Zebrafish Egg Bath Infection Model for Investigating Pathogenesis of Fungal Pathogens

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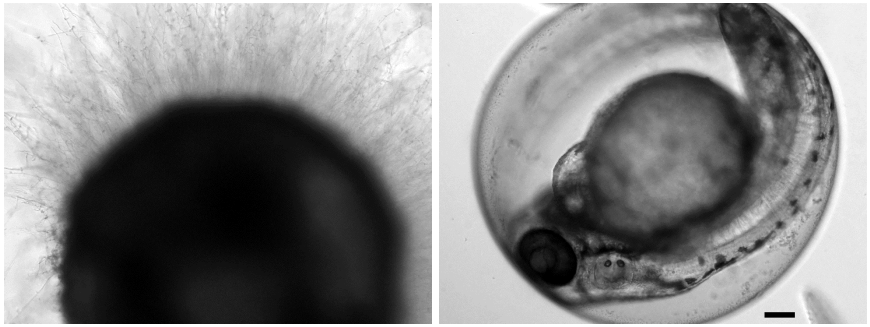
National Health Research Institutes



There Are Good and Bad Fungi

Mushroom

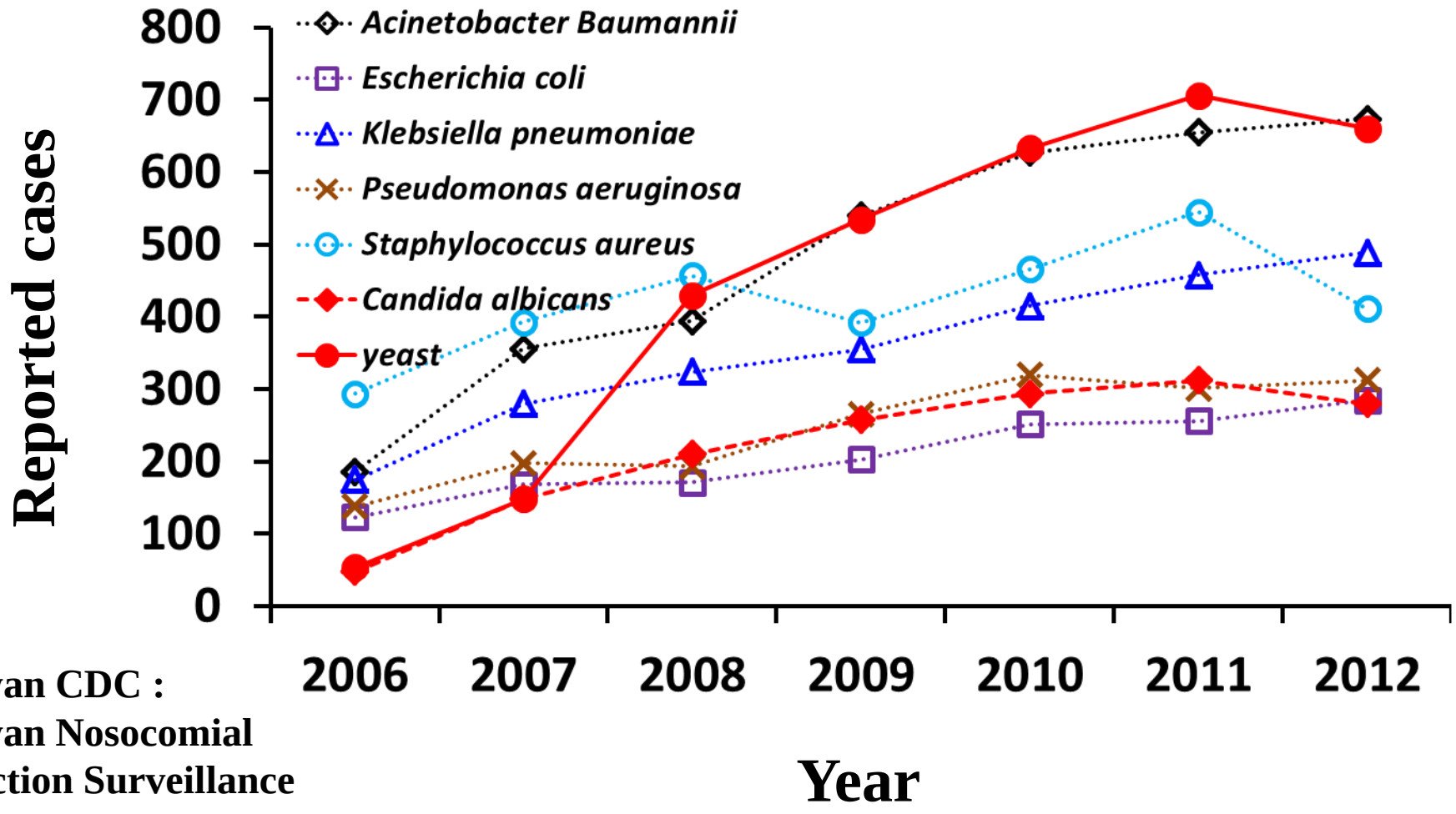
Candida, Aspergillus, Cryptococcus, etc.

Local or systemic infections

Saccharomyces cerevisiae

Beer and Bread

Yeast Infections Increased Significantly



Taiwan CDC : Taiwan Nosocomial Infection Surveillance (TNIS) inpatients in intensive care unit (ICU)

Fungal Infections: Emerging Threats to Human Health

- 1,000,000,000 skin infections
- 100,000,000 Mucosal infections
- 10,000,000 Allergies and SAFS
  (Severe asthma with fungal sensitization)
- 1,000,000 death
  = tuberculosis
  > malaria
  > breast cancer

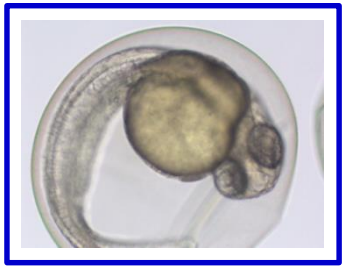
To develop new effective antifungal drugs is needed.

Animal Models for Fungal Infections






Rabbit **Mouse** **Fruit Fly** **Nematodes** **Wax Moths** **Zebrafish**



Zebrafish Embryo

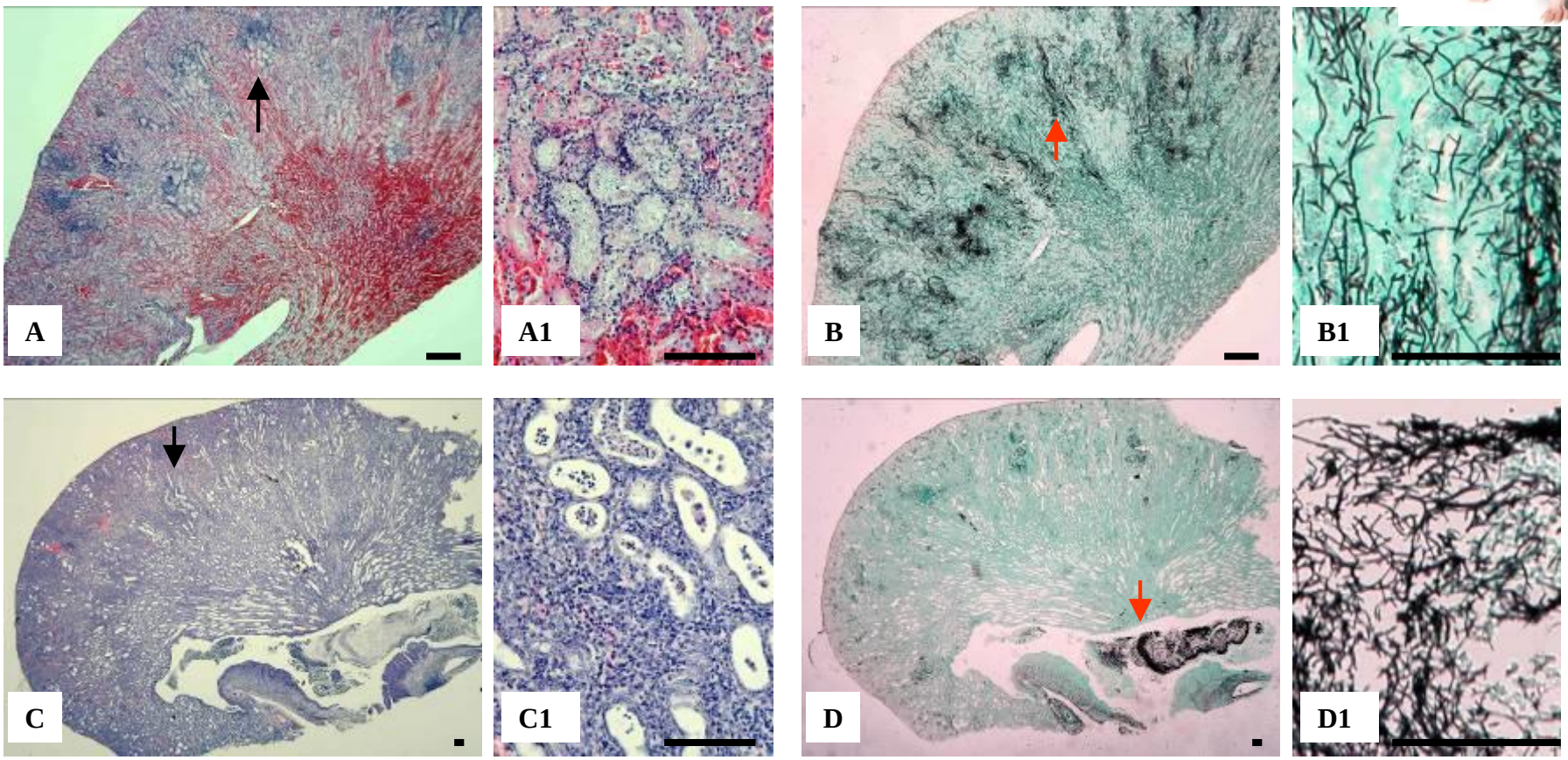
- **Mouse models are predominantly used.**
- **Several invertebrate models (conserved innate immunity and inexpensive care systems and enable experiments to be performed on a large scale)**
- **Zebrafish (drug administration, prolific fecundity, optical transparency)**

