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Review Article

REVIEW ARTICLE

Fluorescent toys 'n' tools lighting the way in fungal research

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One sentence summary: Fungal infections such as those caused by *Candida* species impose serious health problems to patients, and using state-of-the-art fluorescence techniques, it is possible to pave the way towards novel, highly-needed, druggable targets.

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ABSTRACT

Although largely overlooked compared to bacterial infections, fungal infections pose a significant threat to the health of humans and other organisms. Many pathogenic fungi, especially *Candida* species, are extremely versatile and flexible in adapting to various host niches and stressful situations. This leads to high pathogenicity and increasing resistance to existing drugs. Due to the high level of conservation between fungi and mammalian cells, it is hard to find fungus-specific drug targets for novel therapy development. In this respect, it is vital to understand how these fungi function on a molecular, cellular as well as organismal level. Fluorescence imaging allows for detailed analysis of molecular mechanisms, cellular structures and interactions on different levels. In this manuscript, we provide researchers with an elaborate and contemporary overview of fluorescence techniques that can be used to study fungal pathogens. We focus on the available fluorescent labelling techniques and guide our readers through the different relevant applications of fluorescent imaging, from subcellular events to multispecies interactions and diagnostics. As well as cautioning researchers for potential challenges and obstacles, we offer hands-on tips and tricks for efficient experimentation and share our expert-view on future developments and possible improvements.

Keywords: mycology; fluorescence imaging; molecular cell biology; fungal pathogenesis; microscopy

INTRODUCTION

Fungal infections pose a serious problem to human health and welfare. Where some fungal pathogens cause mild superficial infections of skin and mucosal tissue, others can cause severe disease and high lethality through systemic dissemination (Brown *et al.* 2012). Apart from the very few therapies available, most of these drug strategies suffer from inefficacy or host toxicity. Furthermore, as is the case for bacterial pathogens, both acquired and inherent resistance against antifungal drugs are on the rise. To contain infections and safeguard the health of infected patients, novel drug strategies have to be developed (Roemer and Krysan 2014). In order to keep expanding our knowledge on the fundamental processes of microbial infections, it is essential to keep track of novel techniques and use

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the proper tools for our research questions. *Candida* species are among the fungi that are most often isolated from infections. Being opportunistic pathogens, they possess commensal as well as pathogenic traits, taking advantage of a weakened host immune system to make the transition between states (Cauchie, Desmet and Lagrou 2017). *Candida* research differs significantly from other eukaryotic organisms because general genomics, life cycle, metabolism, etc. are quite distinct even from its close relative and model system baker's yeast. Several of these differences hamper the heterologous expression and easy adaptation of techniques within the *Candida* research community. For example, *Candida albicans* is generally encountered as an obligate diploid, therefore adaptations to the genome must often be applied to both alleles. Baker's yeast shows a very clear and distinct life cycle with sexual mating between haploid **a** and **α** mating types, allowing easy genetic experimentation. On the other hand, in *C. albicans* and *Candida glabrata* no sexual cycle seems to be apparent (Magee and Magee 2000; Boisnard *et al.* 2015). Additionally, the CTG clade containing many *Candida* species such as *C. albicans, Candida parapsilosis, Candida tropicalis* and the recently emerged and multidrug resistant *Candida auris* translate the CUG codon as serine instead of leucine, further complicating the heterologous expression of systems adapted for *Saccharomyces cerevisiae* or mammalian cells (Butler *et al.* 2009; Miranda *et al.* 2013). Due to these significant differences, specific optimization and adaptation steps are required in the development of tools and techniques for this human fungal pathogen.

Fluorescence-based techniques possess the unique quality to visualize processes in real time and in a dynamic manner inside cells and communities. They allow us to image interactions between molecules, cells and organisms, provide information on subcellular and organismal localization, report on niche-specific parameters such as pH and oxygen level and can even be used to assess enzymatic activity inside the cell. It is thus evident that fluorescence investigation of microbial and specifically fungal pathogenesis allows for rapid progress in the field and paves the way to the elucidation of novel druggable targets. In this review, we will focus on the fluorescence-based techniques applied to fungal pathogenesis and infections, from a micro to a macro scale, as depicted in Fig. 1.

WHAT IS AVAILABLE?—FLUOROPHORES

Being able to perform the fluorescence-based techniques depicted in Fig. 1 across the different scales of imaging requires an extensive library of fluorophores. So far, the available fluorophores are fluorescent proteins, organic dyes or bioluminescent enzymes, but as science continues to progress and only a fraction of life on earth has been described, additional classes will be discovered. Because fluorophores are the basic requirement for the tools and techniques described in this review, we will start by exploring them before applying them to *C. albicans* tools.

Fluorescent proteins

At the advent of fluorescence—a Candida optimized GFP

The discovery of the wild type Green Fluorescent Protein (GFP) from *Aequorea victoria* and the practicality of using a genetically encoded tag to fluorescently label targets-of-interest with low toxicity, led to an enormous boost in life sciences research. Until now, all fluorescent proteins (FPs) for *Candida albicans* are structurally homologous, containing an 11-stranded β-barrel with an α-helical strand in the middle of the barrel enclosing the chromophore (Shimomura, Johnson and Saiga 1962; Prasher *et al.* 1992). Five years after the initial attempts to heterologously express GFP in *C. albicans*, the first of many species-specific modifications were made to the protein. The first adaptation consisted of replacing the CTG codons, which encode serines in *C. albicans* instead of leucines. As detailed in the *Challenges* section at the end of this manuscript, it is essential to avoid CTG codons in heterologous expression, to ensure proper translation, folding and functionality of the protein. Apart from codon optimization, two residues were mutated, S65G and S72A (Cormack *et al.* 1997). These mutations were shown to increase fluorescence intensity 20-fold compared to enhanced GFP in *Escherichia coli* due to a shift in absorption maximum towards 488 nm (Cormack, Valdivia and Falkow 1996). This mutant, called yeast enhanced GFP3 or YeGFP3, was also inserted in a set of plasmids for use in *C. glabrata* (Zordan *et al.* 2013). Independently from this first study, researchers from another laboratory replaced the CTG codons as well, but also introduced two sets of mutations consisting of either S65T or a combination of S65A, V68L, S72A and Q60R. The latter mutant was termed GFPmut2 (Ullah *et al.* 2013a). These mutants outperformed the wild type chromophore (Ene *et al.* 2016). As is the case for many FPs, specific properties of GFP, such as quantum yield, photostability and expression levels were continuously upgraded in other fields, such as mammalian, plant and bacterial research. By translating certain mutations obtained in *E. coli* to *C. albicans*, Zhang and colleagues were able to construct a significantly improved monomeric GFP version called γmGFP (Crameri *et al.* 1996; Zhang and Konopka 2010). Recently, we showed that further codon optimization using different dedicated algorithms does not improve the *in vivo* brightness of γmGFP, leading us to state that this variant is currently recommended as green FP in *C. albicans* research (Van Genechten *et al.* 2020).

Over the rainbow—expansion of the palette

After initial optimization of the green FP, an expansion of colors, based on mutagenesis of GFP, took place (Fig. 2A). By combining mutations S65G, V68L and S72A, which improve the folding of GFP (Cormack, Valdivia and Falkow 1996), with T203Y, a significantly red-shifted GFP variant was obtained. This variant, termed Yellow Fluorescent Protein (YFP), is spectrally distinguishable from GFP (Ormo *et al.* 1996). On the other hand, a blue-shifted version of GFP, Cyan Fluorescent Protein (CFP), was obtained by a single mutation of tyrosine 66 to tryptophan inside the chromophore (Heim and Tsien 1996). Versions of YFP and CFP were engineered in PCR-based cassettes for convenient cloning and transformation in *C. albicans* (Gerami-Nejad, Berman and Gale 2001). Other *Candida* species such as *Candida glabrata* are able to stably maintain plasmids and therefore use a different set of YFP and CFP expression vectors (Yanez-Carrillo *et al.* 2015). The original YFP and CFP were improved by the development of so-called next generation FPs. Introducing a single F46L mutation into the YFP template, leads to an acceleration of the oxidation step in the chromophore formation. This resulted in the development of Venus which has an enhanced maturation speed at 37°C (Nagai *et al.* 2002). In FRET-pairs, as introduced later, often circular permuted (cp) Venus is used. A circular permutated protein contains a different order of amino acids but a very similar structure as the original one (Kostyuk *et al.* 2019). The N- and C-terminus of a protein are linked together while new termini are positioned close to the chromophore, forming the cp variant of the protein. These variants are generally more flexible thereby allowing optimization of FRET efficiency.

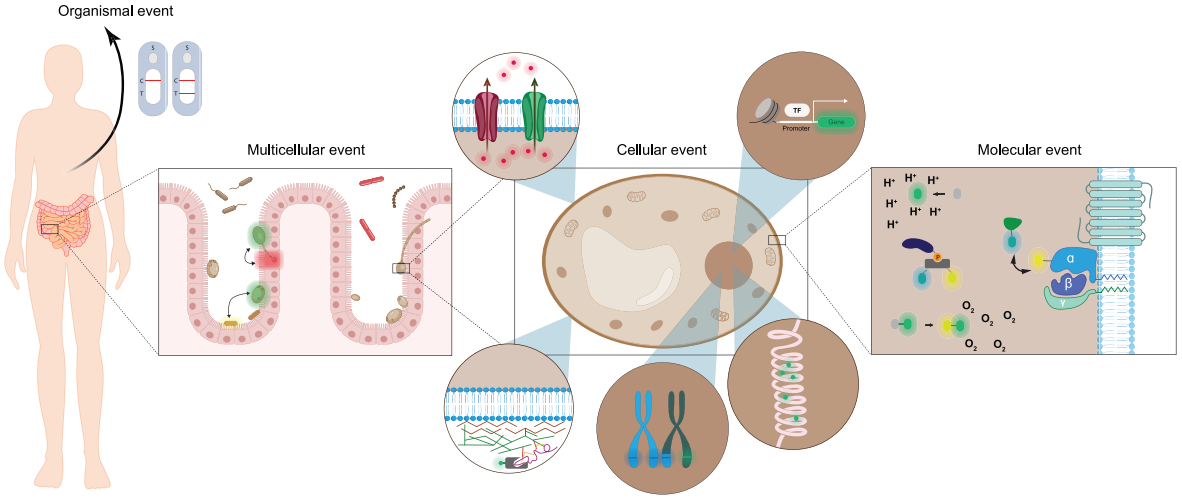

Figure 1. Schematic overview of fluorescent applications in fungal pathogenesis. Fluorescence techniques can be applied in mycology on several levels. Molecular interactions and activity, cellular phenomena, multicellular connections and organismal events can be investigated with the wide array of fluorescent techniques presented in this manuscript.

mTurquoise2 is another next generation FP that is the result of many incremental improvements of mVenus to SCFP1, -2 and -3 (Kremers *et al.* 2006). Within this SCFP3 template one single mutation, T65S, was able to increase brightness of the FP by 50%. This version, called monomeric Turquoise or mTurquoise, has a quantum yield of 0.84 and was then subsequentially improved via a I146F mutation to promote better packing of the bulky chromophore. The quantum yield is the ratio of the number of emitted photons to the number of absorbed photons, and is thus a measurement of the efficiency of the fluorescent protein. Table 1 provides a list of terminology related to fluorescence. The resulting mutant, mTurquoise2, has improved stabilization of the chromophore which leads to less non-radiative decay and thus an improved quantum yield of 0.93 (Goedhart *et al.* 2012). These two next generation FPs, mTurquoise2 and Venus, were codon-optimized and characterized for *C. albicans* (Van Genechten *et al.* 2020). Other FPs based on GFP are pHluorin and its successor pHluorin2, with the latter having an eight-fold increase in fluorescence due to the F64L folding enhancer mutation, available for *C. glabrata* and *C. albicans*. Both are pH-sensitive FPs that can be used to estimate the pH in the direct environment of the FP by ratiometric measurement of emission upon excitation at 395 and 475 nm (Ullah *et al.* 2013b; Liu and Kohler 2016; Tournu *et al.* 2017). The pHluorin2 codon sequence was optimized using a guided random algorithm based on the codon biases of five highly expressed genes in *C. albicans*, *RPL29, RPL32, RPL39, ACT1* and *ENO1*. This optimization method was also applied to other FPs, such as YemVenus, mTurquoise2 and mScarlet, but did not result in a significant improvement of the brightness (Tournu *et al.* 2017).

Apart from the well-known jellyfish-derived FPs, coral-based FPs were rapidly discovered and adapted for *C. albicans*. RFP and mCherry from the Cnidarian *Discosoma sp.* were codon-optimized for *C. albicans* and *C. glabrata* (Gerami-Nejad, Dulmage and Berman 2009; Yanez-Carrillo *et al.* 2015). Altering 218 codons by replacing C or G at the third position by A or T and replacing CTG codons produced a yeast-enhanced mRFP (YemRFP) (Keppler-Ross, Noffz and Dean 2008). The advantage of these RFPs is the nearly complete lack of overlap in emission spectra with GFP and CFP. Recently, a synthetically engineered RFP called mScarlet was published. This synthetic construct was based on a template of mCherry, but gave rise to a significantly improved quantum yield and maturation speed (Bindels *et al.* 2017). One version of mScarlet, mScarlet-I, was independently adapted for *C. albicans* by the research groups of Van Dijck and Bennett (Frazer, Hernday and Bennett 2019; Van Genechten *et al.* 2020). This mScarlet-I was preferred over the mScarlet-H version because it has an improved maturation speed due to the T74I mutation, with a small decrease in quantum yield (Bindels *et al.* 2017). Further expansion of red FPs is ongoing with the recent optimization of mKate2 for *C. albicans* and its application within a heme biosensor (Weissman *et al.* 2020). A blue FP or BFP, was similarly obtained from the Anthozoan sea-anemone *Entacmaea quadricolor* and optimized for *C. albicans*. Interestingly, its emission spectrum can be distinguished completely from GFP with Fluorescence Activated Cell Sorting (FACS) (Loll-Krippleber *et al.* 2015). The most recently developed FP from a non-jellyfish origin is mNeongreen, a yellow-green FP, derived from *Branchiostoma lanceolatum* which also proved to be more photostable than γmGFP (Frazer, Hernday and Bennett 2019). So far, all the FPs that have been described, have emission spectra within the visible area. Infra-red FPs, however, have the added advantage of allowing deep tissue imaging of cells, because of the transparency of tissue to this type of light. These FPs originate from bacterial phytochrome photoreceptors and after several rounds of random mutagenesis iRFP702 was obtained. Further directed mutagenesis of residues at positions 180, 202, 203 and 254 led to spectral shifts resulting in iRFP670 with an emission maximum at 670 nm (Shcherbakova and Verkhusha 2013). This collection of iRFPs requires bilirubin as a co-factor which acts as the phytochrome required for fluorescence. An iRFP670 has been developed for deep tissue imaging of *C. albicans* in zebrafish (Bergeron *et al.* 2017).

