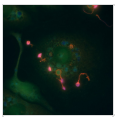

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Fluorescent in vitro model to assess invasion and intracellular matruation of Bd in A6 cells (Plos One)

PLOS One

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1 Works for me dx.doi.org/10.17504/protocols.io.8ishuee

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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.

EXTERNAL LINK

https://doi.org/10.1371/journal.pone.0225224

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Verbrugghe E, Rooij PV, Favoreel H, Martel A, Pasmans F (2019) *In vitro* modeling of *Batrachochytrium dendrobatidis* infection of the amphibian skin. PLoS ONE 14(11): e0225224. doi: 10.1371/journal.pone.0225224

MATERIALS

NAME	CATALOG #	VENDOR
Distilled Water		
Goat anti-Rabbit IgG (H L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 568	A11011	Thermo Fisher Scientific
HBSS with calcium and magnesium		
HBSS without calcium and magnesium		
Triton X-100	93426	Sigma
Fetal bovine serum		
Paraformaldehyde	P6148	Sigma Aldrich
Leibovitz's L-15 Medium	11415049	Thermo Fisher
CellTracker™ Green CMFDA Dye	C7025	Thermo Fisher
ProLong™ Glass Antifade Mountant	P36980	Thermo Fisher
Rat Tail Collagen Coating Solution	122-20	Sigma Aldrich
Calcofluor White stain	18909-100ML-F	Sigma Aldrich
polyclonal antibody against Bd raised in rabbit	View	

MATERIALS TEXT

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 ^{o}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
- Resuspende 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
- Afther 2 hours, wash the cells with 70% HBSS+
- The cells are now ready to be exposed to *Bd* spores

IMORTANT 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 X days (depending on the experimental setup) at 20°C, 5% CO2

IMPORTANT NOTE: *Bd* zoospores lose their motility when exposed to cell medium A.
IMORTANT NOTE: Include a control well that can be used to check the motility of the spores throughout the entire protocol via light microscopy.

6 **Staining of *Bd*-infected A6 cells: (Work in the dark)**
- Wash the infected cells 3 times with 70% HBSS+
- Stain the infected cells with 3 µM celltracker green CMFDA in cell medium A for 45 min at 20°C, 5% C02

7 **Staining of extracellular *Bd*: (Work in the dark)**
- Remove the green celltracker and wash with 70% HBSS+
- Add 200 µL CalcoFluor White (10 µg/mL in cell medium A) for 10 min
- Wash 3 times with HBSS+

8 **Fixation and staining of *Bd* : (Work in the dark)**

- 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% trition in 70% HBSS+ on room temperature during 2 min.
- Wash 2 times with 70% HBSS+
- Incubate 1 hour (up to 1.30 hour) with a primary antibody (1/1000):
    * Anti chytrid antibody produced in rabbit -> Thomas et al. 2018

Thomas V, Blooi M, Van Rooij P, Van Praet S, Verbrugghe E, Grasselli E, et al. Recommendations on diagnostic tools for *Batrachochytrium salamandrivorans*. Transbound. Emerg. Dis. 2018; 65: e478-e488.

- 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+
- Mount the coverslips using ProLongGold antifade mountant
- Use fluorescence microscopy to analyse *Bd*-A6 cell interactions