy counting or colorimetric assay.

**Keywords**: *Batrachochytrium dendrobatidis*, viability, methylene blue, zoosporangia

**Main text:** The amphibian chytrid fungus, *Batrachochytrium dendrobatidis,* has caused widespread loss of biodiversity, impacting hundreds of amphibian species (Scheele et al., 2019) since spreading from Asia (O'Hanlon et al., 2018). It ranks among the world's worst invasive species, but research to develop a solution has been challenging as it is an unusual pathogen and the first from this phylum to cause disease in vertebrates. Hence, optimising new methods for experimental research is important.

*B. dendrobatidis* infects skin and disrupts osmoregulation leading to cardiac arrest and death in susceptible species (Voyles et al., 2009). The life cycle of *B. dendrobatidis* starts with a small, motile infective zoospore, which develops into a zoosporangium (Longcore et al., 1999) after it invades an epidermal cell via a germ tube (Van Rooij et al., 2012). The zoosporangium then produces further infective zoospores, releasing them through discharge tubes into the environment or to re-

infect their amphibian host. Laboratory experiments are essential to improve our understanding of this pathogen. Despite its pathogenicity, *B. dendrobatidis* is easily killed by heat, desiccation (Johnson et al., 2003), disinfectants (Webb et al., 2012), and salt (Stockwell et al., 2012). Therefore, it is often necessary to perform viability estimates on laboratory cultures to determine the proportion of viable cells before commencing experimental work. Viability estimates are essential in fungal susceptibility trials.

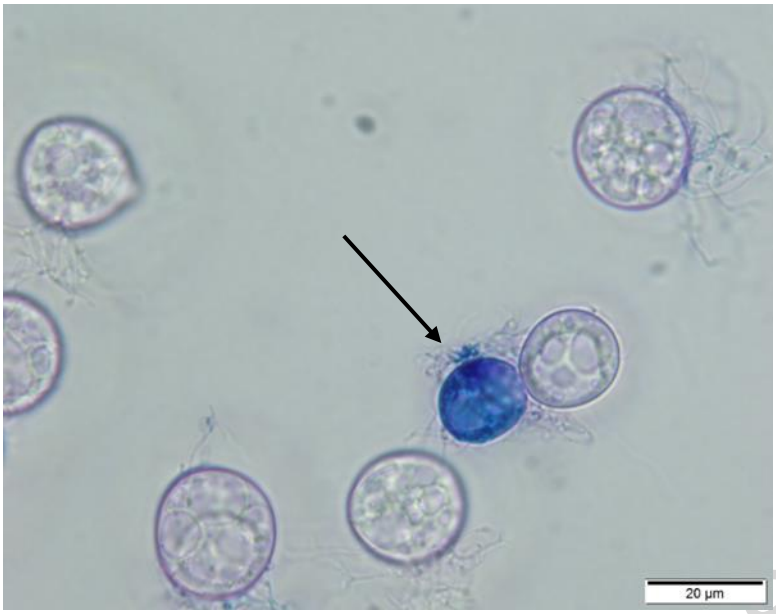
Two viability stains have been validated for use on the zoospore stage – trypan blue (McMahon and Rohr 2014), which is readily available; and SYBR green/propidium iodide, which is less subjective, but more expensive and requires specialized equipment (Stockwell et al., 2010). Both these techniques rely on the exclusion of dye by intact zoospore membranes, so that viable cells remain unstained. For this reason they are unsuitable for the zoosporangial life stage, because the discharge tubes allow the dye to enter the cell regardless of cell viability. This is supported by McMahon and Rohr 2014, who found that trypan blue stained 100% of zoosporangia in a healthy culture, indicating falsely that they were all dead. Zoosporangial growth can be estimated by measuring optical density (OD) over time (Rollins-Smith et al., 2002); however, this method cannot distinguish between live and dead cells. Recently, an optimized MTT assay has been developed which enables more accurate measurements of zoosporangial growth and viability (Lindauer et al., 2019). MTT stains viable cells purple due to the reduction of the reagent by cell metabolic activity, and can be quantified using spectrophotometry (Mosmann, 1983). However, the MTT assay is lengthy (2 h), requires hazardous reagents (dimethylformamide), and does not measure zoospore viability. Here we assessed methylene blue as a safe, rapid and inexpensive method for assessing the viability of both zoospores and zoosporangia, which can be completed in the absence of a spectrophotometer using manual cell counting, or as a colorimetric assay.

Methylene blue differs from other vital stains in that it relies on the ability of viable cells to reduce the stain to a colourless substance via enzymatic activity, rather than relying on membrane impermeability (Bapat et al., 2006, Painting and Kirsop, 1990, Thomson et al., 2015). Methylene blue has a long history as a viability stain (Borzani and Vairo, 1958), and it is still commonly used to estimate yeast viability (Feizi et al., 2016). However, the mechanism of action is still not completely understood. It is likely that due to the positive charge of methylene blue in the oxidized (blue) state, its permeability is excluded in live, but not dead,

cells (Chilver et al., 1978, May et al., 2003). Some reports suggest that cell surface or transmembrane reductases can reduce methylene blue to the colourless form, which can then permeate into live cells and be reoxidised intracellularly to the blue form (Bapat, Nandy, Wangikar and Venkatesh, 2006, Bongard et al., 1995, Merker et al., 1997).