Eye - Fungal Infections of the Cornea

Cambridge Ophthalmological Symposium
Eye (2003) 17, 852–862. doi:10.1038/sj.eye.6700557
Fungal infections of the cornea
Proprietary interests: Nil Presented at the Cambridge Ophthalmological Symposium, 4–6 September 2002

The *cph1 efg1* Mutant Cells Were Capable of Establishing Restricted Zone of Infection

Wild-type



Mutant

A Fruit Fly Infected with *Beauveria bassiana*

Fruit flies that developed in space showed weakened immunity to fungal infections post-spaceflight.
Image Credit: Deborah Kimbrell (PLoS One 2014)

Brunke et al., Of mice, flies – and men? Comparing fungal infection models for large-scale screening efforts, Disease Models and Mechanisms 2015 8: 473-486

Glittenberg et al., Wild-type *Drosophila melanogaster* as an alternative model system for investigating the pathogenicity of Candida albicans Disease Models and Mechanisms 2011 4: 504-514

A Warm Infected with *Beauveria bassiana*

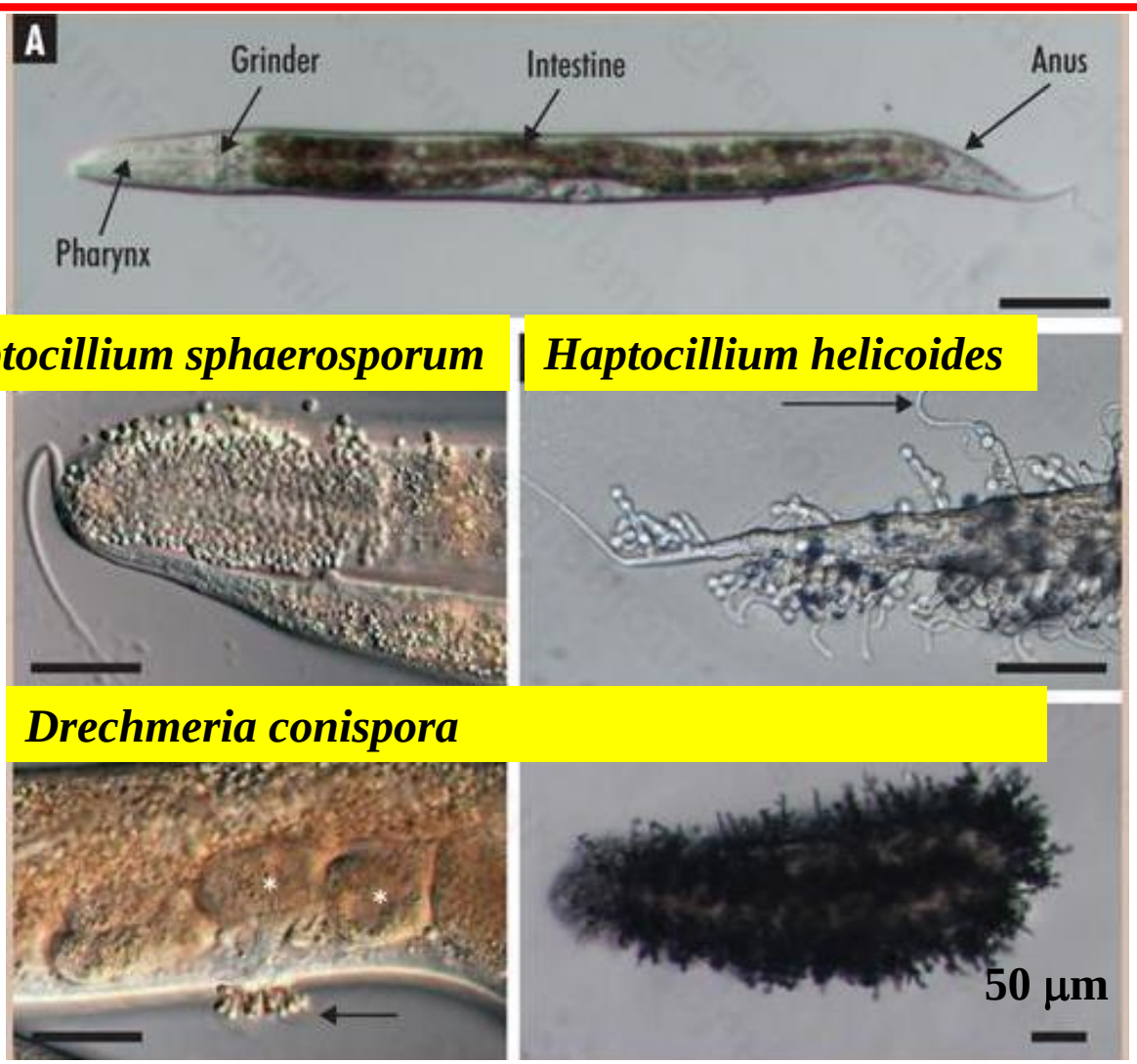


Image courtesy of Remedica Journals
http://www.remedicajournals.com/The-Journal-of-Invasive-Fungal-Infections/Browsel ssues/Volume-5-Issue-4/Article-Caenorhabditis-elegans-Antifungal-Defense-Mechanisms

Caenorhabditis elegans

A Warm Infected with *Candida albicans*

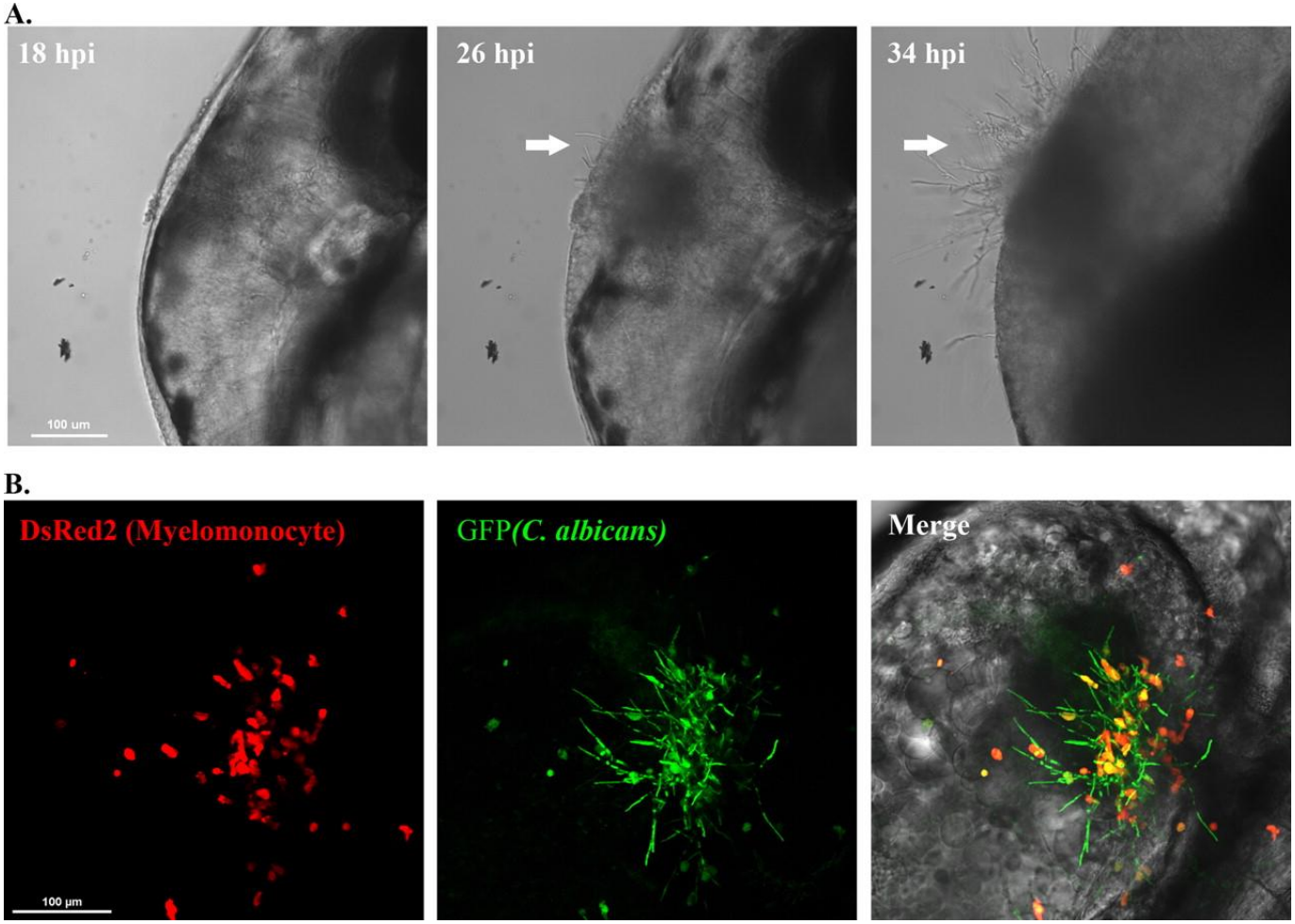
Galleria mellonella

Cowen et al., Harnessing Hsp90 function as a powerful, broadly effective therapeutic strategy for fungal infectious disease“ PNAS, February 24, 2009