Specialized FPs overcoming practical limitations

While all previously described FPs show classical behavior, i.e. they bleach mono-exponentially, a novel realm of smart FPs is rapidly developing with non-classical behaviors such as reversible and irreversible on-off switching, emission spectrum shifts, etc. Fast folding (ff)Dronpa is the first smart FP optimized for *C. albicans* (Van Genechten *et al.* 2020). It is a negative photoconvertible FP (PCFP) which can be switched to the

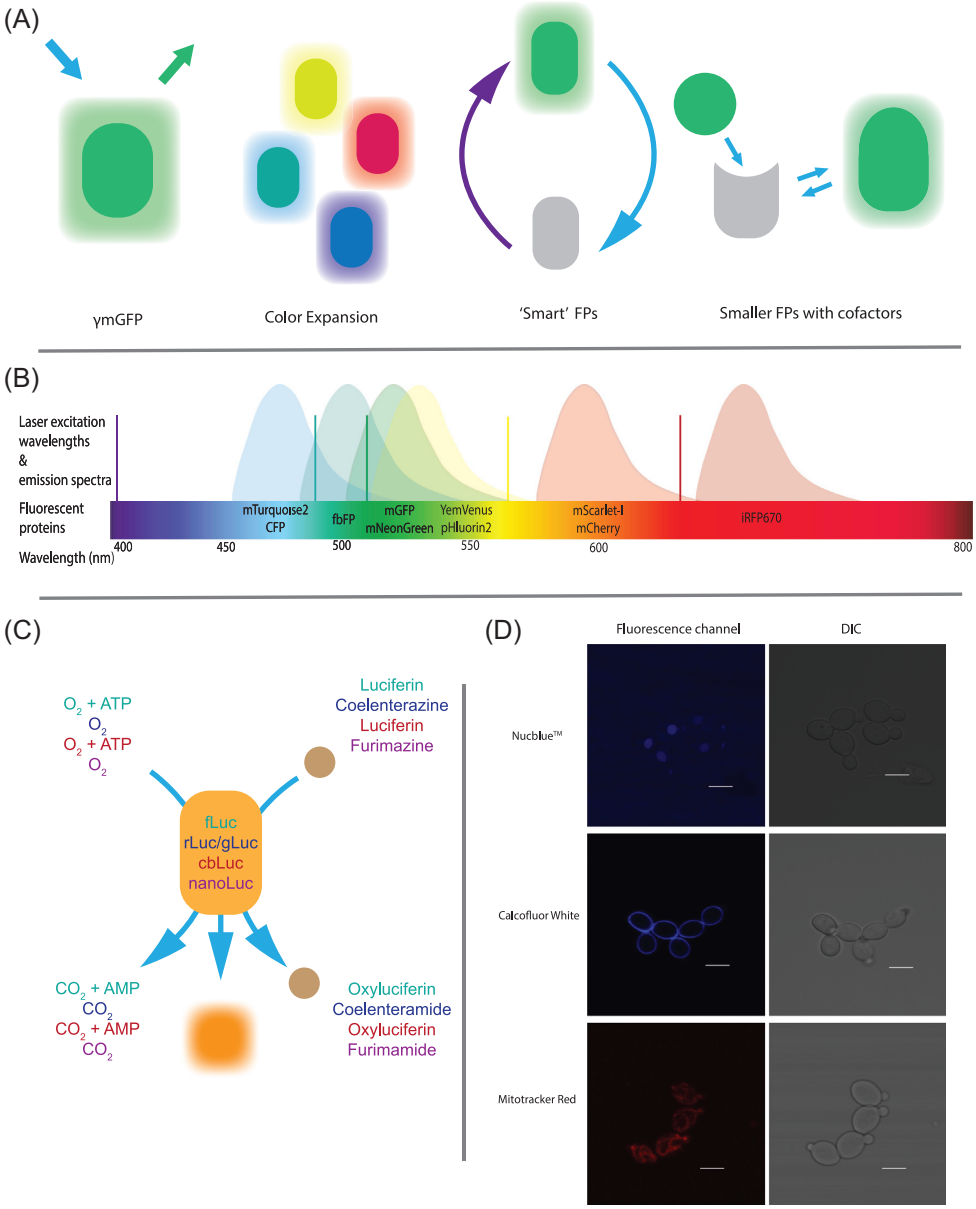

Figure 2. Schematic overview of available *Candida*-optimized fluorophores. Panel **A** provides a schematic overview of the available mechanisms and incremental enhancements of the fluorescent proteins available for *Candida* research. From the optimization of GFP to an extensive color expansion and subsequent development of 'Smart' and smaller FPs. Panel **B** depicts the common excitation wavelengths available in a fluorescence microscope setup as vertical lines. A combination of gas lasers such as a krypton, argon and helium-neon laser provide 405, 488, 515, 568 and 640 nm as possible excitation wavelengths. A simplified depiction of emission spectra of *Candida*-specific fluorescent proteins indicates the range available for research and the possible spectral overlap. **C** represents the palette of available luciferases for *Candida* research. Substrate, oxygen and/or ATP requirements are color-coded according to their respective luciferase. Panel **D** are examples of three common organelle dyes used in *C. albicans* research. In the topmost panel Nucblue™ is an example of a nuclear staining, with a singular lobe within each cell. Middle panel contains the fungal cell wall staining Calcofluor White. Bottom panel depicts the linear structures obtained after staining the mitochondria using Mitotracker Red.

on-state using UV light and reverted to the off-state using blue light as depicted in Fig. 2A. This photochromic behavior makes it suitable for super-resolution localization microscopy such as Stochastical Optical Fluctuation Imaging (SOFI) or Optical Lock-in Detection (OLID) to reduce the background autofluorescence and thus improve the signal-to-noise ratio (Marriott *et al.* 2008; Dedecker *et al.* 2012).

A drawback of all earlier described FPs is that the chromophore within the beta-barrel is formed by a reaction that requires molecular oxygen. These FPs can thus not be used in hypoxic conditions. This problem is circumvented by the *C. albicans*-adapted FMN-based FP (*Candida*-optimized FbFP), a FP that does not require molecular oxygen during maturation, but requires flavin as a co-factor. Even though FbFP requires flavin, which is only present at low concentrations in the nucleus, a targeted localization to the nucleus is functional in *C. albicans* in both normoxic and hypoxic conditions (Eichhof and Ernst 2016). Furthermore, CaFbFP is applied in an oxygen biosensor, which is further elucidated in part 3.3 on monitoring cellular activity. Functionality of this *Candida*-optimized FbFP is based on the light-oxygen-voltage-sensing domains of photoreceptors from *Bacillus subtilis* and *Pseudomonas putida* (Drepper *et al.* 2007;

Table 1. Definitions of commonly used terminology related to fluorescence.

Term	Explanation
Codon Adaptation Index (CAI)	Measure for codon usage bias. When CAI is 1, only high frequency codons are utilized.
Chromophore	Part of the fluorophore that is responsible for the emission
Point Spread Function (PSF)	Describes the pattern acquired after a single emitter is visualized through the objective
Circular permutation	Relationship between proteins in which the order of amino acids is different but the structure the same
Quantum Yield	Ratio of emitted photons over the absorbed photons. Thus, a measurement of efficiency of this conversion process.
Maturation speed	Relative time it takes to form a functional fluorescent protein
Extinction Coefficient	Measure for the absorption efficiency of light by the object
Photostability	Describes the stability or restistance of degradation when the object is illuminated with light
Phototoxicity	Laser illumination leads to the formation of Reactive Oxygen Species (ROS) which are toxic to the cell.
Autofluorescence	Fluorescence from inherent natural structures.
Photoconversion	Process of spectral changes under illumination
Scattering	Light rays deviate from their central path by interaction with an object
Resolution	Minimal disctance between two seperate points that can be distinguished by the optical device
Differential Interference Contrast (DIC)	Microscope technique utilizing the refractive index differences to visualize the object.

Tielker *et al.* 2009). The emission spectra for *Candida*-optimized FbFP and all other previously described FPs are depicted in Fig. 2B and the state-of-the art FPs for *C. albicans* and *C. glabrata* for each color are represented in Table 2.

Heading towards smaller FPs

Because of the relatively large size of all presently-available *Candida*-optimized FPs, each FP is approximately 250 amino acids long, a fusion construct with a protein-of-interest may impose a significant strain on the folding of this protein. Smaller FPs are, therefore, an interesting lead to follow up on (Fig. 2A). One such option is Y-FAST, this protein is approximately half the size of GFP-like fluorescent proteins. Y-FAST is functional as an extracellularly attached protein in *S. cerevisiae* and intracellularly in mammalian cells. Y-FAST in itself is not fluorescent, but it binds a fluorogen 4-hydroxybenzylidene-rhodanine (HBR) or 4-hydroxy-3-methylbenzylidene-rhodanine (HMBR), which stabilizes upon binding, leading to an increase in the quantum yield and a red-shift of the fluorogen (Plamont *et al.* 2016). Another option for a smaller FP in *Candida* research is UnaG, a 139 amino acid long protein isolated from Japanese eel. It is already utilized in mammalian cell lines and the mouse brain. In contrast to GFP, it is functional in anaerobic environments, but just like iRFP it requires bilirubin as a co-factor (Kumagai *et al.* 2013).

Self-labeling proteins

When you decide to fuse a fluorescent tag to your protein of interest, the color of your tag is often the determining factor. Self-labeling proteins offer a solution by providing flexibility in color even after the genetic fusing. Self-labeling proteins are genetic tags to which an exogenous substrate containing a fluorophore can bind covalently. These exogenous substrates have multiple fluorophores available, allowing quick and easy adaptations for multi-colour imaging. Three systems of the same principle are available so far. SNAP-, CLIP- and Halo-tag and have been optimized in *S. cerevisiae* (Keppler *et al.* 2003; Gautier *et al.* 2008; Los *et al.* 2008; Stagge *et al.* 2013). Optimization was required to allow the entrance of the labeling substrate into the cell. This required an additional electroporation step. These optimizations will also be necessary if the system were to be adapted for *C. albicans* research. These self-labeling protein systems can be used simultaneously allowing the researcher to perform multi-colour imaging.

Do-it-yourself

Selecting an optimal FP for your experiments

It is clear that the selection of FPs available for use in *C. albicans* and *C. glabrata* is growing. For each color, the optimal FP is represented in Table 2. Fluorescent proteins in the complete range of the visual spectrum as well as the infra-red region, are available, allowing for researchers to pick any FP with desirable traits or several FPs for multi-color imaging. When choosing fluorescent proteins to answer a specific research question, it is always useful to check all characteristics of the preferred FP, such as the possible cofactors, oxygen requirements and subcellular location. Each experimental condition has a particular set of physical properties which require specific FPs. Some FPs will have different maturation times at 30°C or 37°C and, as will be discussed in later chapters, some infection models require a specific type of FP for visualization. As presented above, FPs in a wide range of colors and with specific adaptations are available. Of note is that N-terminal tags often mask the signal sequences present near the N-terminus of a protein-of-interest and thus hamper the correct localization of the protein (Palmer and Freeman 2004).

Bioluminescence

Bioluminescence is the emission of light upon enzymatic processing of a substrate that is administered to the cells. The enzymes that catalyze the reactions are called luciferases and are generally encoded genetically. It is a common phenomenon in nature which can observed in fireflies or the jellyfish *Aequorea victoria* (Shimomura, Johnson and Saiga 1962). One of the advantages of bioluminescence compared to fluorescence is the lack of the need for excitation, which can be a limiting factor when your sample is prone to scattering of shorter wavelengths. The

Table 2. Overview of fluorescent proteins, dyes and biosensors available for use in *Candida* species. Several fluorescent proteins, dyes and biosensors are available for experimentation in *Candida* species. FP, fluorescent protein; FMN, flavin mononucleotide; FRET, fluorescence resonance energy transfer; QY, quantum yield.