As an initial test, we grew zoosporangia as a monolayer on glass slides, which were then stained with methylene blue dissolved in water. Zoospores were obtained by flooding a 3 day old tryptone, hydrolysed gelatin and lactose (TGhL) agar culture (Longcore, Pessier and Nichols, 1999). The zoospore solution was added to centrifuge tubes containing a sterilized glass slide, and incubated at 21°C. At one, two and three days post encystation, slides were half dipped in ethanol for 30 seconds (Johnson, Berger, Philips and Speare, 2003) to kill zoosporangia attached to the lower half. The entire slide was rinsed with water and the stained with 0.01 mg/ml methylene blue for 2 minutes (Kwolek-Mirek and Zadrag-Tecza, 2014). Examination under a compound microscope revealed that all killed zoosporangia on the lower end of the slide were stained bright blue. Zoosporangia on the upper half of the slide not killed by ethanol remained colourless. There was a transition zone in the middle of the slide containing a mixture of stained and unstained zoosporangia (Figure 1). In addition, empty, effete zoosporangia that had released zoospores were easily distinguishable from killed zoosporangia, as their walls stained blue, but not their contents.

Figure 1: Methylene blue exposure at 0.01 mg/ml for 2 minutes clearly differentiates between dead (arrow) and live zoosporangia adhered to a glass slide.




Next, we aimed to quantify percent zoosporangia viability in a TGhL liquid culture (zoosporangia in suspension) with methylene blue, using methods adapted from McMahon and Rohr 2014. An actively growing culture containing a mix of life stages was concentrated by centrifugation, vortexed, and split equally into two tubes. One tube was killed, using either heat (10 minutes at 50°C) or flash freezing in liquid nitrogen (McMahon and Rohr, 2014). Suspensions containing 0, 25, 50, 75 and 100% dead zoosporangia were prepared. To these 0.02 mg/ml methylene blue was added 1:1 to the suspension to give a final concentration of 0.01mg/ml and samples were incubated for 2 minutes. The proportion of stained and unstained cells in each suspension was determined at least twice using a haemocytometer, and corrected using the 0% dead treatment. Each experiment was conducted using two global Pandemic Lineage isolates of *B. dendrobatidis* from Australia (Mitta Mitta-L. spenceri-2018-LB and Ethel creek–Lnannotis-2013-LB). The proportions of stained zoosporangia matched the known proportion of dead zoosporangia, regardless of the isolate, or the killing method. Paired T-tests (SPSS version 25) comparing the proportion stained and the proportion killed were not significantly different (heat killed $t=-0.259$ $df=23$ $p=0.798$, frozen $t= 0.888$ $df=25$ $p=0.383$). These mixed cultures inevitably contained some dead zoosporangia from the original inoculum used for subculturing. For example, in the "0% dead" treatment we counted up to 10% dead, stained zoosporangia. This highlights the importance of assessing *B. dendrobatidis* viability, as even actively growing cultures will contain some unviable cells.