Candida albicans in Zebrafish

清大 藍忠昱及莊永仁

Danio rerio

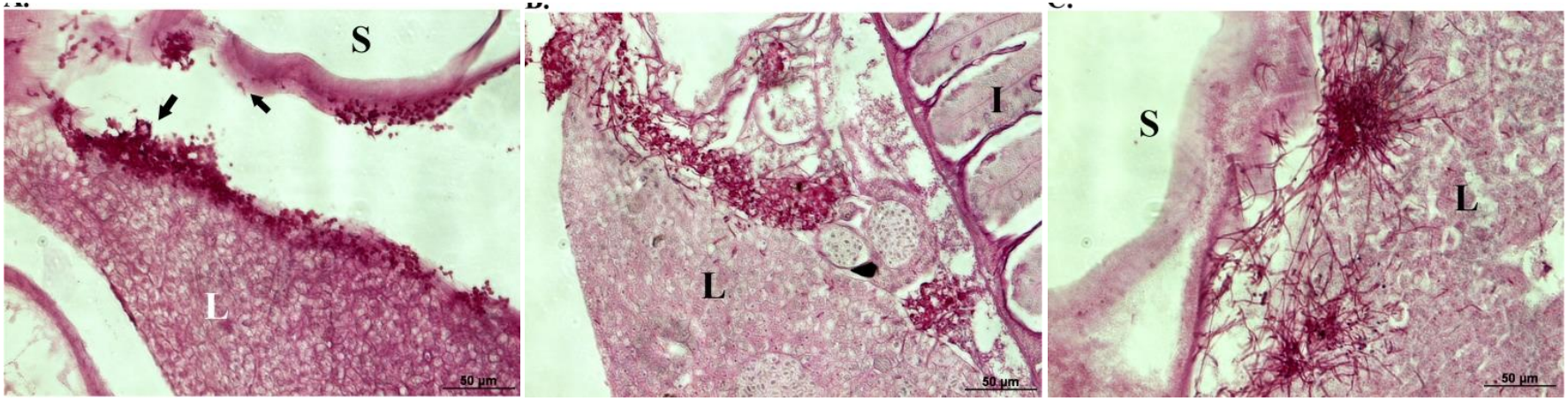


Chao et al., Zebrafish as a Model Host for *Candida albicans* Infection Infect Immun. 2010 Jun; 78(6): 2512–2521.

Progression of *C. albicans* Hyphal Formation in Zebrafish

清大 藍忠昱及莊永仁

Danio rerio



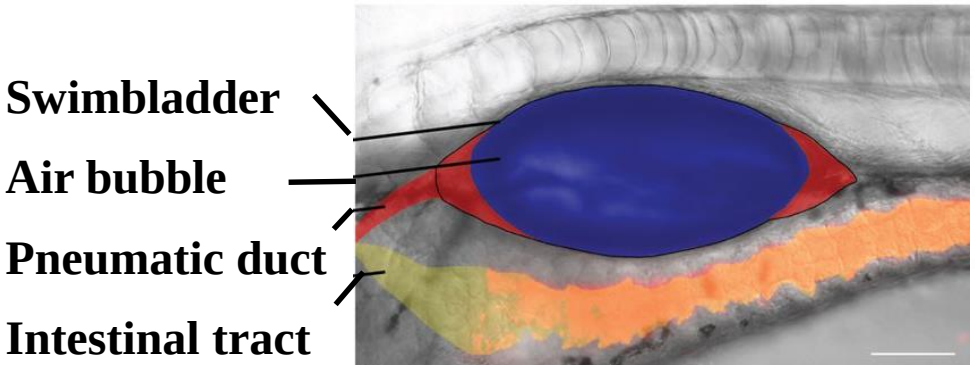
2 **8** **15** **hpi**

Arrows indicate C. *albicans* cells. L, liver; S, swim bladder; I, intestine

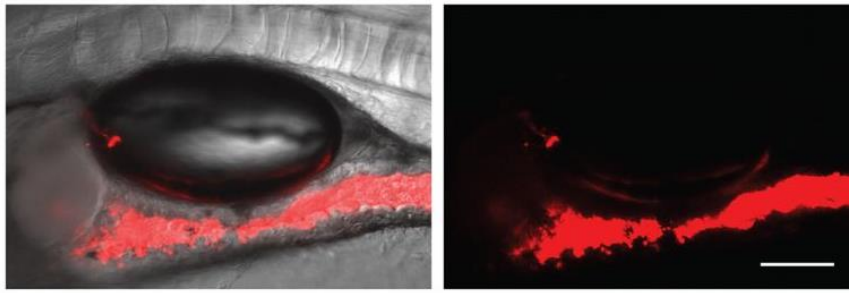
Chao et al., Zebrafish as a Model Host for *Candida albicans* Infection Infect Immun. 2010 Jun; 78(6): 2512–2521.

http://www.jove.com/video/52182/modeling-mucosal-candidiasis-larval-zebrafish-swimbladder

Candida albicans Infects the Swimbladder of Juvenile Zebrafish

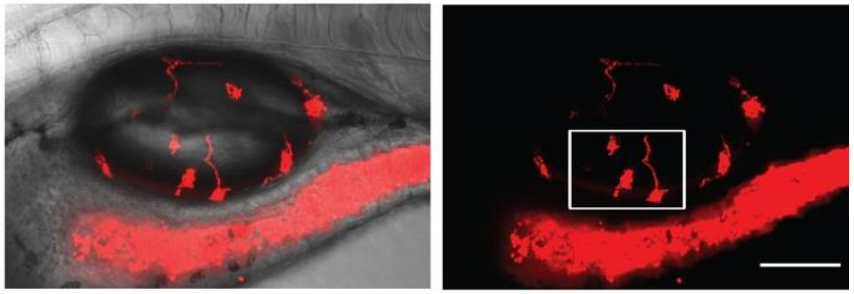


1-20 *C. albicans* cells

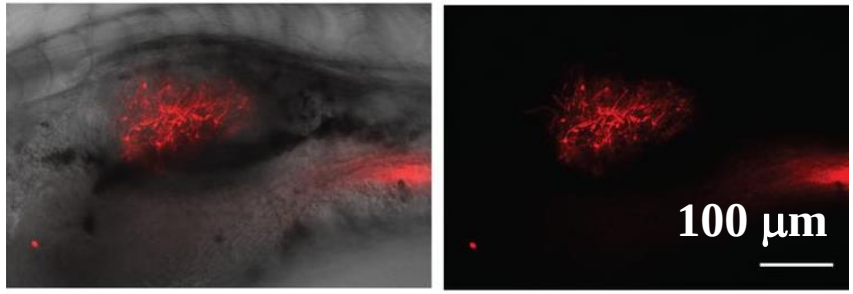


> 20 *C. albicans* cells

inflated



non-inflated



Gratacap et al., Mucosal candidiasis elicits NF-κB activation, proinflammatory gene expression and localized neutrophilia in zebrafish. Disease Models and Mechanisms 2013 6: 1260-1270.

3 dpf

References for Zebrafish for Antifungal Drug Development, Host-pathogen Interaction, and Pathogenesis of Fungal Pathogen

Synthesis and anti-Candida activity of novel benzothiepino[3,2-c]pyridine derivatives. Božinović et al., Chem Biol Drug Des. 2016 Jun 18. [Epub ahead of print]

Phenotypic Plasticity Regulates *Candida albicans* Interactions and Virulence in the Vertebrate Host. Mallick et al., Front Microbiol. 2016 26;7:780.

A Systems Biology Approach to the Coordination of Defensive and Offensive Molecular Mechanisms in the Innate and Adaptive Host-Pathogen Interaction Networks. Wu & Chen, PLoS One. 2016, 16;11(2):e0149303.

Synthesis and evaluation of thiophene-based guanylhydrazones (iminoguanidines) efficient against panel of voriconazole-resistant fungal isolates. Ajdačić et al., Bioorg Med Chem. 2016, 15;24(6):1277-91.

Mechanism-specific and whole-organism ecotoxicity of mono-rhamnolipids. Johann et al., Sci Total Environ. 2016, 548-549:155-63.

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Myeloperoxidase-deficient zebrafish show an augmented inflammatory response to challenge with *Candida albicans*. Wang et al., Fish Shellfish Immunol. 2015, 44(1):109-16

Robustness analysis on interspecies interaction network for iron and glucose competition between *Candida albicans* and zebrafish during infection. Lin et al., BMC Syst Biol. 2014, 8 Suppl 5:S6.

Modeling mucosal candidiasis in larval zebrafish by swimbladder injection. Gratacap et al., J Vis Exp. 2014, (93):e52182.

The role of TGF-β signaling and apoptosis in innate and adaptive immunity in zebrafish: a systems biology approach. Lin et al., BMC Syst Biol. 2014, 8:116.