Fluorescent protein	Excitation maximum (nm)	Emission maximum (nm)	Excitation laser (nm)	Specifics	*Candida* species	Reference
BFP	381	445	405	Blue FP	*C. albicans*	Loll-Krippleber *et al.*, 2015
FbFP	449	495	458	FMN-based FP, independent of oxygen, requires flavin	*C. albicans*	Tielker *et al.*, 2009
CFP	456	480	458	FRET pair with yellow FP	*C. albicans, C. glabrata*	Gerami-Nejad *et al.*, 2001; Yanez-Carrillo *et al.*, 2015
mTurquoise2	434	474	458	Next-generation, highest QY of cyan FPs	*C. albicans*	Van Genechten *et al.*, 2020
ɣmGFP	485	510	488	Widely used	*C. albicans*	Zhang & Konopka, 2010
YeGFP3	485	510	488		*C. glabrata*	Yanez-Carrillo *et al.*, 2015
mNeonGreen	506	518	488	Next-generation, enhanced photostability compared to eGFP	*C. albicans*	Frazer *et al.*, 2019
YFP	513	527	514	FRET pair with Cyan FP	*C. albicans, C. glabrata*	Gerami-Nejad *et al.*, 2001; Yanez-Carrillo *et al.*, 2015
mVenus	515	528	514	Bright, FRET pair with cyan FP	*C. albicans*	Van Genechten *et al.*, 2020
pHluorin	475/395 (acidic/alkaline)	509	488/405	pH sensor	*C. albicans, C. glabrata*	Liu & Kohler, 2016; Ullah et al., 2013
pHluorin2	475/395 (acidic/alkaline)	509	488/405	pH sensor	*C. albicans*	Tournu *et al.*, 2017
mCherry	587	610	568		*C. albicans, C. glabrata*	Gerami-Nejad *et al.*, 2009; Yanez-Carrillo et al., 2015
mScarlet-I	569	593	568	Next-generation, highest QY of cyan FPs	*C. albicans*	Frazer *et al.*, 2019; Van Genechten *et al.*, 2020
mKate2	588	633	568	Utilized in heme sensor	*C. albicans*	Weismann *et al.*, 2020
iRFP670	643	670	632	infrared FP	*C. albicans*	Bergeron *et al.*, 2013

Fluorescent dye	Excitation maximum (nm)	Emission maximum (nm)	Excitation laser (nm)	Organelle	*Candida* species	Reference
DAPI	358	461	405	Nucleus	*C. albicans, C. glabrata*	Brimacombe *et al.*, 2019; Yanez-Carrillo *et al.*, 2015
Hoechst 33342	361	497	405	Nucleus	*C. albicans*	Subotic *et al.*, 2017
Calcofluor white	347	475	405	Cell wall	*C. albicans, C. glabrata*	Han *et al.*, 2019; Dagher *et al.*, 2018
Dansyl dye-conjugated azole	340	520	405	Mitochondria and Fluconazole	*C. albicans*	Benhamou *et al.*, 2017
Bodipy 493/503	493	503	488	Lipid Droplets	*C. albicans*	Nguyen *et al.*, 2011
ER Tracker Green	504	511	488	Endoplasmic reticulum	*C. albicans*	Jain *et al.*, 2018
Mitotracker Green	490	516	488	Mitochondria	*C. albicans, C. glabrata*	Benhamou *et al.*, 2017

sensitivity of each luciferase differs, yet, in general, they provide a more sensitive reporter system with a better signal-to-noise ratio compared to fluorescent proteins and would even allow for monitoring of transcriptional regulation (Hirose *et al.* 2002).

There are several *Candida*-optimized and validated systems available, each with their own advantages and disadvantages. The first attempt to apply bioluminescence in *C. albicans* was based on the green-yellow firefly luciferase (fLuc). However, the system suffered from an extremely low signal (Srikantha, Chandrasekhar and Soll 1995). It appears that a single CTG codon within the firefly luciferase gene was sufficient to completely block its function. Because of this issue, another luciferase from the sea pansy *Renilla reniformis* (rLuc) was introduced successfully in *C. albicans* as a reporter system for white-opaque switching (Srikantha *et al.* 1996). This luciferase emits blue light upon administration of coelenterazine in the presence of oxygen.

The discovery that the CTG codon in *C. albicans* is translated as serine instead of leucine led to the codon optimization of several FPs, as previously discussed, but also of the firefly luciferase (fLuc). The firefly luciferase is preferred over the *R. reniformis* luciferase because the substrate of rLuc, coelenterazine, is less stable than the substrate of the firefly luciferase, luciferin. Nine CTG codons within the firefly luciferase were exchanged for TTG codons, rendering the construct completely functional and thus emitting green-yellow light upon addition of luciferin (Doyle *et al.* 2006b). One of the drawbacks of luciferin is its poor ability to penetrate the remodeled hyphal cell wall (Doyle *et al.* 2006a). The combination of poor diffusion of luciferin with the requirement of intracellular ATP, hampers the applicability of fLuc. Another bioluminescent reporter is the *Gaussia princeps* luciferase (gLuc), which, much like the rLuc system, catalyzes the reaction of coelenterazine to produce light. It has the advantage that it does not require ATP, yet is still oxygen-dependent. To circumvent the issue of poor penetration of its substrate, gLuc was expressed extracellularly by attaching it to a GPI-linked cell wall protein (Enjalbert *et al.* 2009). This problem highlights the main drawback of bioluminescent systems, the necessary optimization of substrate administration and its poor diffusion across the cell membrane (Gaur *et al.* 2017).

As mentioned before, an advantage of bioluminescence is that it does not require excitation, thereby circumventing the inefficient penetration of light through tissues. Especially light of shorter wavelengths is unable to penetrate tissue, due to scattering and absorption by blood hemoglobin, collagen, etc. (Avci *et al.* 2013). Emitted light from the luciferase reaction is also severely attenuated by tissue depth, therefore a more red-shifted luciferase would allow for deeper tissue imaging. By introducing S284T, L295F, T214A and A215L mutations in the gLuc optimized gene, a red-shifted thermostable mutant was acquired. This luciferase is significantly more sensitive *in vivo* in a systemic and oropharyngeal candidiasis (OPC) model compared to the original gLuc (Dorsaz, Coste and Sanglard 2017). Another red- and green-emitting luciferase system was adapted from the click beetle *Pyrophorus plagiophtalamus*. These click beetle luciferases (cbLuc) use luciferin as a substrate in the presence of ATP and oxygen. Because these luciferases use the same substrate as the firefly enzymes, it is possible to spectrally separate both luciferases after activation with one substrate. However, they have not been applied in an *in vivo* infection model yet (Kapitan *et al.* 2016).

As is the case for fluorescent proteins, luciferases are larger proteins, ranging from 311 to 550 amino acids. Overexpression of genes encoding such proteins can diminish the amount of tRNAs and amino acids available for efficient translation of essential proteins. It can also perturb cellular processes, such as folding and degradation processes in the endoplasmic reticulum. A smaller 19 kDa luciferase, called NanoLuc, has been reported and adapted for *C. albicans*. It uses the commercial Nano-Glo containing furimazine as a substrate and is ATP-independent (Luna-Tapia *et al.* 2015; Masser *et al.* 2016). The emission maximum of NanoLuc, 460 nm, does not make it suitable for *in vivo* imaging. An overview of the luciferases with their requirements and substrate is presented in Fig. 2C.

None of the luciferases presented above are optimal for every situation. Therefore, when selecting a luciferase for an experiment, the sensitivity, thermostability and emission wavelength should be taken into account. Immediate further improvements would be an increase of signal strength of the red-shifted luciferases so that they have a similar performance as the green-emitting luciferases. Decreasing their size would be an added advantage. Another significant improvement to every luciferase system would be the engineering of *C. albicans* to make the substrate itself, thereby omitting the need for external administration and circumventing the poor diffusion of the substrate into the cell.

Dyes

In fungal research, dyes are mainly used to provide cellular context or to investigate certain characteristics of a cell, such as ploidy, live/dead ratios and cell wall remodeling. In this section, we discuss the dyes that perform their function as such and do not need to be conjugated to an antibody. Nuclear dyes, such as DAPI (4',6-diamidino-2-phenylindole) and Hoechst 33342, bind AT-rich regions of DNA. These are blue dyes that are excited with UV light. Because of the direct binding to the DNA, the dyes are toxic. They have been applied successfully in *C. albicans* and *C. glabrata* and are ideal for multi-color imaging with any green and/or red FP (Subotić *et al.* 2017; Brimacombe *et al.* 2019; Yanez-Carrillo *et al.* 2015). An example of a Hoechst 33342 staining (Nucblue) is shown in Fig. 2D and an overview of the dyes discussed in this review, is given in Table 2.

The cell wall is an organelle that differentiates *Candida* from mammalian cells and is therefore a preferred target for antifungal agents. The echinocandins inhibit beta-glucan synthase and thereby limit the production of this cell wall component. Another component of the cell wall is chitin. Even though a cell wall staining often uses antibodies or lectins to bind the carbohydrates that make up the brunt of the cell wall, a specific dye is available to perform this without any need for conjugation and extra washing or blocking steps. Calcofluor White is a dye specifically binding glucans and chitin, which are prominent in the fungal cell wall. Similar to the nuclear dyes described above, it is a blue dye that is excited with UV light. It has been utilized in the study of cell wall composition and estimation of chitin levels of *C. albicans* (Ballou *et al.* 2016; Dagher *et al.* 2018; Han *et al.* 2019).

The target of fluconazole is lanosterol 14α-demethylase encoded by *ERG11*. This target is localized to the endoplasmic reticulum. The endoplasmic reticulum is therefore an important cell organelle in antifungal drug research. Using a combination of fluorescence or Förster resonance energy transfer (FRET) and the ER Tracker staining, the interaction between Ras1 and Gpi1 was proven to be localized to the ER (Jain *et al.* 2018). ER Tracker specifically binds to K^+-channels which are present on the ER membrane and the plasma membrane. Two different colors of BODIPY® ER Tracker dyes, red and green, have been applied in *C. albicans* (Shah *et al.* 2015; Jain *et al.* 2018).

The last organelle that can be specifically stained without using antibodies or lectins is the mitochondrion. This organelle is stained by the Mitotracker dyes, which encompass green, orange, red and deep red colors, allowing researchers to pick one that is compatible with their setup. Mitotracker dyes diffuse passively over membranes and sequester into the mitochondria, where they are oxidized and retained because of their reaction with active thiol-groups (Keij, Bell-Prince and Steinkamp 2000). Using Mitotracker Red, localization of several proteins to the mitochondria was shown (Ferrari *et al.* 2011; Dana *et al.* 2019). We show the localization pattern of Mitotracker Red in Fig. 2D. Two other dyes were engineered that localize to the mitochondria. These dyes are based on an azole backbone to which a Dansyl or Cy5 is attached, emitting green and far-red light, respectively. They were shown to co-localize significantly with Mitotracker green proving that azoles are sequestered in mitochondria and that these azole-based dyes can be used as a mitochondrial stain (Benhamou *et al.* 2017).

Finally, BODIPY 493/503 is a specific stain for lipid droplets. Although initially lipid droplets were not considered to be a true classical organelle, their extensive role in lipid trafficking and metabolism indicated their role as a conserved organelle. Lipid droplets are shown to be necessary for full virulence of *C. parapsilosis* in a murine infection model (Nguyen *et al.* 2011).

Other cell organelles do not have a specific dye that can be applied in *Candida* research. There are, as far as we know, no dyes available to stain the Golgi apparatus. The dyes presented above can be applied in live cell imaging, but have some toxicity that could lead to imaging artefacts such as highly fluorescent vesicles. Organic dyes show low overall cell toxicity and are a preferred option if available. When dyes are utilized in experiments, control experiments should be performed to check the possible harmful effects.

WHAT IS POSSIBLE?—SUBCELLULAR IMAGING

The basic application of fluorescence in fungal research is subcellular imaging of proteins or structures. To achieve information on the presence and localization of certain components, the target-of-interest should be fused to a fluorescent label through appropriate linkers. Whilst many methods are available to monitor the fluorescent signal coming from fluorophores within *Candida* species, microscopy remains the most prominent technique. Further technical advancements have made it possible to image in a time- and space-resolved manner. Apart from cellular structures, also protein-protein interactions and cellular activity can be imaged.

Imaging subcellular structures

Design of linker sequences

Several types of linkers that connect proteins to the above-mentioned fluorescent tags exist, such as flexible, rigid and cleavable linkers. Dependent on the type of study, the linker sequence should maintain a certain level of flexibility (Kerppola 2009). Generally, linkers are composed of small, polar or non-polar amino acids, such as the widely used $(Gly\text{-}Gly\text{-}Gly\text{-}Gly\text{-}Ser)_n$ linkers (Ene *et al.* 2013). Glycine residues are small, allowing for flexibility, while serine aids in maintaining the stability by forming hydrogen bonds with the surrounding water molecules, thereby reducing unfavorable interactions with the protein moiety.

Widefield and confocal microscopy

Widefield microscopy is one of the basic methods of microscopy and therefore widely applied in fungal research. However, one needs to understand the basic principle of fluorescence widefield microscopy in order to make further improvements and adaptations. In widefield microscopy the whole sample, a fluorescent *Candida* cell, is illuminated evenly by the excitation light passing through the objective. The entire excited sample emits light which passes through the objective and is focused on the camera. This basic microscopy setup was originally used to visualize the *C. albicans* optimized GFP, YFP, CFP, mCherry and is still applied regularly due to its relatively low cost and high effectiveness (Cormack *et al.* 1997; Gerami-Nejad, Berman and Gale 2001; Gerami-Nejad, Dulmage and Berman 2009). The resolution of widefield microscopy is limited to approximately half of the wavelength of the excitation light, being about 200nm. However, it is possible to image the subcellular localization of proteins using this setup because a *Candida* cell is around 4–10 μm in size and hyphae can easily stretch out to 30–50 μm. Since the whole sample is illuminated, out-of-focus emission light is also captured by the objective, causing background signal. Furthermore, this background signal is further increased by autofluorescent molecules such as riboflavin, causing again a drop in the effective signal-to-noise ratio (Demuyser *et al.* 2020).

