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Fluorescent caspase-3 staining to assess induction of apoptosis in A6 cells (Plos One)

PLOS One

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ABSTRACT

The largest current disease-induced loss of vertebrate biodiversity is due to chytridiomycosis and despite the increasing understanding of the pathogenesis, knowledge unravelling the early host-pathogen interactions remains limited. *Batrachochytrium dendrobatidis* (*Bd*) zoospores attach to and invade the amphibian epidermis, with subsequent invasive growth in the host skin. Availability of an *in vitro* assay would facilitate in depth study of this interaction while reducing the number of experimental animals needed. We describe a fluorescent cell-based *in vitro* infection model that reproduces host-*Bd* interactions. Using primary keratinocytes from *Litoria caerulea* and the epithelial cell line A6 from *Xenopus laevis*, we reproduced different stages of host cell infection and intracellular growth of *Bd*, resulting in host cell death, a key event in chytridiomycosis. The presented *in vitro* models may facilitate future mechanistic studies of host susceptibility and pathogen virulence.

OPEN ACCESS

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MATERIALS

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MATERIALS

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29258

Keywords: A6 cells,
Batrachochytrium
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☒ Distilled Water **Contributed by users**

☒ Goat anti-Rabbit IgG (H L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 568
Thermo Fisher Scientific Catalog #A11011

☒ HBSS with calcium and magnesium **Contributed by users**

☒ HBSS without calcium and magnesium **Contributed by users**

☒ Triton X-100 **Sigma Catalog #93426**

☒ Fetal bovine serum **Contributed by users**

☒ Paraformaldehyde **Sigma Aldrich Catalog #P6148**

☒ Leibovitz's L-15 Medium **Thermo Fisher Catalog #11415049**

☒ Hoechst 33342, Trihydrochloride, Trihydrate, 100 mg **Thermo Fisher Catalog #H1399**

☒ ProLong™ Glass Antifade Mountant **Thermo Fisher Catalog #P36980**

☒ Rat Tail Collagen Coating Solution **Sigma Aldrich Catalog #122-20**

☒ anti-caspase-3 antibody **Sigma Aldrich**

24-well tissue culture plates

glass coverslips

1 **Prepare Cell Medium A:**

L15 medium: 70%

Distilled water: 20%

Fetal bovine serum 10%

2 **Prepare Cell Medium B:**

L15 medium: 40%

Distilled water: 55%

Fetal bovine serum: 5%

3 **Coat coverslips with Rat tail collagen:**

Add glass coverslips in a 24-well tissue culture plate. Coat the glass coverslips at 37°C for 2 hours. Therefore, carefully aspirate the Collagen Coating Solution and add 200 µl per well (so per coverslip). After 2 hours, rinse the coated surface twice with HBSS-. Coated tissue culture ware may be used immediately or air-dried and stored at 4 °C for up to one week.

4 **Seeding A6 cells:**

- Detach A6 cells from a cell culture flask using trypsin
- Centrifuge for 5 min at 1500 rpm
- Wash the cells with 70% HBSS- and in meantime count the cells
- Centrifuge for 5 min at 1500 rpm

- Resuspend the cells in cell medium A to reach a concentration of 10e5 cells per mL
- Seed the cells at a concentration of 10e5 cells per well (so add 1 mL) which contains a collagen-coated glass coverslip
- Let the A6 cells attach for 2 hours at 26°C and 5% CO₂
- After 2 hours, wash the cells with 70% HBSS+
- The cells are now ready to be exposed to *Bd* spores

IMPORTANT NOTE: Include a control well that can be used to check the cells throughout the entire protocol via light microscopy.

5 ***Bd* infection of A6 cells:**

- Isolate *Bd* zoospores and spin them down for 5 min at 3000 rpm (20°C)
- Remove the supernatant, resuspend them in cell medium B and count the spores
- Dilute the spores to a concentration of 10e6 spores/mL in cell medium B
- Add 1 mL of the spores suspension (= 10e6 spores) to the wells containing A6 cells (on a coverslip). As such the spores are seeded at a MOI of 10:1.
- Incubate for 2 hours at 20°C 5%CO₂.
- After 2 hours gently wash the infected cells three times with 70% HBSS+ to remove non-adherent spores
- Replace the cell medium B with cell medium A for 4 to 6 days (depending on the experimental setup) at 20°C, 5% CO₂

IMPORTANT NOTE: *Bd* zoospores lose their motility when exposed to cell medium A.

IMPORTANT NOTE: Include a control well that can be used to check the motility of the spores throughout the entire protocol via light microscopy.

6 **Fixation and staining of apoptotic cells: (Work in the dark)**

- Gently wash the infected cells 2 times with 70% HBSS+
- Fix the infected cells with 0.5 mL of 3% paraformaldehyde for 10 min
- Wash 2 times with 70% HBSS+
- Permeabilise with 200 µL of 0.1% triton in 70% HBSS+ on room temperature during 2 min.
- Wash 2 times with 70% HBSS+
- Incubate 1 hour with a primary antibody (1/1000):
 - * Anti-caspase-3 antibody produced in rabbit -> Sigma-Aldrich
- Wash 3 times with 70% HBSS+
- Incubate for 1 hour with a secondary antibody (1/500):
 - * Alexa Fluor 568 goat anti-rabbit IgG
- Wash 3 times with 70% HBSS+
- Incubate with Hoechst for 15 min
- Wash 3 times with 70% HBSS+
- Mount the coverslips using ProLongGold antifade mountant
- Use fluorescence microscopy to analyse apoptosis