A systems biology approach to study systemic inflammation. Chen & Wu, Methods Mol Biol. 2014, 1184:403-16.

Macrophage-pathogen interactions in infectious diseases: new therapeutic insights from the zebrafish host model. Torraca et al., Dis Model Mech. 2014 , 7(7):785-97.

Functional characterization of chitinase-3 reveals involvement of chitinases in early embryo immunity in zebrafish. Teng et al., Dev Comp Immunol. 2014, 46(2):489-98.

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Essential functional modules for pathogenic and defensive mechanisms in *Candida albicans* infections. Wang et al., Biomed Res Int. 2014, 2014:136130

Generating a battery of monoclonal antibodies against native green fluorescent protein for immunostaining, FACS, IP, and ChIP using a unique adjuvant. Sanchez et al., Monoclon Antib Immunodiagn Immunother. 2014, 33(2):80-8.

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NADPH oxidase-driven phagocyte recruitment controls Candida *albicans* filamentous growth and prevents mortality. Brothers et al., PLoS Pathog. 2013, 9(10):e1003634.

Dynamic transcript profiling of *Candida albicans* infection in zebrafish: a pathogen-host interaction study. Chen et al., PLoS One. 2013, 8(9):e72483.

Interspecies protein-protein interaction network construction for characterization of host-pathogen interactions: a *Candida albicans*-zebrafish interaction study. Wang et al., BMC Syst Biol. 2013, 7:79.

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Mucosal candidiasis elicits NF-κB activation, proinflammatory gene expression and localized neutrophilia in zebrafish. Gratacap et al., Dis Model Mech. 2013, 6(5):1260-70.

Diverse Hap43-independent functions of the *Candida albicans* CCAAT-binding complex. Hsu et al., Eukaryot Cell. 2013, (6):804-15.

Identification of infection- and defense-related genes via a dynamic host-pathogen interaction network using a Candida albicans-zebrafish infection model. Kuo et al., J Innate Immun. 2013, 5(2):137-52.

Non-invasive imaging of disseminated candidiasis in zebrafish larvae. Brothers & Wheeler, J Vis Exp. 2012 , 65.

Live imaging of disseminated candidiasis in zebrafish reveals role of phagocyte oxidase in limiting filamentous growth. Brothers et al., Eukaryot Cell. 2011, 10(7):932-44.

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Differential modulation of Burkholderia cenocepacia virulence and energy metabolism by the quorum-sensing signal BDSF and its synthase. Deng et al., J Bacteriol. 2009, 191(23):7270-8.

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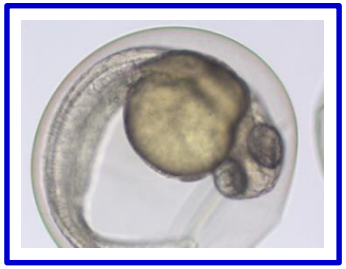
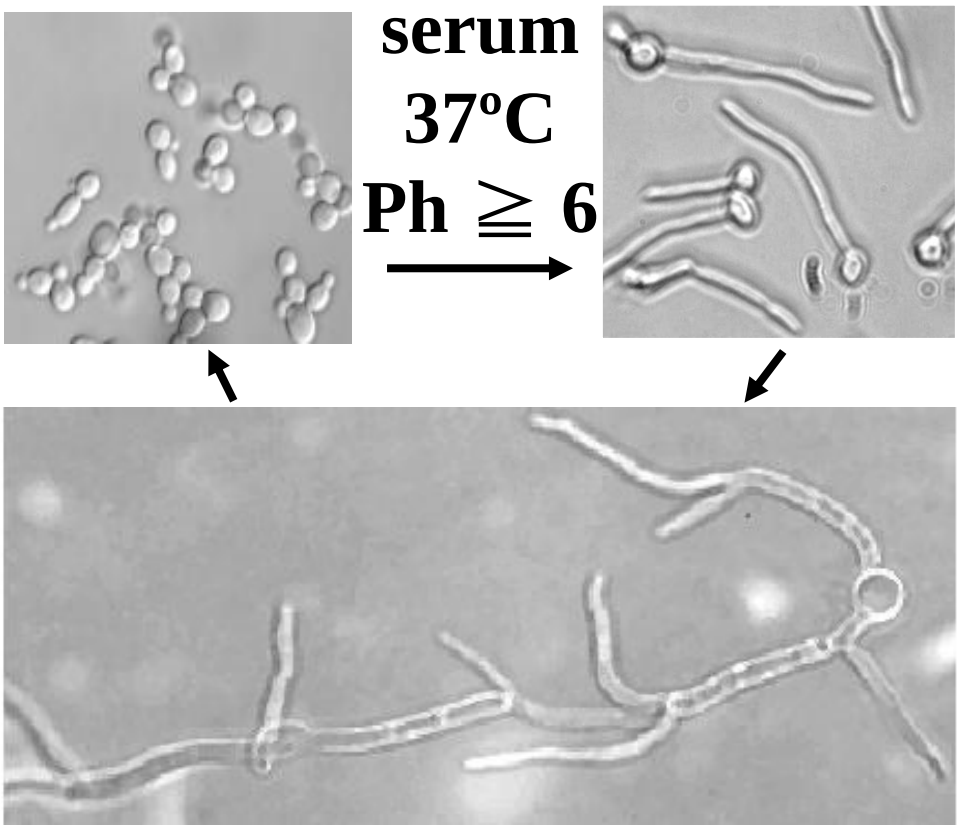


Zebrafish Embryo

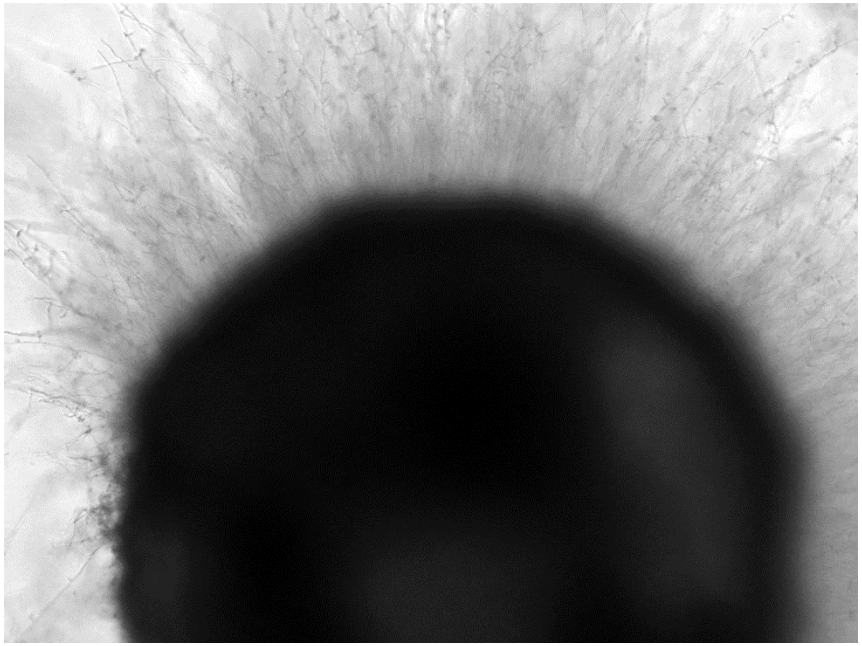
- **Mouse models are predominantly used.**
- **Several invertebrate models (conserved innate immunity and inexpensive care systems and enable experiments to be performed on a large scale)**
- **Zebrafish (drug administration, prolific fecundity, optical transparency)**

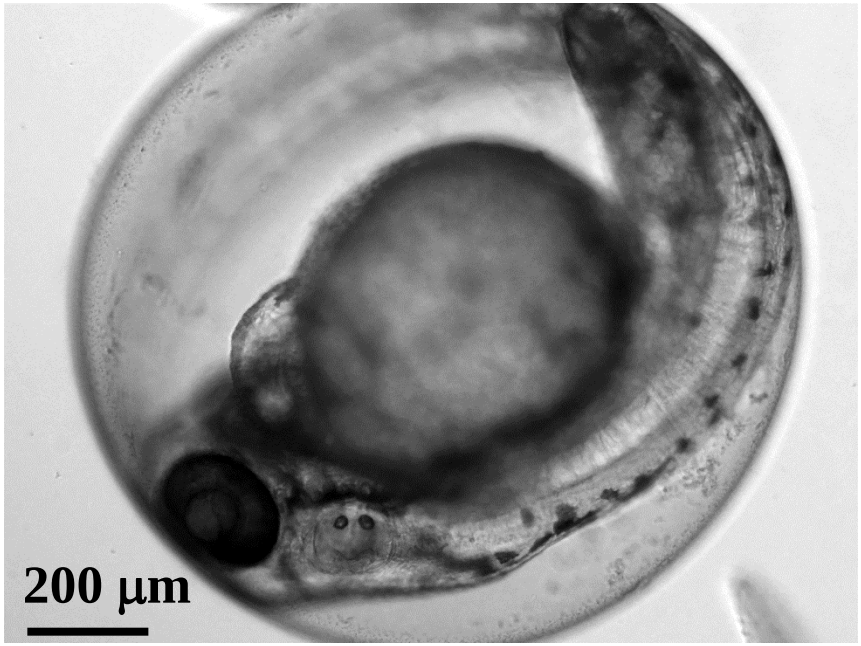
Candida albicans Switch between Yeast Form and Hyphal Form



The ability to switch is important for its virulence.