Using confocal microscopy can partially solve the issue of background noise, by specifically illuminating a certain area in a single plane of the sample. This is done by focusing excitation light onto a single area in the sample and consequently scanning the entire sample to construct the complete image. The emitted light from a single pixel area passes through a pinhole which reflects out-of-focus light. The selected photons are captured by a photomultiplier tube (PMT) or an avalanche photon detector (APD) instead of the CCD or CMOS cameras used for widefield microscopy. Additionally, it is possible to change the plane of illumination, therefore allowing to image at different depths within the sample. A reconstruction of several images at different depths can result in a 3D-model of the sample, which is particularly interesting when studying biofilm structures (Tsui, Kong and Jabra-Rizk 2016). Even though scanning speeds can often be adjusted in software, it remains a rather slow method. By using spinning disk confocal microscopy one can increase the speed at which a confocal image can be acquired. This technique uses a spinning disk containing multiple holes instead of one pinhole. Since excitation light is allowed to pass through multiple holes of the spinning disk, an image can be reconstructed much faster (Davidovits and Egger 1971). Since confocal microscopy is able to reject much of the out-of-focus autofluorescence background of *Candida* species, it is the preferred method for subcellular imaging and colocalization studies of proteins-of-interest.

Super-resolution microscopy

The methods described above are limited by Abbe's law, which states that the resolution is controlled by diffraction according to:

$$d = \frac{\lambda}{2NA}$$

Where d is the smallest distance between two resolvable objects, λ is the wavelength of the emitted light and NA is the numerical aperture of the objective. In practice, this results in a resolution limit of near 200 nm. As already mentioned earlier, this resolution is sufficient for many applications in *Candida*

research, but further enhancements would potentially increase our understanding of certain molecular mechanisms.

In the chapter on fluorophores, we already discussed ffDronpa, a smart fluorescent protein. This protein can switch reversibly between a bright and a dark state. This specific behavior is a prerequisite for single molecule localization microscopy (SMLM). This method is based on the fact that if two emitters are in close proximity, they cannot be discerned due to the overlap of their PSFs. However, if two images are obtained sequentially where only one of the emitters is in the bright state, it is possible to pinpoint the center of the emitter using the gaussian nature of the PSF. Several SMLM techniques are available, PALM, (d)STORM and pcSOFI, each requiring a certain induced behavior of the fluorescent protein (Betzig *et al.* 2006; Rust, Bates and Zhuang 2006; Dertinger *et al.* 2009). The codon-optimized ffDronpa is particularly suitable for pcSOFI and this would be a robust method able to enhance the localization of fluorophores and reduce the significant background autofluorescence of *C. albicans* (Dedecker *et al.* 2012; Moeyaert *et al.* 2014).

Another way to provide sub-diffraction resolution, however not performed with *Candida* species yet, is structured illumination microscopy (SIM). Here, the sample is illuminated with a specific sine pattern which, in combination with Fourier transformation, leads to a diffraction limit of approximately 100 nm. This technique has been applied on yeast species to image the spindle pole body. SIM was even combined with light-sheet-based fluorescence microscopy (LSFM) yielding csiLSFM by which the resolution of a GFP-tagged ER structure was significantly enhanced (Burns *et al.* 2015; Chang, Perez Meza and Stelzer 2017). Both studies utilized classical FPs of which optimized versions are available for *C. albicans* suggesting that these methods can be applied on this pathogenic yeast as well. Much like SIM imaging, stimulated emission depletion microscopy (STED) requires a specific microscope setup that produces a carefully constructed illumination pattern. Similar to laser scanning microscopy, one specific area in the sample is excited. Around this excitation area a 'donut' shaped depletion ring reduces the excitation point spread function (PSF), the function describing the blurring of a point by the optics of the system, by switching off the fluorophores on the outer side of the excitation area. This results in a smaller excitation area and thus an increase in resolution (Klar *et al.* 2000). A combination of STED and the Snap, Halo and CLIP-labeling system was successfully applied in *S. cerevisiae* and resolved details in the cell cortex that were not distinguishable using confocal microscopy (Stagge *et al.* 2013).

Light-sheet microscopy

Even though confocal microscopy is a significant improvement over widefield microscopy and the excitation light is focused onto a small area of the sample, out-of-focus laser light is still able to excite and bleach dyes and FPs outside of the focal plane. This leads to a decreased signal when another plane of the sample is imaged. Light-sheet-based fluorescence microscopy (LSFM) offers a solution for this issue. The advantages of this technique for deep-tissue imaging will be further discussed in the section on host-pathogen imaging, but the implementation of LSFM can also be useful for imaging single yeast cells. As the name implies, a sheet of excitation light is created by focusing a collimated laser beam onto a thin plane by a cylindrical lens (Greger, Swoger and Stelzer 2007). This cylindrical lens is orthogonal to the objective lens which focuses the emitted signal from the sample onto the detector. Since only a small part of the sample is illuminated, there is no need for a pinhole to remove out-of-focus excitation light. The method is able to capture images much faster than a confocal microscope since the sample is not completely scanned, but all pixels are excited and imaged in parallel by the same type of camera as in widefield microscopy (Reynaud *et al.* 2008). Using this setup, embryos of zebrafish, tumor cells and amoebae have been visualized (Huisken and Stainier 2007; Lorenzo *et al.* 2011; Takao *et al.* 2012). It has also been successfully applied to image organic dyes in baker's yeast, indicating that this technique could also be applied on *Candida* species (Reynaud *et al.* 2008).

Do-it-yourself

Selecting the best microscopy technique for your research

The limiting factor in subcellular imaging of *Candida* species is often the fluorescent probe instead of the microscopy setup. Choosing your fluorescent protein or dye wisely is thus of uttermost importance. Nevertheless, some critical differences between a widefield and a confocal microscope should also be considered when choosing the best method for a certain experiment. If your target-of-interest is a highly mobile protein, a confocal microscope will not be able to construct an image fast enough. Therefore, the movement of this protein will cause a smear of fluorescent signal in your final image. Even parts of the cytoskeleton such as alpha-tubulin are continuously degraded and re-assembled, and therefore highly mobile in live cells. Since the speed of widefield microscopy relies on the camera acquisition rate, mobile targets can be imaged using such a setup. Fixating the cells is another option to image mobile proteins, yet comes at a cost of introducing artefacts, decreasing fluorescence from fluorescent proteins and losing the capacity to perform time-lapse experiments.

As mentioned earlier, many stress factors introduce elevated autofluorescence in the fungal cell, these experiments would benefit from confocal microscopy to reject most of the out-of-focus fluorescence. Especially when the signal from the sample is sparse, reducing the out-of-focus fluorescence is of utmost importance to improve your signal-to-noise ratio. Confocal imaging is also the method of choice in imaging *C. albicans* biofilms or other thicker samples, because these are more prone to scattering. Another way to resolve this, but at a greater cost, is light-sheet microscopy, which is able to combine the fast image acquisition with selective illumination to reduce out-of-focus fluorescence.

If your signal is sparse, increasing excitation power can overcome this issue, but is not always beneficial due to phototoxicity and increased photobleaching as discussed later. In confocal microscopy or widefield microscopy, respectively decreasing the scanning speed or increasing the exposure time of the camera and thus acquiring more signal from all single points within the sample is another way to boost the signal obtained from the fluorophore. Furthermore, utilizing low-fluorescent medium based on yeast nitrogen base where folic acid and riboflavin are omitted, diminishes the amount of autofluorescence and is thus an enhancement for both widefield and confocal imaging.

Imaging protein-protein interactions

When studying an organism's signaling pathways and metabolism in order to discover novel antifungal drug targets, it is essential to take into account the numerous protein-protein interactions (PPIs). However, for *C. albicans*, and even more so for other *Candida* species, protein-protein interactions have been largely neglected in the drug discovery process. Moreover, very few of the documented interactions have been gathered

in accessible databases. Recently, colleagues from our laboratory have performed an exhaustive literature search of all known PPIs in *C. albicans*, thereby greatly updating the existing databases (Schoeters and Van Dijck 2019). Depending on the research question, several techniques can be used for the study of PPIs in *C. albicans*.

For high-throughput screening of PPIs, the yeast two-hybrid (Y2H) technique is mostly used. However, since many genes contain CTG codons, the use of *S. cerevisiae* as model system for *C. albicans* PPI studies is complicated. To circumvent this issue, the Vesicular Capture Interaction system was developed in *C. albicans* in 2009 (Boysen *et al.* 2009). In this assay, the bait consists of one protein-of-choice, fused to Vps32, a subunit of the endosomal sorting complex required for transport (ESCRT). In the prey construct, another protein-of-choice is fused to GFP. By using a *vps4* mutant strain, in which vesicular accumulation of Vps32 is stimulated, a positive interaction between both proteins-of-interest will be visible as bright fluorescent spots. It is, however, still questionable to which extent this assay can be used in high-throughput setups. For this reason, colleagues from our laboratory developed a novel *Candida* two-hybrid system to be able to perform high-throughput PPI screenings directly in the pathogenic organism (Stynen, Van Dijck and Tournu 2010; Schoeters *et al.* 2018). Alternatively, when not so specifically interested in high-throughput screenings, yet more in the specific interaction between multiple proteins, techniques such as co-immunoprecipitation (co-IP) or tandem-affinity purification can be used (Alkafeef *et al.* 2018; Sellam *et al.* 2019). The major disadvantage of such techniques is the lack of an *in vivo*, cellular context and any spatial information on the native location of the interaction inside the cell.

BiFC

Bimolecular Fluorescence Complementation or BiFC and Fluorescence/Förster Resonance Energy Transfer or FRET are *in vivo* techniques that can provide information on the native localization of the interaction. BiFC is a technique whereby a fluorescent protein is split into two complementary parts, an N- and C-terminal fragment. Both parts are fused to two proteins of which interaction is under investigation (Kerppola 2009). When the proteins interact, the fluorescent protein fragments are brought together irreversibly and a fully functional and excitable FP is reconstituted. A few years ago, we developed the first BiFC system, based on YemVenus, optimized it for use in *C. albicans* and showed its functionality by visualizing interaction of Snf4-Kis1, Bcy1-Tpk1/2 and Gpr1-Gpa2 protein pairs (Subotić *et al.* 2017). When performing PPI studies, it is important to confirm an interaction with more than one technique. Subotić *et al.* therefore used the established *Candida* two-hybrid system from our laboratory to confirm the interactions earlier observed by BiFC. In the same year, a similar BiFC assay was optimized for *C. albicans*, using the *S. cerevisiae* system as described in Sung and Huh 2007 (Sung and Huh 2007; Mamouei *et al.* 2017). The non-yeast optimized fluorescent protein Venus was used, in which all CTG codons were mutated to TTG by site-directed mutagenesis. The physical interaction between several ferroxidases and permeases was assessed by means of this setup. Very recently, a novel Tet-on-BiFC system was established in which mCherry is used as a fluorescent protein (Lai, Sun and Shieh 2020). Researchers validated the system by showing interaction between the rho-type GTPase Cdc42 and the putative rho GDP dissociation factor Rdi1. In this system dominant markers were used, whereas the system we developed, is based on the use of auxotrophic markers. Furthermore, the Tet-on system allows for regulated expression of both genes-of-interest. The main disadvantage, however, is the usage of mCherry, which has a lower quantum yield in *C. albicans*, compared to YemVenus and is therefore not always suitable for endogenous expression of tagged genes. Based on the *S. cerevisiae* system, researchers also used a BiFC assay to detect PPIs in *C. glabrata* (Sung and Huh 2007). Heterodimer formation between mating type proteins *Cga*1 and *Cg*alpha2 as well as *Cg*alpha3 homodimer formation were detected using a Venus-based BiFC assay (Robledo-Márquez *et al.* 2016). Positive signals were detected and quantified by flow cytometry. Both interactions were confirmed by co-IP using Flag-tagged and cMyc-tagged proteins. However, not all co-IP detected interactions could be confirmed by BiFC analysis. Likely certain interactions that are detected *in vitro*, are less relevant under *in vivo* conditions, where also other factors influence the interaction. This indicates that assessing interactions in a cellular context is always preferable to evaluate the relevance of the PPI.

FRET

Due to the irreversible character of FP complementation, BiFC-based methods lack the potential to report on the PPI in a dynamic, more physiologically-relevant way. FRET, on the contrary, holds the unique quality to display an interaction *in vivo* and in real-time in the cell, while providing information on spatiotemporal dynamics. The process is based on the non-radiative transfer of energy between two fluorophores which are in close proximity. Excitation of one FP (donor FP) leads to emission of this FP. If another FP (acceptor FP) is nearby, part of the excitation energy from the donor FP, is transferred to the acceptor FP, eventually leading to emission of this latter FP. The theoretical FRET efficiency (E_{FRET}) can be calculated as follows: $E_{FRET} = 1/(1+(r/R_0)^6)$, where r represents the distance between the fluorophores and R_0 the distance at which the FRET efficiency is 50% (Bajar *et al.* 2016). The most common FPs to be combined as a FRET pair are cyan ECFP—yellow EYFP or green EGFP—red mCherry, as the spectral overlap between the donor emission spectrum and the acceptor excitation spectrum is considerably high(Bajar *et al.* 2016).