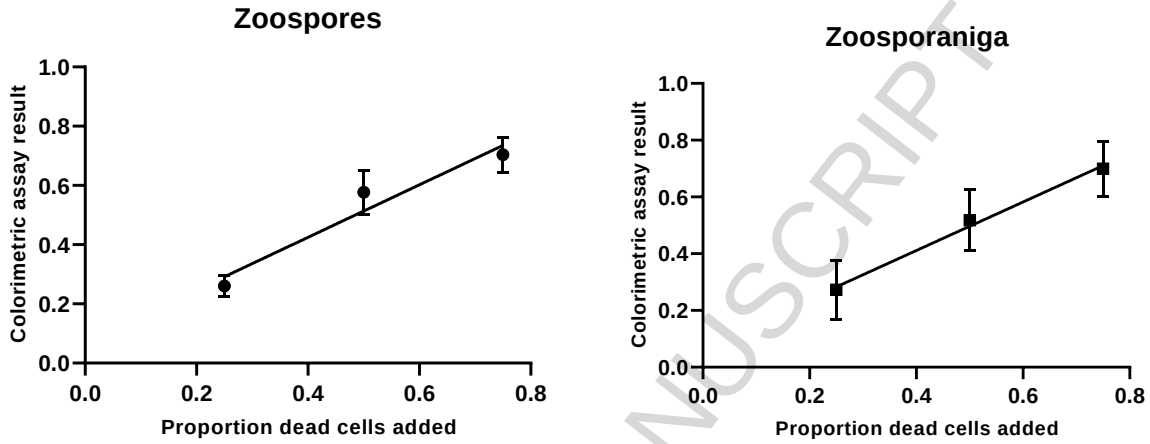
As manual cell counting via hemocytometer can be time consuming and subjective, we also developed a rapid colorimetric assay to determine the viability of *B. dendrobatidis* zoospores and zoosporangia. Suspensions containing 0%, 25%, 50%, 75% and 100% heat-killed zoosporangia or zoospores from an actively growing Mitta Mitta isolate were prepared. After staining, excess dye was removed by centrifugation and rinsed once with Milli-Q water. The washed pellet was re-suspended in 0.1 M HCl (Dent et al., 1995) for 15 minutes to elute the methylene blue. The amount of HCl can be adjusted to suit the cell concentration, here we used between 60 µl-100 µl. The eluent was centrifuged to pellet cells, the supernatant removed and absorbance was measured twice at 650 nm using an Omega Polarstar spectophotometer (using 0.1 M HCl as a blank). To test that this method is applicable over different cell concentrations likely to be used in experiments, we used a range of total zoosporangia per treatment ($6.7 \times 10^3$, $6.5 \times 10^4$, $1.2 \times 10^5$, $3.2 \times 10^5$). However, due to the small size of zoospores (3-5 µm) compared to zoosporangia (40 µm) (Longcore, Pessier and Nichols, 1999), the amount of dye that can be eluted from zoospores is low. Therefore, we tested this method three times using high concentrations of zoospores per treatment ($1.5 \times 10^6$, $1.8 \times 10^6$, $2.4 \times 10^6$). We found strong linear relationships between absorbance and the proportion of dead zoosporangia ($R^2$ =0.979, 0.955, 0.9946, 0.976 respectively), and zoospores ($R^2$ =0.980, 0.981, 0.978 respectively) added to solution. Absorbance of eluted methylene blue can therefore serve as a predictor of non-viable zoosporangia and zoospores. Because the eluted methylene blue is proportional to the total number of dead cells, we recommend adding as many cells as possible to ensure the dye can be detected by the spectrophotometer.

This colorimetric assay could be used to estimate sub-lethal effects on *B. dendrobatidis* cultures of either life stage, especially in experiments with large sample numbers, or where inter-researcher consistency in estimating viability is important. Experiments should include an untreated negative control to estimate background cell death (Bonora and Mares, 1982). Because methylene blue stains non-viable cells, it has an advantage over MTT, in that a control aliquot can be prepared by killing all the cells using heat or flash freezing. The absorbance of each treatment (adjusted for background cell death) can then be divided by the absorbance of the killed control (100% dead) to give a percentage of cells that have died as a result of treatment. We found a strong linear relationship between the proportion of dead cells predicted using this method, compared to the proportion of

dead cells added to the solution (zoospores $R^2$= 0.88, zoosporangia $R^2$= 0.78) (Figure 2)

Figure 2: Absorbance of eluted methylene blue can be used to estimate the proportion of dead zoosporangia or zoospores using the equation:

$$Proportion\ dead\ cells = \frac{Absorbance\ unknown - Absorbance\ untreated\ control}{Absorbance\ killed\ control}$$




Care must be taken when using methylene blue as a viability stain, as methylene blue is a fungicide, and mildly toxic to *B. dendrobatidis*. However, the concentration used here is tenfold lower than the minimum concentration needed to cause zoospore death in 30 min (Berger et al., 2009). We recommend counts are performed within 5 min, or by removal of excess methylene blue at the conclusion of the staining period, to remove the possibility of mortality due to the stain affecting the results. It is also possible that cells might be misclassified as dead if the dye is re-oxidized inside live cells, leading to the re-appearance of the blue colour. (Bapat, Nandy, Wangikar and Venkatesh, 2006). To address this possibility in *B. dendrobatidis*, we monitored live cells treated with methylene blue over time and found that they remained unstained, indicating that the assay was stable for at least 60 min. It is not known conclusively whether zoosporangia exclude the dye or reduce it extracellularly, but it is likely that live cells do not take up appreciable amounts of either oxidised or reduced methylene blue. Regardless, our assay is not affected by either possible mechanism of action as we wash the cell pellets and elute the dye from inside dead cells.

Our results indicate that methylene blue staining is a useful tool for determining zoosporangia and zoospore viability. It is a readily available, stable, inexpensive and rapid stain that can be quantified by manual counts via microscopy, or by or

colorimetric assay using a spectrophotometer. These methods could be used to check that handling procedures have not harmed cultures before conducting research such as infection experiments or lymphocyte proliferation assays, or to assess sensitivity of *B. dendrobatidis* to disinfectants or other treatments. The colorimetric assay is quicker and likely more consistent than manual counts. We hope that the viability assays described here will allow increased accuracy in experiments, enabling a better understanding of this devastating fungal disease.

## Conflicts of interest

None

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**Declaration of interests**

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Highlights:

- Viability estimates for the pathogen *B. dendrobatidis* are hindered by the presence of discharge tubes, precluding the use of most vital dyes.
- Methylene blue stains dead cells of both life stages, leaving viable cells unstained.
- This protocol can be used to calculate proportion of viable cells using either microscopy or colorimetric assay.