First Try
Zebrafish Bath Infection Model





Wild-type ***cph1/cph1 efg1/efg1***

SC5314 cells formed hyphae & killed embryos.
***cph1/cph1 efg1/efg1* cells is not lethal to embryos.**
Optimal conditions?

Optimal Conditions?

To define the optimal conditions for zebrafish embryo bath infection, we co-incubated wild-type *C. albicans* cells, SC5314, with 1-day post-fertilization embryos

for various periods of time (1 or 4 hours),

at various shaking speeds (0, 80, 180 rpm),

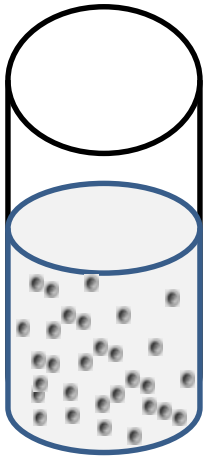
and in various media [egg water (0.03% sea salt), egg water/10% serum, RPMI, RPMI/10%serum]

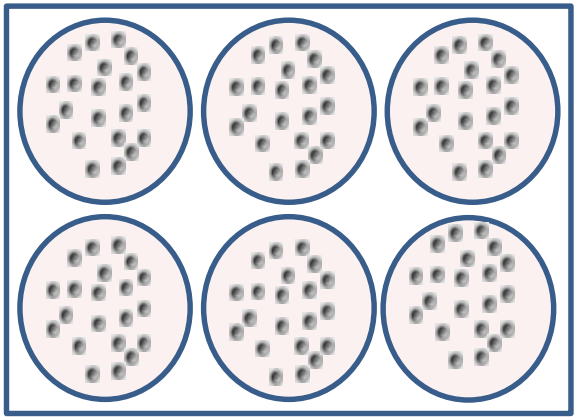
Procedures

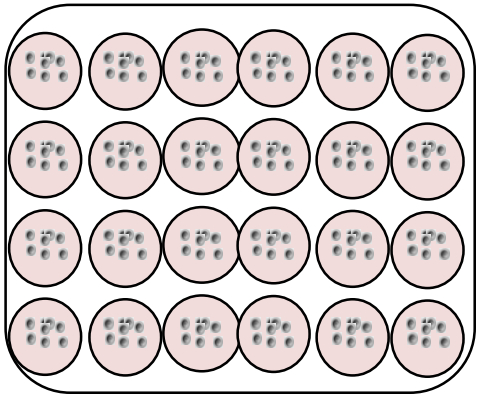
Sterilize 1-day post-fertilization embryos with 0.028% bleach

Co-incubate embryos with *C. albicans* cells in 6-well plate containing 4 ml of different medium at 0, 80, or 180 rpm and 30 ℃ for 1 or 4 hours

1. Removed un-adhered *C. albicans* cells
2. Incubate embryos in 24-well plate in 1 ml of egg water at 30 ℃ for 2 days







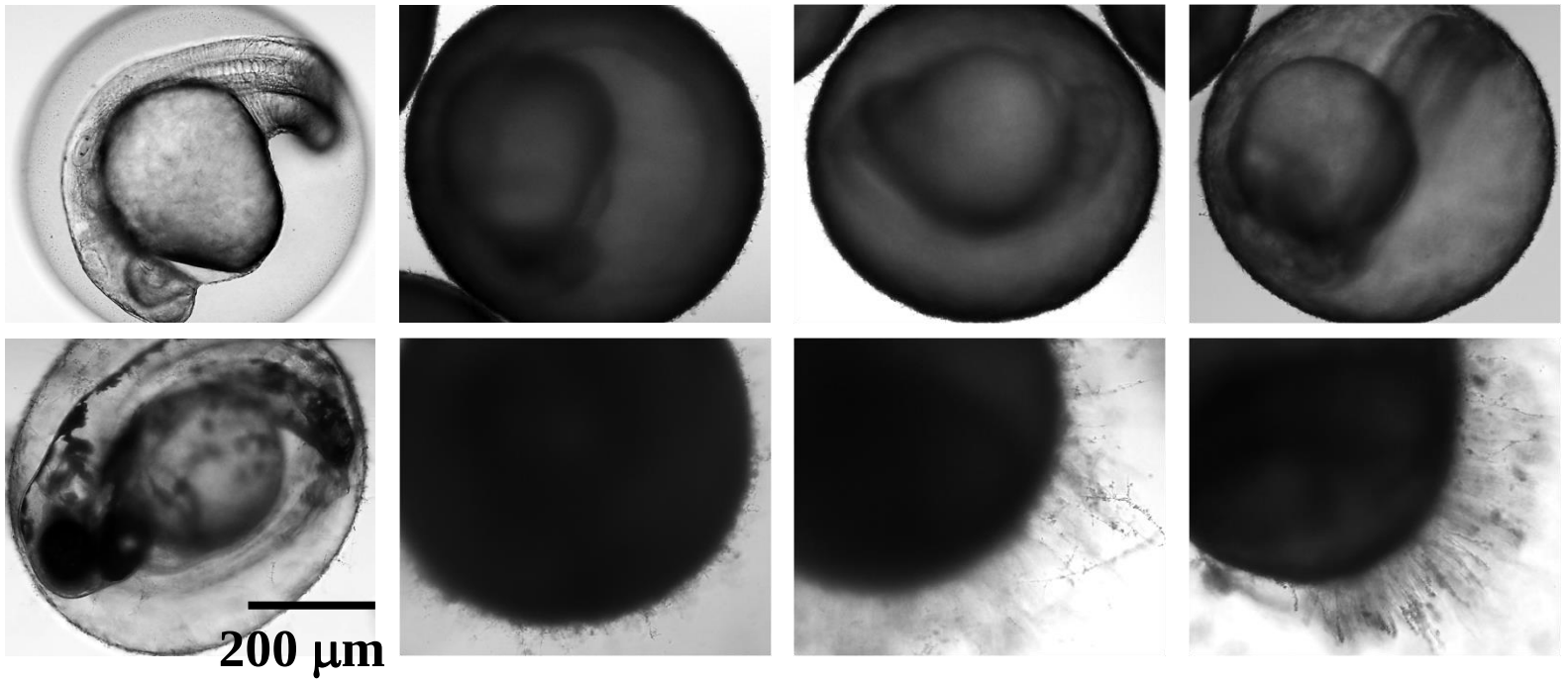
The Conditions for the Zebrafish Egg Bath Infection Model

Medium/	4-h co-incubation		
	Shaking speed (rpm)		
inoculum	0	80	180
	2-day additional incubation		
EW/10^7	100	100	100
EW+S/10^7	62 ± 8.7	0	45 ± 50.7
R/10^7	0	0	0
R+S/10^7	3 ± 5.8	0	29 ± 41.9
R/10^6	0	0	37 ± 50.1
R+S/10^6	15 ± 13.8	0	91 ± 16.2

EW: egg water; R: RPMI; S: 10% serum; 10^6:1 X 10^6 ells/ml; 10^7:1 X 10^7 cells/ml; *Survival rate ± standard deviation

4 h co-incubation, 80 rpm

Candida Cells Adhered the Chorion and Formed Hyphae/Biofilms



	Egg water	Egg water/ serum	PRMI	PRMI/ serum
Survival rate	100	0	0	0

Lowest Inoculum?

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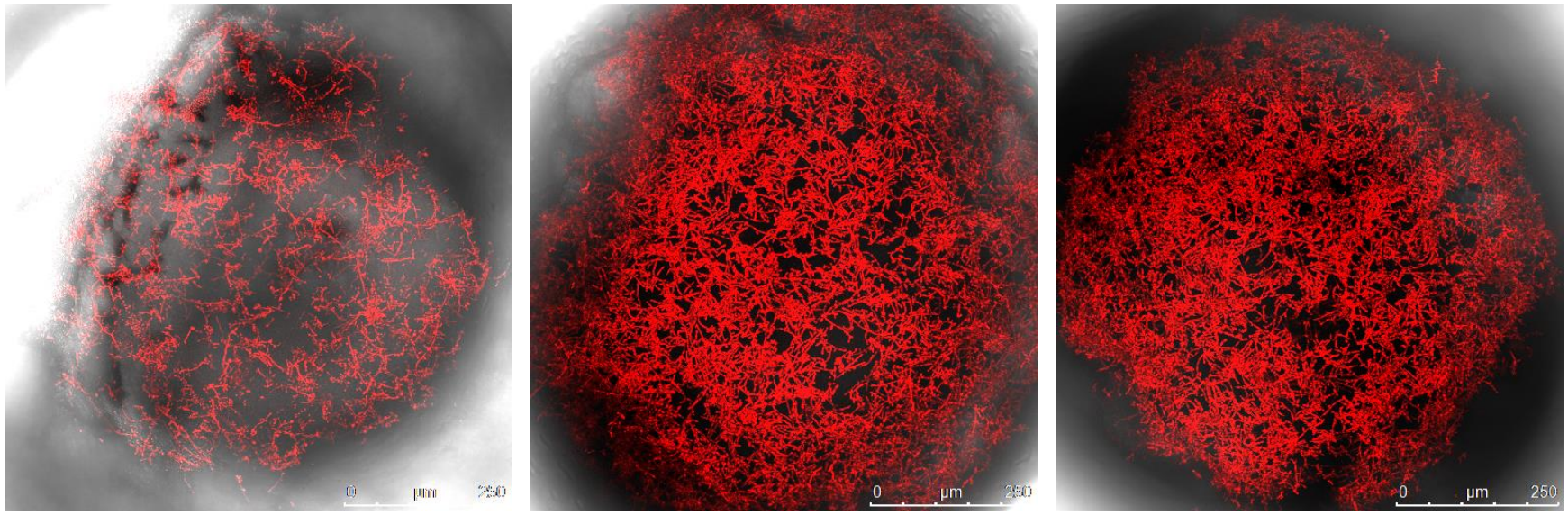
EW: egg water; R: RPMI; S: 10% serum; 10^6:1 X 10^6 ells/ml; 10^7:1 X 10^7 cells/ml; *Survival rate ± standard deviation

Lowest Inoculum

1×10^5 5×10^5 1×10^6



Lowest Inoculum: 5×10^5 cells/ml

CAF2-dTomato: Brothers et al. (2013) PLoS Pathog 9: e1003634.