Several methods can be used to monitor FRET inside a living cell and are categorized, based on whether E_{FRET} or a change in E_{FRET}, for instance upon addition of an inducer, is measured. A first method to directly measure E_{FRET} is acceptor photobleaching. If energy transfer happens from the donor to the acceptor, photobleaching the acceptor will cause the donor to retain more energy, and an increase in donor emission will be observed (Bajar *et al.* 2016). Using this technique, researchers showed interaction between the GTPase Ras1 and the GPI-N-acetylglucosaminyl transferase Gpi2 in the endoplasmic reticulum of *C. albicans* (Jain *et al.* 2018). Another technique to measure FRET is fluorescence lifetime imaging (FLIM)-FRET, in which the fluorescence lifetime (which is the time a FP spends in the excited state before emitting a photon and returning to the ground state) of the donor is measured in the absence and presence of the acceptor. The fluorescence lifetime of the donor will decrease if FRET occurs (Piston and Kremers 2007; Bajar *et al.* 2016). A second category of methods is mostly used to measure relative changes in FRET efficiency, for example upon addition of an inducer, rather than the absolute FRET efficiency. In sensitized emission-FRET, the donor is excited and emission of both donor and acceptor is monitored (van Rheenen, Langeslag and Jalink 2004). Ratiometric FRET is derived from sensitized emission-FRET. It calculates the change in E_{FRET} over the change in donor emission. This is often the preferred technique

when unimolecular FRET-based biosensors are used, as illustrated in the section 'Monitoring cellular activity'. Finally, certain commercial assays also make use of FRET as a readout for specific biochemical or biological processes. One example is the LIVE/DEAD® *Funga*Light™ Yeast Viability Kit from ThermoFisher, in which two fluorescent probes are used, SYTO®9 and propidium iodide. SYTO®9 labels nucleic acids fluorescently green while PI probes nucleic acid fluorescently red. Both stains, however, differ in their membrane permeability. While SYTO®9 can penetrate both living and dead cells, PI only penetrates cells with damaged membranes. In cells with intact cell membranes, as a result, only green fluorescence can be observed, while cells with damaged membranes fluoresce mainly red due to a FRET-related decrease in green fluorescence if SYTO®9. Using this technique, researchers measured the anti-*Candida* effect of Psd1, an antimicrobial peptide isolated from *Pisum sativum* seeds (Gonçalves *et al.* 2017).

Important disadvantages of using FRET to detect PPIs in living *Candida* cells are the variability in expression of both proteins-of-interest and concomitantly, the donor and acceptor, the high level of autofluorescence and specific cellular context (pH, O_2, presence of quenchers), which often complicate straightforward analysis. Other systems operating in a simpler environment, such as an alternative host organism or an *in vitro* setup, can be used, yet generally at the expense of biological relevance. Researchers recently showed that an antifungal peptide inhibits cell wall biosynthesis in *Candida tropicalis* by binding to β-1,6-glucanase Kre9 using *E. coli* as an alternative host (Li *et al.* 2020). Using an *in vitro* setup, other researchers illustrated the role of critical amino acid residues in ATP binding and hydrolysis by the drug efflux pump Cdr1 in *C. albicans* (Rai *et al.* 2008). They exploited the intrinsic fluorescence of a tryptophan residue and labelled a cysteine residue in the active site with a fluorescent sulfhydryl probe. Proximity of both residues was analyzed under several conditions and their role in Mg^{2+} and ATP binding and hydrolysis was specified. Using another *in vitro* single molecule FRET-based setup, researchers monitored binding of the *C. glabrata* telomere-ending binding protein complex or CST and one of its subunits, Cdc13, to higher order G-tail structures of the telomere ending (Lue and Chan 2013). Using a biotinylated PEG-coated surface, they immobilized biotinylated DNA by binding to neutravidin. The DNA strand was labelled with two fluorescent dyes, Cy3 and Cy5. Upon binding of the CST or Cdc13 proteins to the DNA sequence, the fluorophores set apart and energy transfer between both diminishes.

Improvements for fluorescent PPI assays

Using new techniques or better probes, a number of the shortcomings of standard fluorescent PPI techniques can be overcome. Development of new fluorophores in certain organisms, has led to specific improvements of FRET and BiFC experiments in the respective research fields. A few examples of new adaptations are FRET or BiFC with far-red and/or infrared fluorophores, especially interesting for imaging interactions in deeper tissues; FRET based on FPs with large Stokes shifts, to minimize cross-talk between channels; FRET pairs with a dark acceptor, as such reducing bleed-through and phototoxicity; and multicolor FRET or BiFC, to allow visualization of multiple interactions or processes at the same time (Filonov and Verkhusha 2013; Bajar *et al.* 2016; Fujii, Yoshimura and Kodama 2018). Specifically for BiFC, overcoming the irreversibility of the interaction would be a great improvement. One option here is to replace the GFP-like fluorophore by IFP1.4, an infrared fluorescent protein which is not yet optimized for *Candida* species (Shu *et al.* 2009). Upon reassembly of the two IFP protein parts and addition of the chromophore, biliverdin, fluorescence can be detected, which is reported to be reversible (Tchekanda, Sivanesan and Michnick 2014). The fluorescent protein can also be replaced by a split fluorescence-activating and absorption shifting tag or FAST, which allows for binding and stabilization of the fluorogen HBR. Upon complementation of the FAST parts, and binding of HBR, the fluorescence of the latter increases significantly. Most interestingly, the splitFAST system is fully reversible which is a major advantage over the classical FP-based fluorescence complementation (Tebo and Gautier 2019). Both for FRET and BiFC, a fluorescent protein could be replaced by a luciferase enzyme. Since no excitation light is needed here, problems such as photo-toxicity, autofluorescence, photobleaching and cross-excitation are avoided to a large extent (Sun *et al.* 2016). BiFC analysis could be performed using a luciferase enzyme instead of a fluorescent protein, in this case the assay is called a split-luciferase system (Fukutani *et al.* 2017). BRET or bioluminescence resonance energy transfer resembles FRET, with the exception that the donor fluorophore is replaced by a bioluminescent protein, a luciferase. This leads to an increase in signal-to-noise ratio and thus sensitivity of the measurements. As mentioned earlier, quite a few bioluminescent proteins have been established for *Candida* species, yet BRET has never been reported. In *S. cerevisiae* the system has proven to be efficient in screening PPI inhibitors in the context of anticancer drug development (Corbel *et al.* 2017).

Other more recent advances making use of resonance energy transfer are single molecule FRET (smFRET), in which total internal reflection microscopy is integrated to overcome the limited spatial resolution, and sequential RET, in which a combination of BRET and FRET allows for assessment of the physical interaction between three proteins (Carriba *et al.* 2008). A similar combination between FRET or BRET and BiFC can be made to monitor formation of multiprotein binding complexes (Cui *et al.* 2019). Finally, also colocalization analysis can be used to assess interaction between proteins (Cui *et al.* 2019). The main limitation here is spatial resolution, restricted among others by the resolution of the light microscope and the wavelength of the light. One option is to use super-resolution microscopy, as detailed in the previous section, in dual color imaging, by STED, PALM or STORM. Alternatively, also 3D imaging can improve the accuracy of interaction assessment through colocalization. Mapping PPIs in *Candida* species can be of utmost importance to unravel signaling cascades and discover novel antifungal drug targets (Schoeters *et al.* 2018). Due to increased automatization and establishment of high-content imaging, many of the above-mentioned techniques could be used in a high-throughput setup as is already the case for other organisms (Song, Madahar and Liao 2011; Miller *et al.* 2015; Corbel *et al.* 2017).

Monitoring cellular activity

Apart from proteins or interactions, also cellular activity can be visualized, using fluorescence microscopy, or quantified, using FACS. Biosensors are molecules that allow for monitoring of either chemicals, conditions, enzymatic activity or physicochemical properties. An overview of all discussed biosensors, is given in Table 2.

pH

An important biochemical characteristic for cells is the pH, both intracellular as well as extracellular. *Candida albicans* is able to adapt to completely different niches with the pH ranging from 4 to 8 and monitor changes in the external pH. One of the pathways involved in monitoring acidification or alkalization of the

environment is the Rim101 signaling pathway. Alkalization of the environment is sensed at the plasma membrane by Rim21 and signaled intracellularly, leading to filamentation (Obara and Kihara 2014). On the other hand, the intracellular pH is an important factor of niche adaptation. A first version of the ratiometric pH sensor, pHluorin, was adapted for *C. albicans* in 2016. pHluorin is a pH-sensitive fluorescent protein which undergoes a change in excitation maximum upon acidification or alkalization, with a fixed emission maximum at 509 nm. Depending on the acidity of the cellular environment, excitation at 475 nm and 395 nm increases and decreases or *vice versa*. The ratio of the emission intensity at these two excitation peaks is used to calculate the intracellular pH using a calibration curve. Using this assay, the group of Köhler has shown that, upon administration of omeprazole to the cells, the cytosolic pH dropped due to the inhibition of the plasma membrane proton ATPase, Pma1 (Liu and Kohler 2016). Similar experiments were performed in *C. glabrata* where the pHluorin from *S. cerevisiae* proved to be functional, albeit in a tandem copy setup, to improve brightness. In these experiments, it was shown that *C. glabrata* maintains a higher intracellular pH compared to *S. cerevisiae* and that the administration of fluconazole to the cells did not affect the intracellular pH, while echinocandins and amphotericin B did (Ullah *et al.* 2013a). An enhanced version of this pH-sensitive FP is pHluorin2. This version has improved fluorescence at 37°C due to the folding enhancing mutation F64L (Mahon 2011). Attaching pHluorin2 to Cpy1 or Pga59 of *C. albicans*, localized this sensor to the vacuole or the external surface of the cell, respectively. Using this sensor, it was shown that there is a significant difference in vacuolar pH across clinical isolates and that azoles further acidify the vacuole. Contradictory, in a previous study it was shown that the cytoplasmic pH did not change upon administration of fluconazole (Tournu *et al.* 2017). Beside pHluorin, also other pH probes or dyes can be used. Many were already used in *S. cerevisiae*, such as the fluorescent proteins RaVC and pHRed and dyes Lysosensor Yellow/blue, C-fluorescein and C-SNARF-4, as reviewed elsewhere (Valkonen *et al.* 2013). Using the latter dye, of which the emission maximum wavelength changes upon altering the pH, several fungal biofilms were visualized and pH analyzed (Schlafer, Kamp and Garcia 2018).

Oxygen

Another biochemical parameter that has an influence on *C. albicans* virulence is oxygen (Grahl *et al.* 2012). The availability of oxygen is inversely linked to CO_2 in eukaryotic cells, since metabolism consumes O_2 and produces CO_2. This leads to the generation of a hypoxic environment in the direct vicinity of the cells. CO_2 has a well-known effect on the virulence of *C. albicans*, since it induces hyphae through activation of the PKA pathway (Klengel *et al.* 2005). During infection, oxygen levels drop due to metabolic activity and damage to epithelial cells. *Candida albicans* is able to withstand these low oxygen conditions and continues growing, albeit at a lower rate. Low oxygen availability also induces hyphal formation through the transcriptional regulator Ace2 (Mulhern, Logue and Butler 2006). This illustrates that, next to the pH, oxygen is another important biochemical parameter sensed by *C. albicans* to adapt to host niches. The group of Ernst developed a reporter for molecular oxygen, utilizing the oxygen-requirements of FbFPs and classical GFP-like fluorescent proteins. When cells are grown in a hypoxic environment, the chromophores of classical FPs, such as YFP, are unable to mature and are therefore not fluorescent. FbFP, however, does not require oxygen and is able to emit light under these conditions. A tandem of YFP and two copies of *Candida*-optimized FbFP form a functional FRET pair that is able to transfer energy from *Candida*-optimized FbFP to YFP under normoxic conditions, whilst no fluorescence from YFP is detected under hypoxic conditions. The presented fusion construct can thus be used as an *in vivo* oxygen sensor, termed YFOS for yeast fluorescent oxygen sensor (Eichhof and Ernst 2016).

cAMP-PKA activity

In 2018, we established the first FRET-based biosensors in *C. glabrata* (Demuyser *et al.* 2018). Based on the AKAR3 and EPAC2 biosensors developed for mammalian cells and *S. cerevisiae*, we created the *C. glabrata* variants. AKAR3 is an A-type kinase activity reporter which measures phosphorylation activity by protein kinase A (PKA). ECFP is used as donor and cpVenus as acceptor. As mentioned earlier, circular permutations generally render an FP more flexible. It was shown that certain circular permutations of Venus resulted in significantly increased FRET efficiencies (van der Krogt *et al.* 2008). The cp^{173}Venus variant was selected in this respect (Allen and Zhang 2006). Upon activation of PKA, the sensor domain is phosphorylated, bringing together donor and acceptor. EPAC2 is an intracellular cAMP sensor with ECFP-EYFP as FRET pair. Upon binding of cAMP, both FPs are set apart, leading to a reduction in FRET. For both sensors, ratiometric FRET was used to show a concomitant increase in cAMP and activation of PKA upon addition of glucose to starved *C. glabrata* cells.

Heme iron

Recently, the group of Prof Dr James Kronstad developed a heme sensor (HS1) for use in the fungus *Cryptococcus neoformans* (Bairwa *et al.* 2020). This sensor fuses the red FP, mKate2 to an EGFP in which a heme-binding domain is embedded. When heme binds the sensor, the EGFP fluorescence is quenched and a decrease in FRET signal is perceived. This sensor was also adapted for *C. albicans* where it was utilized to investigate iron homeostasis and the role of heme oxygenase in extracellular heme toxicity(Weissman *et al.* 2020). This heme sensor will enable more in-depth analysis of the function of iron and heme in azole susceptibility (Demuyser *et al.* 2017).