Conditions

Inoculum: > 5 x 10^5 cells/ml
Co-incubation period: 4 hours
Medium: RPMI or RPMI/serum
Sharking: 80 rpm
Temperature: 30 ºC

Can this model be applied to study other mutant strains known to be involved in virulence?

Reported Mutant Strains

***bcr1*: decreased adhesion, decreased biofilm formation, normal hyphal growth**

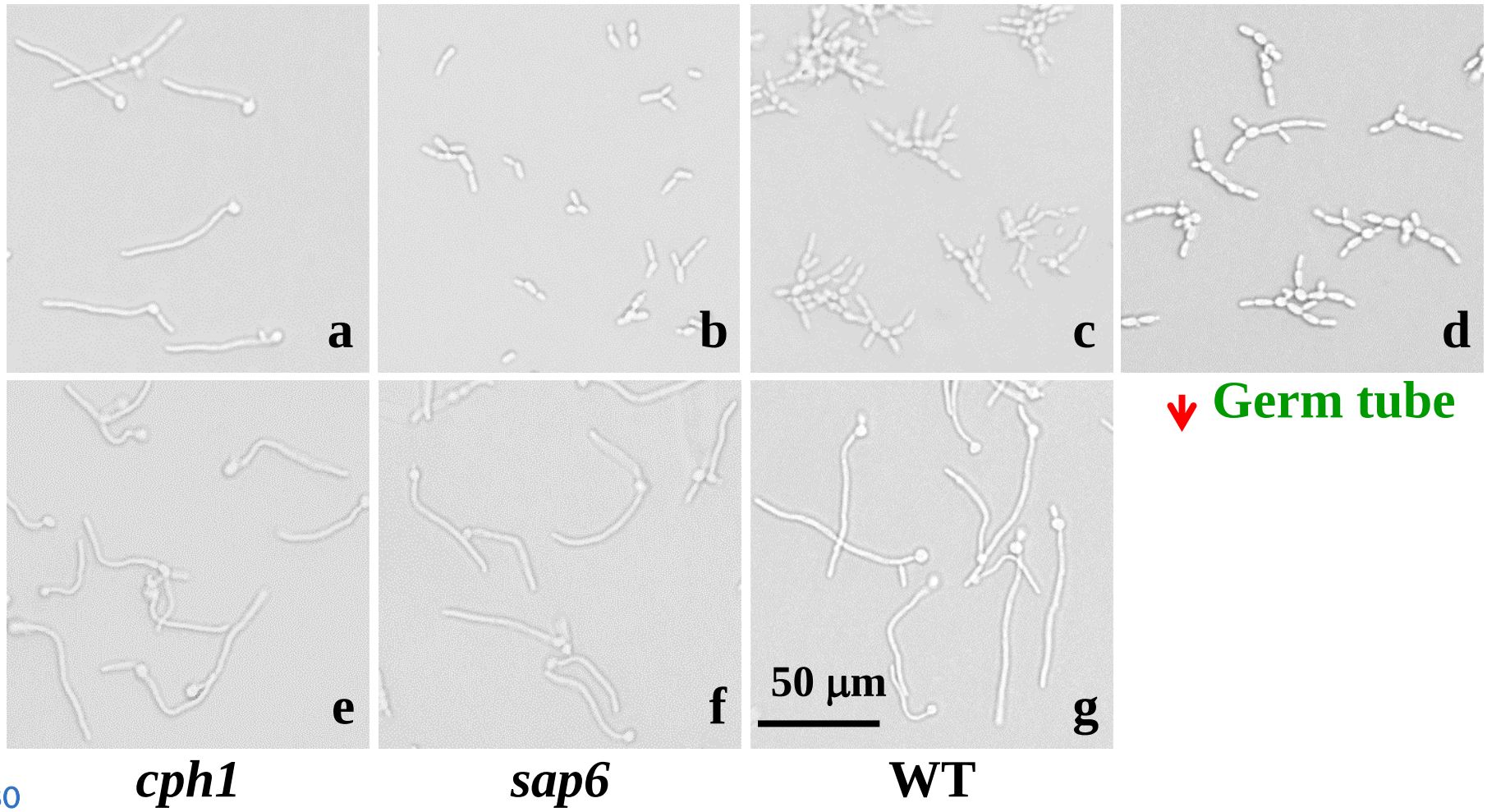
***cph1*: normal germ tube formation and hyphal growth**

***efg1*: decreased adhesion, decreased biofilm formation, no hyphal growth**

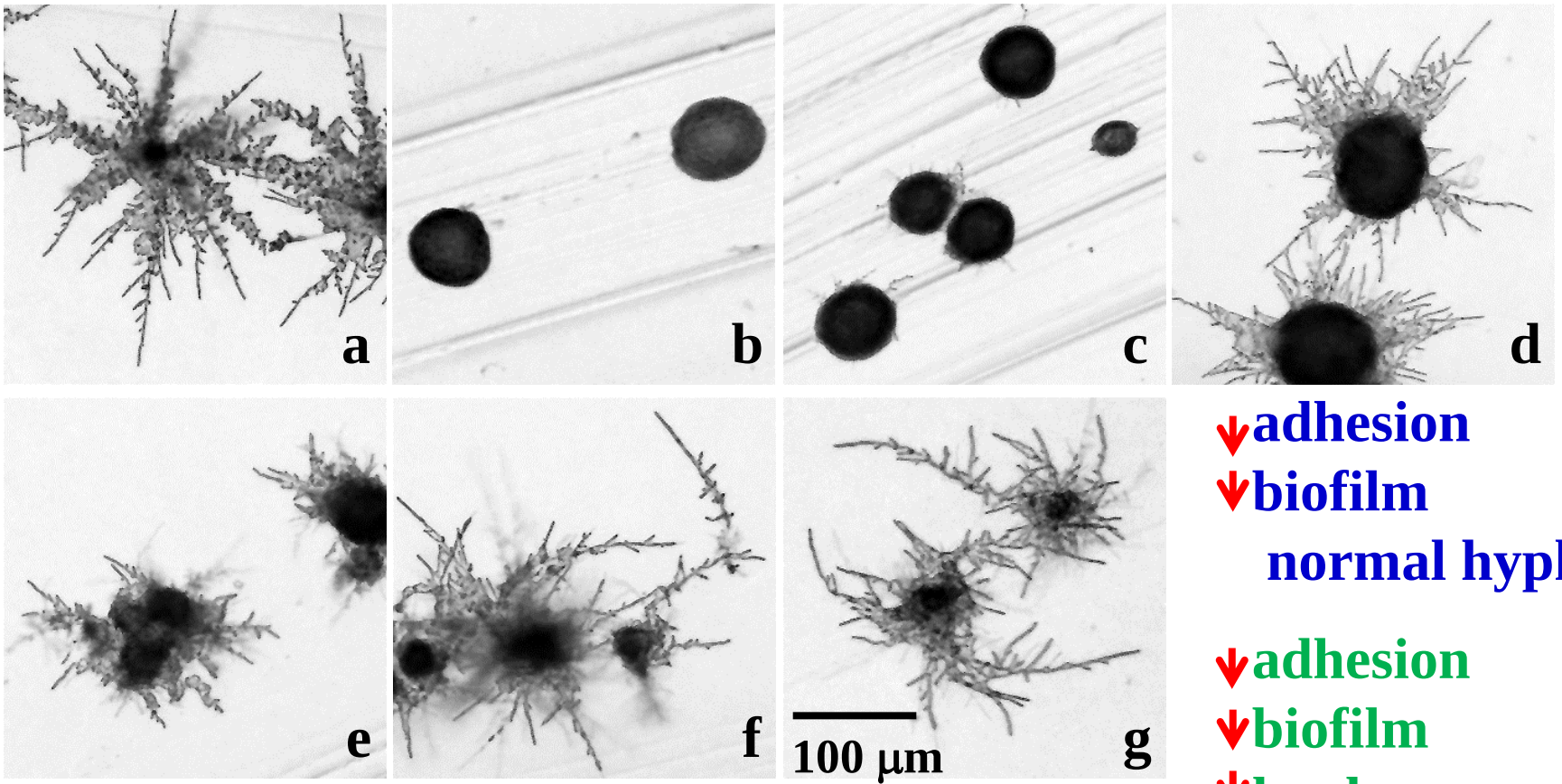
***sap6*: Secreted aspartyl protease; normal hyphal growth and virulence**

***tec1*: decreased adhesion, decreased biofilm formation, normal hyphal growth**

Germ Tube Formation of other Mutant Strains?



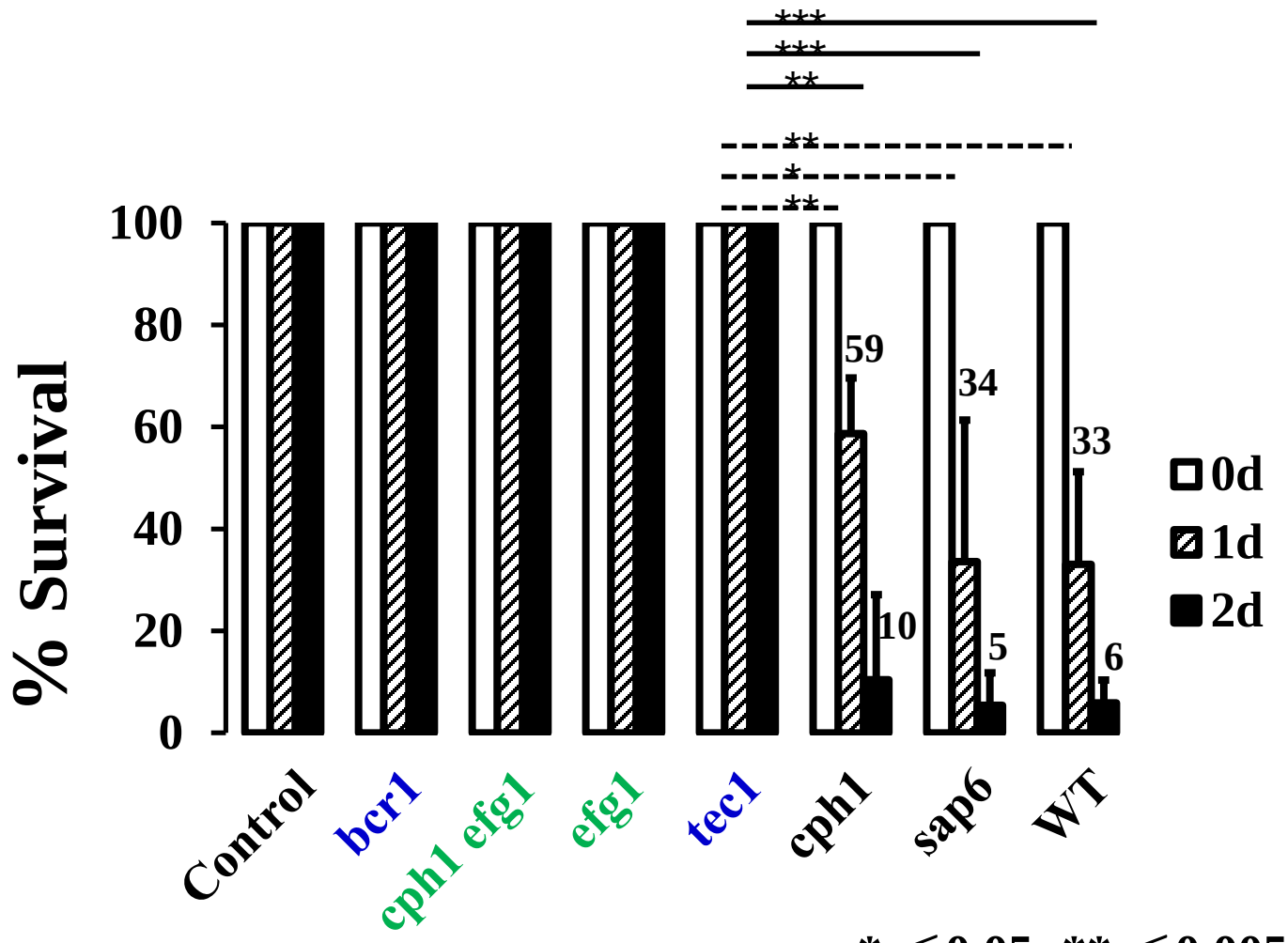
Hyphal Formation of other Mutant Strains?



↓adhesion
↓biofilm
normal hyphae

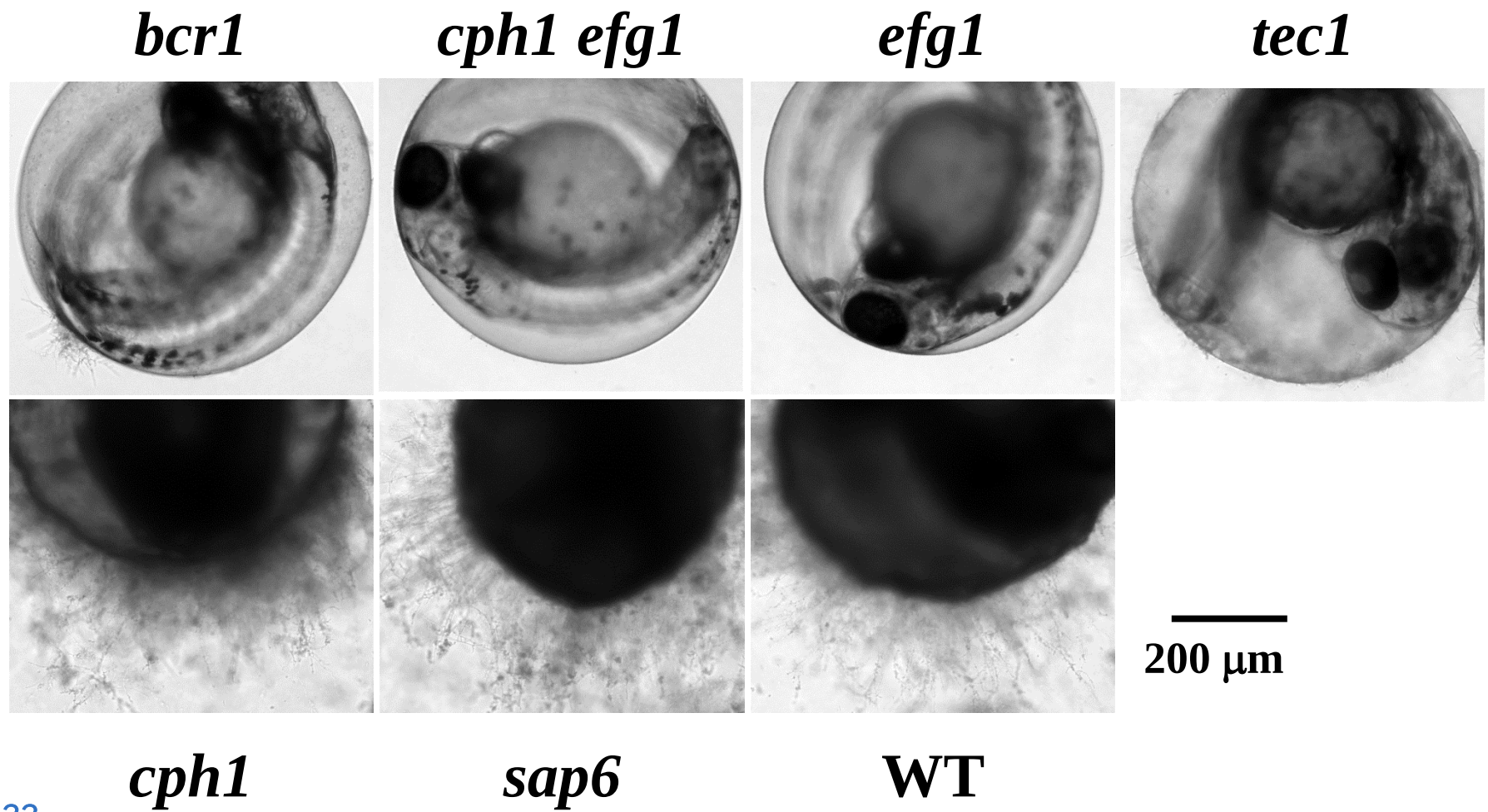
↓adhesion
↓biofilm
↓hyphae

The Results of the Embryo Model Are Consistent with Those in Mouse one



* ≦0.05; ** ≦0.005; *** ≦0.001

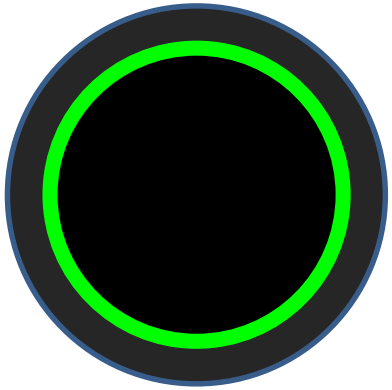
Deletions of *CPH1* or *SAP6* Did Not Have Effect on Adhesion or Hyphal Formation



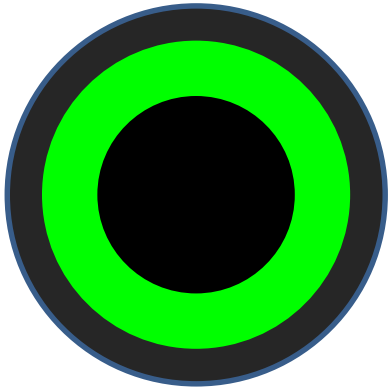
What are the potential causes for the death of embryos?

Did Candida cells direct penetrate into larvae?