Outlook on biosensors

Many biosensors have been developed over the past years, although mainly in mammalian cell systems (Cui *et al.* 2019). Their applications are very diverse and can be, roughly, divided in three categories. An overview of several types of biosensors is given by Terai *et al.* 2019; Miyawaki and Niino 2015 and Skruzny *et al.* 2019 specifically for *S. cerevisiae* (Miyawaki and Niino 2015; Skruzny, Pohl and Abella 2019; Terai *et al.* 2019). Several biosensors measure the presence of small molecules, such as ions, secondary metabolites and metabolic intermediates. Especially interesting for examination of fungal virulence factors, and signal transduction thereof, would be the Ca^{2+}, Zn^{2+} and Cu^{+} sensors, as all of these ions have been shown to be involved in pathogenesis or drug resistance, as reviewed by Li *et al.* 2018 (Li *et al.* 2018). Measuring sterol levels would be of particular interest to the investigation of azole sensitivity in *Candida* species, as both have been extensively studied, yet never in a dynamic and visual manner as is possible with FRET biosensors (Song *et al.* 2015; Aron *et al.* 2016; Chauhan, Jentsch and Menon 2019). Secondary messenger cAMP, plays an essential role in the PKA pathway and related pathogenesis (Hogan and Sundstrom 2009). A cAMP biosensor, EPAC2, was developed for use in *C. glabrata* by our laboratory, although novel types exist and could be tested as well, in several *Candida* species (Surdo *et al.* 2017; Demuyser

et al. 2018; Botman, van Heerden and Teusink 2020). Nutrient sensors that are of particular interest in *Candida* metabolism, are those detecting glucose, sucrose and lactate (Höfig *et al.* 2018; Lager *et al.* 2006; San Martín *et al.* 2013; Van Ende, Wijnants and Van Dijck 2019). Trehalose and trehalose-6-phosphate have been reported to play an important role in fungal metabolism and have been put forward as potential antifungal drug targets (Arguëlles 2017). FRET-based sensors have been generated to measure levels of both sugars in mammalian cells and *S. cerevisiae* (Peroza *et al.* 2015; Kikuta *et al.* 2016). A second category of biosensors are those monitoring enzyme activity, such as the previously described AKAR3 for PKA activity (Demuyser *et al.* 2018). The options here are numerous. Biosensors have been developed for measurement of the activity of GPCRs, proteases, kinases, phosphatases, small GTPases and many others (Miyawaki and Niino 2015; Komatsu *et al.* 2018; Skruzny, Pohl and Abella 2019). Finally, a relatively recent class of FRET-based biosensors that were developed, can measure the physicochemical properties of cells, such as pH, redox state, protein crowding and tension (Oku *et al.* 2013; Morikawa *et al.* 2016; Freikamp *et al.* 2017; Burgstaller *et al.* 2019). Translating these technologies to a *Candida*-directed setup would allow for detailed investigation of several virulence attributes. As an example, FRET-based tension sensors allow for investigation of cell-cell adhesion and cell-matrix adhesion possibly interesting in biofilm formation and multispecies interactions (Freikamp *et al.* 2017).

Apart from the numerous applications of FRET-based biosensors one can think of, several advances have been made recently that aim to lift these technologies to the next level. A first one is monitoring activity of several signaling enzymes in one assay by multiplexing sensors. Lifetime detection of multiple FRET donors at the same time has allowed for simultaneous detection of PKA and ERK kinase activities in the cell (Demeautis *et al.* 2017). In FRET-BRET hybrid biosensors, advantages of both FRET and BRET sensors are combined by simply fusing a bioluminescent protein to a FRET biosensor (Komatsu *et al.* 2018). The main advantages that these hyBRET sensors owe to their BRET character are their applicability in whole-body imaging in model organisms and the ability to read out the signals in standard luminescence microplate readers. However, the HyBRET sensors generally also offer higher signal intensities compared to BRET sensors. Another option to combine FRET-based biosensors with *in vivo* imaging in a multicellular host organism, is the application of intravital imaging. In this setup, host cells, or infecting pathogens, expressing genetically engineered biosensors can be imaged in deeper layers of host tissue, thereby providing unique insights in the processes happening in the otherwise hidden places of the host body (Hirata and Kiyokawa 2016). Finally, it must be said that lately FRET-based biosensors have to compete more and more with single fluorescent protein (SP)-based sensors. Generally, these biosensors need less data analysis and allow for faster recording of the desired signal (Terai *et al.* 2019).

Do-it-yourself

Selection of FPs and linkers for PPI and biosensor assays

Optimization of E_{FRET} can be achieved by using a donor FP with a high quantum yield and an acceptor with a high extinction coefficient. Increased overlap between the donor emission and acceptor excitation spectra will also enhance the efficiency of energy transfer (Piston and Kremers 2007). A very convenient tool to select proper FPs for FRET is fpbase (www.fpbase.org/fret/), where spectra can be displayed and matched and relevant parameters can be calculated. For *Candida* species, we recommend the use of a cyan FP, such as the novel mTurquoise2 and a yellow variant, such as YemVenus. Depending on the research question, a different method for calculation of E_{FRET} should be chosen. For intermolecular FRET measurements, acceptor photobleaching, FRET-FLIM or sensitized emission FRET is preferable, while biosensors can be also monitored by ratiometric FRET. The main difference between both classes is that in intramolecular FRET, the donor and acceptor are always present in equimolecular concentrations, while this is not the case for intermolecular FRET. It is recommended to always confirm the positive FRET signal using at least two different methods.

Importantly, when using BiFC or FRET for the assessment of an interaction, one has to consider the conformation. For BiFC, the chance of reconstituting the fully functional fluorescent protein during an interaction, depends on the orientation of the non-fluorescent fragments. To decrease the chance of false negative results, a few measures have to be taken. First, both C- and N-terminal tagging combinations should be tested, bearing in mind the potential presence of cryptic signaling sites. Secondly, choosing the appropriate linker sequence is vital to allow for flexibility. Similarly, for FRET, the maximal E_{FRET} is reached when the FPs are aligned in parallel (Day and Davidson 2012). Since the orientation of these FP dipole moments can hardly be predicted, the best way to deal with this is to insert a flexible linker between the two FPs or between the protein-of-interest and the FP to allow multiple conformations. In the section on 'Imaging cellular structures', some linkers were already proposed. It was shown that, although serine residues are important for flexibility, FRET efficiency in a simple ECFP-linker-EYFP probe increases with higher Gly/Ser ratios in the linker(van Rosmalen, Krom and Merkx 2017). Other linkers, containing alternative residues, can be used as well (Kerppola 2006). Apart from the sequence, also linker length should be considered. It was shown that E_{FRET} of the ECFP-linker-EYFP probe decreased with longer linker lengths (Evers *et al.* 2006). Komatsu and coworkers also optimized their linker sequence and length, specifically for their GTPase activity FRET biosensor (Komatsu *et al.* 2011). They show that linkers with more Ser-Ala-Gly-Gly repeats make the sensor orientation-independent and lead to an increase in gain. However, implicating extremely long linkers (above 116 amino acids), did not further cause an increase in FRET, confirming the delicate equilibrium between length and flexibility. It is thus of pivotal importance to maintain adequate flexibility and protein functionality, without creating too much distance between the interacting domains. In our *C. albicans* BiFC system, a simple $(Gly\text{-}Gly\text{-}Gly\text{-}Gly\text{-}Ser)_2$ linker proved to be efficient (Subotić *et al.* 2017).

Selection of proper controls for PPI and biosensor assays

Both for BiFC and FRET analyses, it is essential to include the proper controls to exclude false positive and minimize false negative results. In BiFC, nonspecific complementation of the fluorophore fragments often happens to a certain extent. The best negative control, for both BiFC and FRET, is mutation of the interaction domain (Kerppola 2006; Subotić *et al.* 2017). It is essential, however, to verify stability of the fusion proteins under these circumstances. Only when the signal is significantly reduced in the absence of the interacting domain, it likely represents a real interaction. FRET-biosensors can also be verified using such control. For instance, one can mutate the phosphorylation site of the AKAR probe (as explained in the 'Monitoring cellular activity' section) and verify absence of FRET even under PKA activating conditions. In the case where no interacting residues are known, one can screen for mutations that decrease the BiFC or

FRET efficiency. When this is also not an option, you could assess the interaction between a protein-tag fusion and the complementary free tag, which should not produce any significant fluorescent or FRET signal (Subotić *et al.* 2017). In any case, it is important to verify the expression levels of the gene fusions as well as controls. In *C. albicans*, integration of plasmids is highly variable (Demuyser *et al.* 2017). The number of integration events should be the same for all constructs. As a positive control, a known interaction can be assessed. Specifically for FRET measurements, extra controls are necessary depending on the method used (Piston and Kremers 2007). For instance, when acceptor photobleaching is applied, one has to be certain that bleaching the acceptor does not affect donor fluorescence. Using sensitized emission, although the procedure itself is rather simple, many controls need to be tested and integrated in the calculation of E_{FRET}. The main issue here is crosstalk or bleed-through, more precisely the direct excitation of the acceptor using donor excitation light and the emission spectrum of the donor bleeding through in the acceptor emission channel. In all cases, the background effect should be taken into account when calculating E_{FRET}.

WHAT IS POSSIBLE?—SINGLE CELL PHENOMENA

Imaging of cells on a subcellular level allows researchers to investigate localization of proteins, detection of interactions and other molecular targets using fluorescent proteins. On a slightly larger scale, using fluorescent techniques to analyze complete or even many cells, researchers are able to draw conclusions about phenomena averaged over individual cells and accumulating to entire populations.

Techniques based on fluorescent proteins

Loss of heterozygosity

Candida albicans has a very dynamic and unstable genome. With genomic duplications, chromosome loss and other genomic rearrangements occurring frequently (Hickman *et al.* 2013). Loss of Heterozygosity (LOH) is one of these genomic rearrangements which appear in *in vivo* infection models and are able to render strains resistant to antifungals (Coste *et al.* 2006; Forche *et al.* 2009). A LOH reporter using BFP and GFP was developed to rapidly assess the amount of LOH events in a *Candida* population. This reporter system makes use of an additional locus that contains GFP on one chromosome and BFP on another chromosome. Fluorescence of the cells can be easily and rapidly measured using Fluorescence Activated Cell Sorting. Using appropriate gating settings, the number of cells expressing only BFP or GFP are an assessment for the amount of LOH events at that locus within the population. The combination of FACS and the fluorescent marker to assess LOH was verified using the markers that are also integrated at the locus. However, these LOH events are only measurable if they happen at the specific locus where GFP and BFP are inserted (Loll-Krippleber *et al.* 2015).

Phenotypic switching

Next to being genetically flexible, *C. albicans* is also phenotypically flexible. It is able to undergo a switch between a white and an opaque state. These cell states are visually distinguishable by the color and shape of the respective colonies on a rich nutrient medium. White cells form semi-spherical colonies, whilst opaque cells form a grey, flatter colony. This difference in phenotype can also be observed in single cells, where opaque-type cells are larger and bean-shaped compared to white-type cells (Slutsky *et al.* 1987). Using FPs as reporters for gene expression, it is possible to assess the phenotypic state of a single cell. This assay has been altered and optimized by using the next-generation FPs mNeonGreen and mScarlet placed behind the promotors of respectively *WOR4* or *WH11*. If cells are in the opaque state, they fluoresce yellow-green based on expression of mNeonGreen, whilst in the white state they express mScarlet. The proportion of cells in either state can be assessed using FACS and appropriate filter sets. Using FACS in this setting allows for a rapid analysis of the phenotypic state of individual cells, instead of scoring colonies on plates (Frazer, Hernday and Bennett 2019).

Techniques based on organic dyes

Some organic dyes provide cellular context on a subcellular level, these have been discussed in the first chapter. However, these are not the only organic dyes useful for research on the pathogens *C. albicans* or *C. glabrata*. Organic dyes can be a powerful tool when combined with specific antibodies or lectins and provide insight into single cell characteristics within a population.

Dead/Live assays

A first example of such an organic dye is fluorescein diacetate, which is a cell-permeant chemical that is broken down by intracellular esterases into fluorescein, which is a green dye that can be excited with 488-nm light. This Dead/Live assay is thus an alternative for the commercial LIVE/DEAD® *Funga*Light™ Yeast Viability Kit from ThermoFisher that was discussed earlier. Using fluorescein diacetate it was shown that a *C. albicans* biofilm treated with high concentrations of amphotericin B still contains some living persister cells, with esterase activity, within a large complex of dead cells (Wuyts, Van Dijck and Holtappels 2018). The dye was also used in a screening assay for antifungal agents against *C. albicans*, where the absence of fluorescence from fluorescein is linked to cell death. Comparison of this technique to the standard method according to CLSI, revealed that it is suitable for detecting antifungal activity of amphotericin B, miconazole and fluconazole (Brouwer *et al.* 2006). Unfortunately, it was shown that Cdr1, a multidrug transporter, is able to extrude fluorescein diacetate, thereby limiting fluorescence, even from metabolically active cells (Yang *et al.* 2001). Whilst fluorescein diacetate specifically stains living cells, propidium iodide stains dead cells. It is a red dye which undergoes a red-shift in excitation and emission upon intercalating between the bases of DNA. It is only able to penetrate the cells if their integrity is disturbed. Therefore, it has been applied in an antifungal screen for compounds that target the membrane structure and thus lead to membrane permeabilization (Menzel *et al.* 2017). Another study utilized propidium iodide to assess pheromone-induced death of *C. albicans* and showed that approximately 20% of opaque SC5314 cells die upon administration of α pheromone (Alby *et al.* 2010). Both studies harvest the power and simplicity of a simple staining with propidium iodide, counting 10 to 10^5 cells with FACS for rapid data acquisition. Even though propidium iodide is a DNA intercalator and should therefore mainly be localized to the nucleus, significant portions of the dye accumulate in the cytoplasm, which makes it unsuitable for localization microscopy.

DNA content assay

Propidium iodide is often applied in *S. cerevisiae* to assess ploidy by intercalating within the DNA. Similarly, SybrGreen is also a DNA intercalating dye that is widely used in gel electrophoresis to visualize DNA bands under blue light and can also be used to investigate ploidy. Fixed cells that are exposed overnight to SybrGreen can be analysed using FACS by excitation with 488 nm and emission captured through a 520/30-nm bandpass filter. The fluorescence intensity is related to the DNA content, with haploid strains having a mean fluorescence intensity nearly 50% lower compared to diploid strains. Using this simple assay, it was proven that *C. albicans* is not an obligate diploid and that all isolates of the novel emerging pathogen *Candida auris* are haploid (Hickman *et al.* 2013; Bravo Ruiz *et al.* 2019).