Confocal Imaging: Green Candida cells

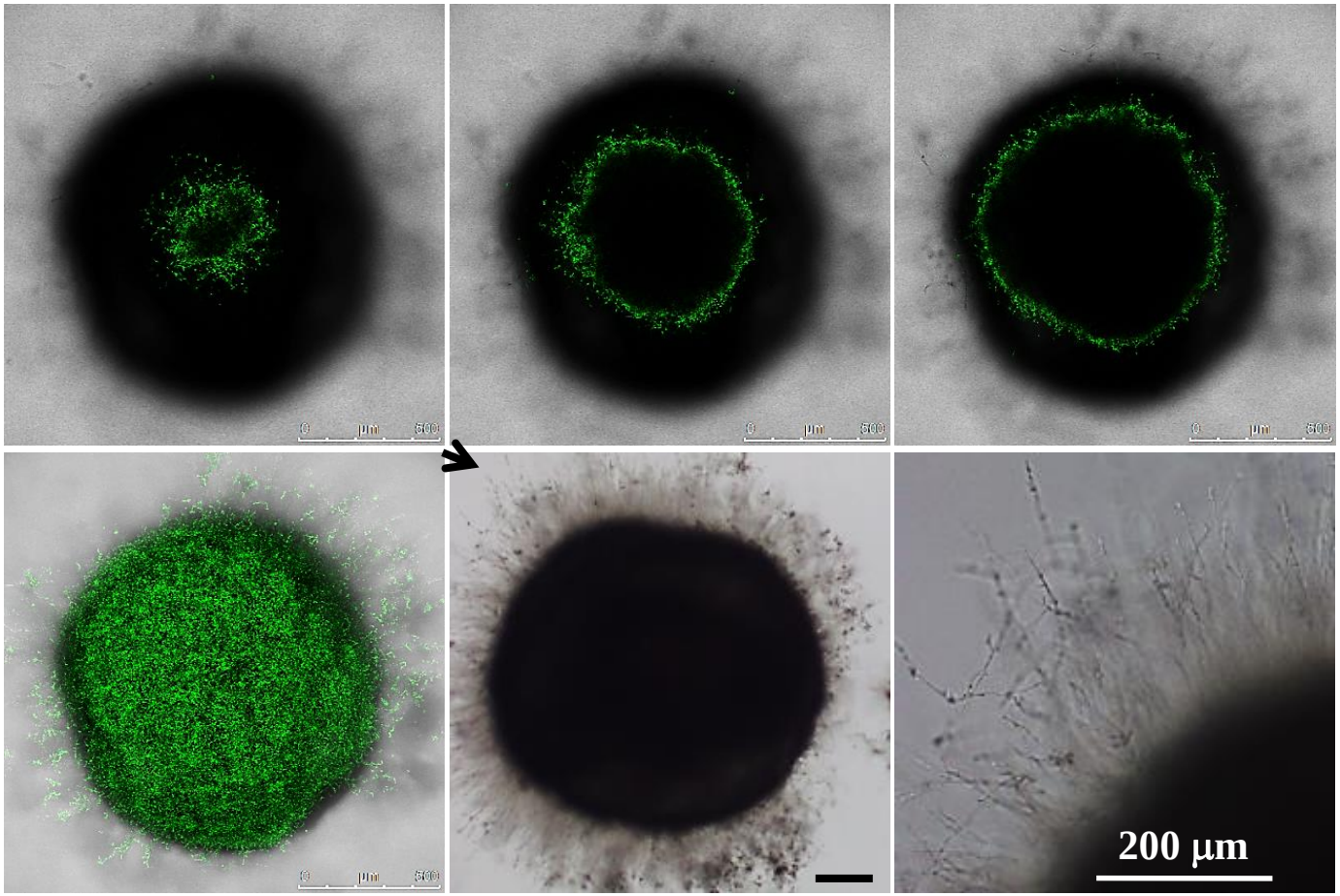


On the chorion



Penetrate chorion

The Cause for the Death of Embryos?



OG1 cells: Chao et al (2010). Infection and Immunity 78: 2512.

Majority of *C. albicans cells* stay on the chorion.

Potential Cause(s) for the Death of Embryos

failure of transporting toxicities,
either secreted by *C. albicans* or
generated by embryos,

and/or lack of oxygen

Any comments/suggestions are highly appreciated.

We have established a protocol in 24-well plate.

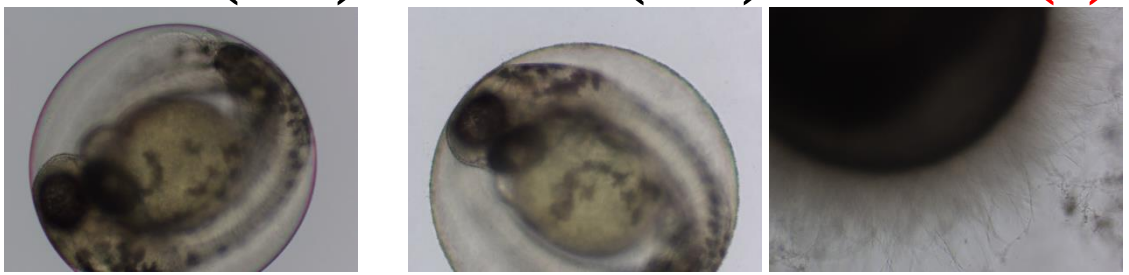
Can this model study pathogenesis of other species?

Candida parapsilosis Caused Death of Embryos

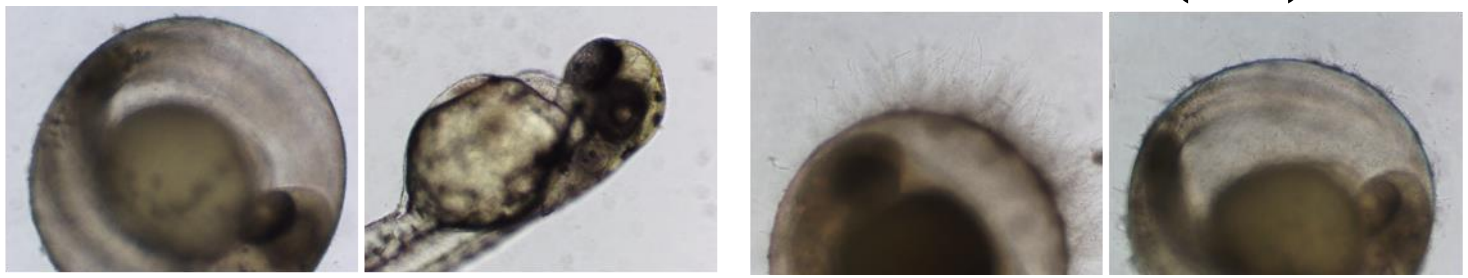
10282015	RPMI 1x10^7 cells/ml	
Co-incubated in RPMI in 24-well for 6 h , washed, EW with 0.5% YPD, survival rate determination		
	24 h	48 h
Control	100	100
C. albicans SC5314	0	0
C. albicans cph1 efg1	100	100
C. krusei	100	100
C. parapsilosis	50	0
C. glabrata	100	90
C. tropicalis	78	78

Survival Rates after 24 h/48 h Additional Incubation

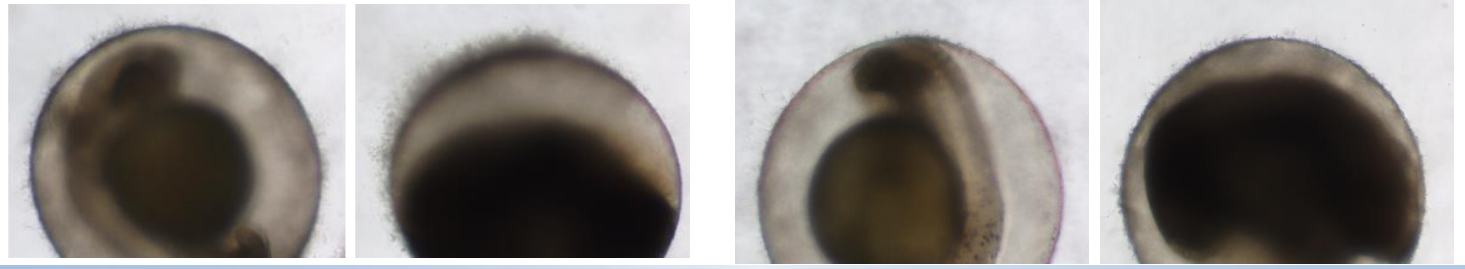
Control (100) **HLC54 (100)** **SC5314 (0)**



***C. glabrata*(100)** ***C. krusei* (100)**



***C. parapsilosis* (50/0)** ***C. tropicalis* (78/78)**



Under the conditions tested,

1. All tested *C. krusei, C. glabrata, and Cryptococcus neoformans* did not cause death of embryos.

2. Some *C. parapsilosis* and *C. tropicalis* strains can cause death of embryos

Acknowledgements

- Dr. C. H. Lin for kindly providing us with *bcr1/bcr1* and *tec1/tec1* strains, Dr. Y. C. Chen for the *sap6/sap6* strain, Dr. C. Y. Lan for the OG1 strain, and Dr. R. Wheeler for the CAF2-dTomato strain.
- NHRI zebrafish core facility for its help in establishing the bath infection model.
- Hard worker: Ms. Yin-Zhi Chen
- Collaborators: Drs. M. S. You and Y. L. Yang
- Supported by NHRI and MOST in Taiwan

I am lucky to have their help.

Thank You for Your Attention