Cell wall assays

One of the most significant differences between *Candida* species and mammalian cells is the fungal cell wall. This structure consists of an outer layer of α- and β-mannans and phosphomannans, with an interwoven matrix of chitins and β-glucan underneath. A common staining method for the mannans is based on Concanavalin A linked to FITC, Texas red, Alexa Fluor dyes, etc. Concanavalin A is a lectin, thus binding to sugars, such as α- and β-mannans in the cell wall. This lectin-based staining is a common 'bread and butter' method and was recently applied to stain cells in a amphotericin B treated biofilm (Wuyts, Van Dijck and Holtappels 2018). The staining is compatible with flow cytometry allowing a rapid assessment of the amount of mannans present in the cell wall in various clinical isolates and under different treatment conditions (Warolin, Essmann and Larsen 2005). A lectin-based assay was also developed to assess the amount of β-glucan masking. This β-glucan assay utilizes the mammalian β-glucan receptor, Dectin-1 as a tool to specifically target the beta-glucans within the cell wall. Soluble Dectin-1 binds to the β-glucans after which an antibody selective for this Dectin-1 and crosslinked with a dye, such as Alexa Fluor 488 or FITC, is able to stain the β-glucan, allowing for localization microscopy or whole cell beta-glucan content analysis using flow cytometry (Graham *et al.* 2006). Using this assay, it was shown that *Candida* strains grown on L-lactate mask their β-glucan to evade the host-immune response (Ballou *et al.* 2016).

Drug efflux pump assays

Research on *Candida* species often revolves around gaining knowledge about this pathogen as a way to develop novel antifungal drugs or to understand the mechanisms in which the pathogen becomes resistant to the antifungals that are available. One of these mechanisms of antifungal resistance is to lower the amount of intracellular antifungal, by means of sequestration or actively removing the drugs using drug efflux pumps. Activity of these drug efflux pumps in *C. albicans* is similar to the pumps of multidrug-resistant tumor cells. To assess the activity of these tumor cell efflux pumps, a yellow mitochondrial dye called Rhodamine 6G has been proven effective. When administered to the cells, it is selectively pumped out of the cytosol and therefore unable to stain the mitochondria of living mammalian cells (Kutushov and Gorelik 2013). Even though Rhodamine 6G was not shown to stain the mitochondria in *C. albicans*, it can be used to assess the activity of fungal drug efflux pumps. Using this assay, it was shown that Cdr1 is able to remove Rhodamine 6G, and that a *CDR1* overexpressing strain, resistant to azoles, showed increased dye extrusion (Maesaki *et al.* 1999; Sharma *et al.* 2009). Another dye shown to be exported by the multidrug pumps Cdr1, Cdr2 and Mdr1, is Nile red. Nile red becomes highly fluorescent in a lipid-rich environment. It is usually excited using 488-nm light in a flow cytometry setup. Rhodamine 6G and Nile red are complementary drug efflux assays since Nile red is exported by all three drug efflux pumps in *C. albicans*, whilst Rhodamine 6G is not exported by Mdr1. This allows for discrimination of the mode-of-action of chemosensitizing compounds in a high-throughput screening setup (Ivnitski-Steele *et al.* 2009).

Combining multiple assays

All assays presented above use simple but clever techniques to exploit the power of fluorescent proteins and dyes to gain insights in the virulence and characteristics of *Candida* species. Each method has its limitations, upon which improvements can be made. It is clear that a combination of two assays is not always possible due to spectral overlap or other practical incompatibilities. When two dye-assays need to be combined, one can easily select a secondary antibody or lectin with alternative conjugated dyes. However, in the case of fluorescent proteins-based assays, such as the pH and oxygen sensing systems, simply choosing another fluorescent protein is not possible. Combining assays within a single strain is thus not always possible. Apart from the obvious option to evaluate the two parameters-of-interest sequentially or in parallel, one can also assess the possibility to acquire a spectral shift in one of the fluorescent proteins, for instance, by random mutagenesis. One could achieve this by error-prone or site-directed PCR on the FP. So far, most of these mutagenesis approaches expressed the mutagenized FP in *E. coli* and read out the fluorescence parameters in this organism. It would be even more relevant, however, to transform the mutagenized set of plasmids directly into *Candida* after which the transformed colonies are screened on plates or in a 96 well format. This way one integrates the possible effects of the organism and even organelle micro-environment in the assay.

WHAT IS POSSIBLE?—MULTISPECIES INTERACTIONS

Apart from visualizing molecular interactions of *C. albicans* on a subcellular level, fluorescence microscopy can also be used to detect and describe multi-species interactions. One can image the interaction between the fungus and its host organism or between the fungus and alternative micro-organisms, such as bacteria or other fungi. Both types of interactions are essential in fungal virulence and the study thereof indispensable for full understanding of pathogenesis and infection.

Host-pathogen interactions

The most relevant way of analyzing mechanisms and processes of fungal pathogens, is during infection of the host species, in many cases human beings. It is needless to say, however, what the difficulties are of such an endeavor. The use of model host organisms can be seen as a valid alternative for studying fungal infections. Fig. 3 represents an overview of model hosts which have been used for *in vivo* imaging of fungal infections. Care should be taken while choosing the model organism for your study, since not all imaging techniques can be used for all species. It goes without saying that the model organism should be relevant for the process under study. Aspects to keep in mind are body temperature, immune system, natural microbiota, etc. The host species should resemble the target host, in many cases human beings, as closely as possible. Furthermore, the animal

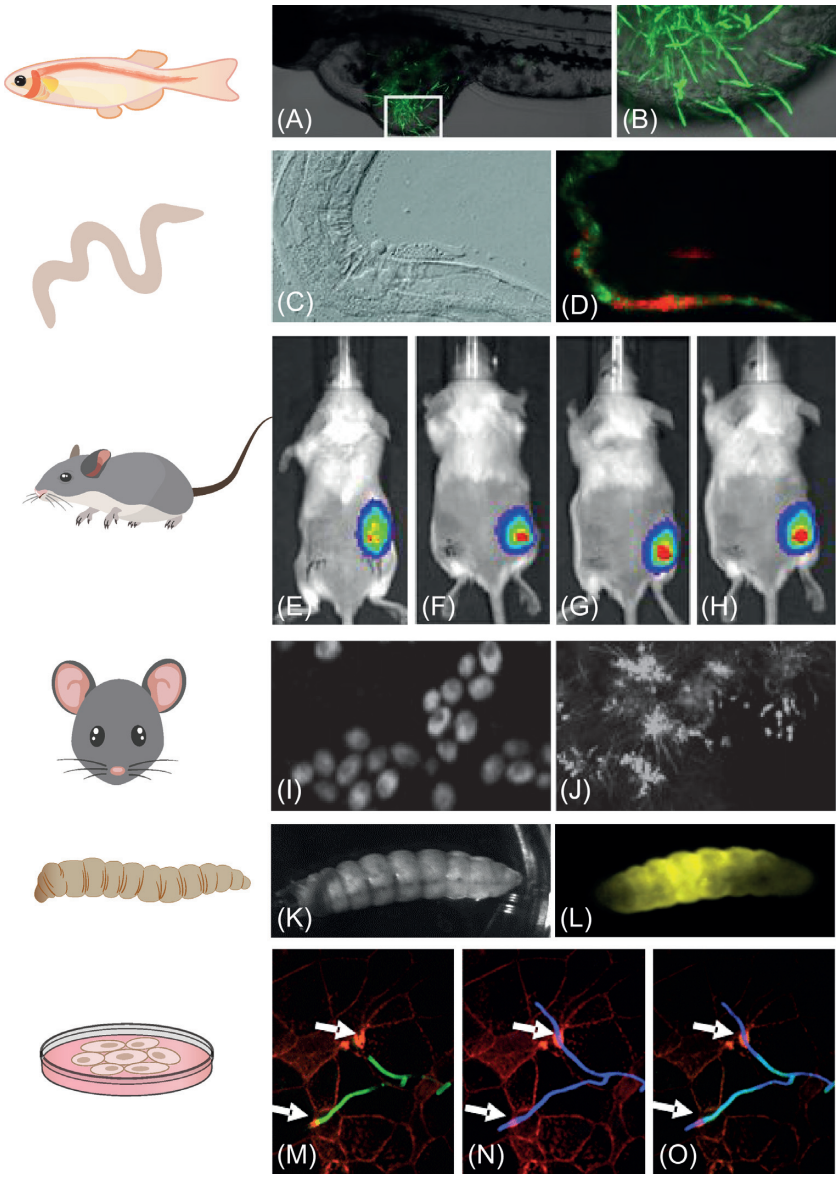

Figure 3. Exemplification of fluorescence application in fungal infections of model organisms. **A** and **B** represent yolk infection of zebrafish larvae with GFP-labelled wild type *C. albicans* cells. **C** and **D** display co-infection of *C. elegans* by *C. albicans* and *E. faecalis*. **E-H** show biofilm-related subcutaneous infection of mice with *C. glabrata*, imaged with bioluminescence over time. **I** and **J** represent images of GFP-labelled *C. albicans* intradermal infection of the mouse ear, 30 min and 24 h post infection, respectively. **K** and **L** show a *C. albicans* infection of *Galleria mellonella* using bioluminescence. **M**, **N** and **O** represent invasion of *C. albicans* hyphae in epithelial cells. Actin filaments of Caco2 cells were stained with phalloidin-Alexa Fluor 596, *C. albicans* was stained with an antibody linked to Alexa Fluor 488 before and Calcofluor White after permeabilization of the epithelial cells. More information as well as references are given in the main text.

should allow easy experimentation. Some practical aspects to consider, are ease of breading, housing and cost.

Using animal models for fluorescence imaging, however, implies some extra features to reflect on. Most importantly, the organism must be transparent for the type of excitation and emission light used during imaging. Certain biological molecules omnipresent in animal tissues, such as hemoglobin, melanin, water, collagen and nucleic acids, absorb photons in the visible and near-visible region, thereby decreasing visualization efficiency of exogenous chromophores (Bachmann *et al.* 2002). Secondly, some host molecules are highly fluorescent on their own, leading to, what is called, autofluorescence. Flavins and NAD(P)H are main causes of such native fluorescence in biological systems, yet also other molecules, such as fatty acids and proteins, emit light upon excitation at a particular wavelength (Croce and Bottiroli 2014). Lastly, biological tissue scatters light, meaning it causes diversion of photons from a straight path (Ntziachristos 2010). All these factors complicate simple and efficient imaging in animal tissues and organisms. Studying an infection in a host environment has several advantages. First, the conditions, comprising amongst others interaction with microbiota and host cells, the available nutrients and physical environment, mimic reality far closer to what is possible *in vitro*. Secondly, using imaging techniques allows the researcher to follow the same sample over time, thereby reducing variation of data which could possibly hide biologically interesting trends. Furthermore, it reduces the number of animals that is used in one study. From an ethical point of view, imaging processes in a living host rather than in postmortem tissue sections, is a great improvement. In the following section, we will

describe a number of interesting animal models that can be and are used for fluorescent imaging of fungal infections.

Zebrafish

Multiple model organisms are easy to breed, allow simple housing and come at a relatively low cost, yet of high importance for imaging are the optical properties of the animal-of-choice. Apart from few pigmented cells, zebrafish larvae of 2–3 weeks of age are completely transparent, allowing for direct imaging of fungal infections (Kimmel *et al.* 1995). While pigmentation increases gradually towards adulthood, transparent adult zebrafish can also be obtained. One can chemically treat the embryo, for instance using 1-phenyl-2-thiourea, to inhibit melanogenesis (Karlsson, von Hofsten and Olsson 2001). Important side-effects of such treatment are impairment of normal behavior and survival of the adult fish (Antinucci and Hindges 2016). Multiple pigmentation mutant zebrafish strains have been generated. The best-known example is the *casper* mutant. This strain combines two mutations, the *nacre* mutation causes absence of melanocytes and the *roy* mutation causes lack of iridophores (White *et al.* 2008). Introducing a third mutation causes, what the researchers describe as, a crystal-clear phenotype because these mutant fish also lack retinal pigmentation (Antinucci and Hindges 2016).

Because of the lack of general pigmentation, virtually any fluorophore can be used to visualize either microbial or host cells. The lab of Prof Dr Robert Wheeler is specialized in imaging fungal infections in zebrafish larvae as a model host. Simply labeling the *C. albicans* strain fluorescently allows for analysis of dissemination and morphology *in vivo* during the infection process, as exemplified in Fig. 3A and B (Brothers, Newman and Wheeler 2011; Seman *et al.* 2018). Researchers have also developed fungal reporter strains, that report on the stresses encountered in the host niche. One such reporter allows for monitoring of oxidative stress and uses a fluorescence marker placed behind an oxidative stress-induced promoter (Brothers, Newman and Wheeler 2011; Brothers *et al.* 2013). Another example is a transcriptional arginine starvation reporter, where the fluorophore is placed behind the *ARG1* or *ARG3* promoter (Jiménez-López *et al.* 2013). By using zebrafish as a model system, one can determine the virulence constituents of the pathogen as well as the host immune factors necessary for efficiently preventing or reducing infection. The innate immune system of zebrafish is very similar to that of humans. The adaptive part, which differs to a larger extent, takes longer to develop in the model organism, allowing, however, for more in depth analysis of the initial immune response upon infection (Tobin, May and Wheeler 2012). One can study phagocyte recruitment by fluorescently labeling the cells-of-interest. Very recently, it was shown that the cytolytic peptide toxin, candidalysin, which is secreted by the fungal hyphae upon infection, does not only induce cell damage, as visualized by the fluorescent nuclear SytoxOrange dye, but also triggers an immune response in the host (Moyes *et al.* 2016). Using a transgenic zebrafish line with GFP-labelled neutrophils and red dTomato-labelled fungal cells, they showed that the epidermal growth factor is necessary for efficient recruitment of immune cells to the site of infection (J. Ho *et al.* 2019). Using a switchable fluorophore, such as Kaede, allows for specifically activating a subset of immune cells and following their movement upon infection (Brothers *et al.* 2013). Obviously, these zebrafish infection models can also be used for studying non-*albicans Candida* infections (Johnson *et al.* 2018; Archambault *et al.* 2019). The team of Prof Dr Jeniel Nett showed recently that contrary to what is seen for *C. albicans*, *C. auris* recruits significantly less neutrophils to the site of infection and that no observable NETs or neutrophil extracellular traps are formed, potentially explaining to some extent the particularly severe character of *C. auris* infections (Johnson *et al.* 2018).

Apart from its transparency, zebrafish are extremely suitable for live imaging because of the ability to immobilize them for a longer period of time and obtain high resolution images. Furthermore, their small size allows for high-content imaging, which could be especially interesting for screening compounds as potential new antifungal drugs or toxicity analyses (Oldach and Zhang 2014; Martinez *et al.* 2015). There are plenty of zebrafish infection models available, each exploiting a different route of infection and resembling an alternative niche in the human host (Rosowski *et al.* 2018; Lim *et al.* 2020). The ability to visualize the entire organism allows for unbiased mapping of pathogen distribution and effects at the host level. Apart from normal fluorescence imaging, zebrafish are also suitable for alternative microscopy techniques. One example is light sheet microscopy, during which only a thin plane of the sample is excited. It also allows for imaging of the entire organism for an extended period of time, due to the relatively low phototoxicity and solid immobilization (Kaufmann *et al.* 2012; Taormina *et al.* 2012). Disadvantages of using zebrafish as a model system, are their lack of certain organs compared to humans and the absence of a proper adaptive immune system. Furthermore, these fish are best kept at a temperature between 22 and 33°C, which is significantly lower compared to the human body temperature at which most fungal pathogens thrive best (Rosowski *et al.* 2018).

Caenorhabditis elegans

Another model organism that meets the criteria of imaging, is *Caenorhabditis elegans*, a nematode worm. Apart from being very small, easy to breed and maintain, and low in cost, importantly, *C. elegans* is also transparent. Furthermore, it is easy to immobilize, using agarose, and obtain high resolution images. Similar to zebrafish, the immune system of *C. elegans* is limited to an elaborate innate part. The nematode feeds on bacteria and also infections are mainly initiated by ingestion of pathogens. Therefore, the intestinal epithelium is an essential barrier against pathogenic micro-organisms (Pukkila-Worley and Ausubel 2012). Luckily, many of the mechanisms involved in this intestinal immunity are conserved and can thus be studied, using this model organism (Aballay and Ausubel 2002). When *C. albicans* cells are fed to the worms, they are ingested and produce hyphae. This results in the disruption of tissue and eventually in the death of the animal (Pukkila-Worley *et al.* 2009). Using fluorescent reporter constructs in *C. elegans*, Pukkila-Worley *et al.* showed that certain nematode genes are specifically upregulated upon bacterial infections and others upon fungal infection, with quite some genes showing opposite expression patterns in both situations (Pukkila-Worley, Ausubel and Mylonakis 2011).

To assess effectiveness of potential antifungal compounds, *C. elegans* can be used as a model host. Using this approach, it was shown that pyridoxatin, a small compound isolated from another fungus, efficiently inhibits virulence of *C. albicans*. One can also setup a high-throughput screening system to identify promising antifungal drugs. Combining this with high-content imaging, either using a fluorescently-labelled pathogen or a live/dead staining, would allow to screen for compounds that reduce virulence and/or mortality of the pathogen (Anastassopoulou, Fuchs and Mylonakis 2011). Using such as setup, researchers found novel compounds that show promising activity in the nematode as well as murine model of candidiasis

(Breger *et al.* 2007). One aspect to keep in mind when using *C. elegans* as a model host for fluorescence analysis of fungal infections, is the relatively high autofluorescence in the blue/green region of the spectrum. Upon stress or ageing, the worm produces lipofuscin, which emits light at the same wavelength as GFP, thereby hampering visualization of weak signals (Pincus, Mazer and Slack 2016). Although the use of certain filters can overcome this issue, often red fluorophores form a safer option (Elkabti, Issi and Rao 2018; Teuscher and Ewald 2018).

Bioluminescence imaging in mice

As discussed at the beginning of this paragraph, certain molecules present in living tissue absorb light, for example melanin and hemoglobin. This is the main reason why using normal excitable fluorophores is commonly not advisable in non-transparent animals. Bioluminescence is a process by which proteins, named luciferases, produce light by oxidation of a substrate, often in the presence of O_2 and/or ATP. As there is no need for excitation, no energy is lost. However, emission light can still be absorbed by the tissue. Since hemoglobin is the major cause of photon absorption in most tissues and it mainly absorbs light below 600 nm, it is advisable to opt for luciferases that emit light above this threshold wavelength (Zhao *et al.* 2005). Multiple luciferase enzymes were already discussed in section 1.2. Bioluminescence. Here, we will specifically link the enzymes to *in vivo* experimentation.

The firefly luciferase (fLuc) originates from beetle species and needs luciferin as a substrate (Wood, Lam and McElroy 1989). ATP is also required, thereby linking bioluminescence to the physiological state of the cell. Emission of the firefly luciferase occurs at wavelengths above 600 nm, which limits absorption by biological molecules such as hemoglobin (Brock 2012). The firefly luciferase system was applied to a vaginal infection model in mice, where a proper correlation was shown with colony forming unit counts. Systemically, a significant signal could not be detected. The authors suggested several reasons for this shortcoming, such as limited access of the substrate to the infected body site, restricted light penetration and lower permeability to the substrate of hyphal cells compared to yeast cells. Researchers of the HKI in Jena completely codon-optimized the luciferase for use in *C. albicans* and the peroxisomal targeting sequence, which is known to impair emission, was removed (Jacobsen *et al.* 2014). Using the optimized gene, these researchers were able to obtain a strong signal in a systemic infection by *C. albicans*. Upon infection of mice via the lateral tail vein, bioluminescent patches became visible in the kidneys, the brain and urinary bladder. Interestingly, using this platform, it became appreciable that, upon treatment of a systemic infection with caspofungin, some fungal cells persisted in the gall bladder, functioning here as a cryptic host niche. This example shows how imaging-based research can offer more insights than originally envisaged in the research question. Recently, the firefly luciferase was further improved (Dorsaz, Coste and Sanglard 2017). Researchers adapted the codon-optimized version (Jacobsen *et al.* 2014) and mutated certain residues to obtain a shift towards the red side of the spectrum and improve thermostability and enhanced light emission. The resulting luciferase showed improved sensitivity of *C. albicans* detection in *in vitro* and *in vivo* setups.

Another relevant luciferase enzyme originates from the sea pansy, *Gaussia princeps*—gLuc, which requires coelenterazine as a substrate. Notably, the emission of light is independent of ATP (Brock 2012). Drawbacks are the very poor distribution of the substrate in living organisms and the auto-oxidation that the substrate tends to undergo, leading to a high background signal. Furthermore, the emission at wavelengths below 600 nm causes high absorbance. Remarkably, the luciferase enzyme is secreted, which allows for improved accessibility of the substrate, yet limits its localization of the fungal cells. To circumvent this limitation, other researchers adapted the luciferase for it to become attached to the fungal cell wall and thus more readily available for its substrate in both yeast and hyphal forms (Enjalbert *et al.* 2009). The applicability of this system in imaging of disease progression, was shown in a murine model of vaginal candidiasis where effectivity of a β-glucan-conjugate vaccine was confirmed (Pietrella *et al.* 2010). Although the system proved fit for use in a mouse model of oropharyngeal candidiasis as well, systemic infections still appeared impossible to image (Mosci *et al.* 2013; Gabrielli *et al.* 2015).

Both the firefly and the *Gaussia princeps* luciferase have advantages and shortcomings. While the optimized firefly luciferase is optimal for systemic and deep-seeded infections, the *Gaussia princeps* luciferase can be used for superficial infections. Colleagues from our laboratory have used the latter luciferase to establish and optimize a platform to visualize biofilm-related *C. albicans* infections in a subcutaneous mouse model (Vande Velde *et al.* 2014; Kucharíková *et al.* 2015). The system outperforms the standard methods used for assessing biofilm biomass as it allows to monitor the fungal burden from the start of the experiment onwards. It was shown that the established platform enables efficient evaluation of antifungal therapies over a long period of time, both *in vitro* and *in vivo* (Vande Velde *et al.* 2018). Very recently, we have established a similar system for monitoring *C. glabrata* biofilm infections, using an optimized firefly luciferase (Fig. 3E, F, G and H) (Persyn *et al.* 2019). Bioluminescence imaging is not restricted to rodents. In 2015, a *C. albicans* strain expressing the optimized firefly luciferase was used in the mini host *Galleria mellonella* (Fig. 3K and L) (Delarze *et al.* 2015). This model organism, although quite rudimentary in anatomy, has a number of advantages over rodent model systems. It is small, cheap and experimentation is ethically less regulated. This makes it better suited for high-throughput screening setups. The researchers also successfully used a novel, water-soluble, formulation of coelenterazine, which is not toxic and thus allows for *in vivo* kinetic studies of the infection.

Alternative imaging options for mice

Contrary to luciferases, standard fluorophores need to be excited by light. This limits their use in *in vivo* model systems because of high absorption of light by animal tissue. Two ways to circumvent this issue, are to image alternative host areas or to use fluorophores that are excited at wavelengths that are not readily absorbed by tissue. In 2010, Mitra *et al.* published an alternative *in vivo* model system in which GFP-tagged *C. albicans* cells can be imaged during a systemic infection in mice (Mitra *et al.* 2010). Injecting a fungal suspension intradermally into mouse ears, allowed to visualize the fungal cells at high resolution at this location by normal confocal microscopy (Fig. 3I and J). Infection of the kidneys, as determined post-mortally, confirmed the systemic character of the infection. The second option to use standard excitable fluorophores in *in vivo* non-transparent model systems, is to use FPs with a longer excitation wavelength. One such FP has already been presented when discussing the possibilities of working in the transparent zebrafish larvae, namely iRFP670. This FP is excited at 643 nm and emits at 670 nm (Shcherbakova and Verkhusha 2013). Light of such high wavelength is only minimally absorbed by hemoglobin and melanin.

Furthermore, this spectral area is interesting due to its low light scattering and autofluorescence properties. Shcherbakova *et al.* show that injection of iRFP670-expressing tumors caused remarkable fluorescence in the intact animal (Shcherbakova and Verkhusha 2013). Using such labels for fungal pathogens could possibly also yield satisfactory results and would, most interestingly, omit the need for addition of exogenous substrates.

Novel in-host imaging techniques

Intravital microscopy (IVM) is an interesting novel tool that holds the promise of generating immense progress in the field of fungal pathogenesis. Using this technology, cell biology can be imaged in the living host at a relatively high resolution (Weigert *et al.* 2010). An image window is implanted in the tissue- or organ-of-interest, allowing for direct and real-time monitoring of cellular processes. Several advances made in the field of microscopy, such as two- and three-photon imaging, have improved the technology in terms of imaging depth and resolution. Due to limitations of intravital microscopy, fibered confocal fluorescence microscopy or FCFM provides some particular advantages (Choi, Kwok and Yun 2015). In this technique, the objective is replaced by a bundle of flexible, optical fibers, which can penetrate the tissue further, compared to what is possible with IVM. This technique has been used to image fungal infection of host organisms, for instance of pulmonary infections, caused by *Aspergillus* or *Cryptococcus* species (Vanherp *et al.* 2018).

As emphasized in a recent review on the matter, techniques combining whole-body imaging with a decent cellular resolution are still largely lacking from the mycologists' toolbox (Van Dyck *et al.* 2020). Some promising technologies are, however, ready to be translated to fungal research. Selective plane illumination microscopy (SPIM), also referred to as light sheet microscopy, allows for imaging of larger, mesoscopic, samples, such as small organisms, organs or tissues with an intermediate to high resolution. Using a sheet of excitation light, planar illumination of a focal plane leads to optical sectioning of the 3D sample. Since only a thin plane of the sample is illuminated, the phototoxicity and background fluorescence is minimized. The immense potential of SPIM in host-pathogen imaging was recently illustrated by Amich *et al.* They used this technique to investigate the interaction between *Aspergillus fumigatus* and host immune cells in whole mouse lungs (Amich *et al.* 2020). The unique combination of whole-body analysis with high resolution imaging, yielded information on the spatial distribution patterns of immune cell populations in response to fungal infection. Alternative to SPIM, one can also use fluorescence optical projection tomography (OPT). In this technique, fluorescent images are captured while rotating the sample over an axis, generating multiple angular projections (McGinty *et al.* 2008). The resolution of the latter is limited in larger samples, yet it can work with fluorescent as well as non-fluorescent contrasts (A. Liu *et al.* 2019). Combining both SPIM and OPT in a hybrid technique, called OPTiSPIM, allows for fluorescent and non-fluorescent, high-resolution analysis of mesoscopic samples (Mayer *et al.* 2014). Both OPT and OPTiSPIM, have been used mainly in developmental biology with few examples of applications in infection biology (Ohtani *et al.* 2014; Schmidt *et al.* 2017).

Imaging microbe interaction with host cells

Working with living animals, although being most relevant, is not always easy nor possible in any laboratory context. Alternatively, one can examine the interaction between fungal pathogens and host cells or tissues in 2D or 3D setups. Here as well, fluorescence microscopy can help in visualizing the multi-species interaction and its characteristics.

Several cell types can come into contact with fungal pathogens, depending on the type and site of the infection. With alternative types of host cells, the type of interaction differs as well. When *C. albicans* colonizes the oral, vaginal epithelium or enterocytes, damage is induced by active penetration or induced endocytosis of host cells by the hyphae. To visualize the invasion process, researchers typically stain the fungal cells before and after permeabilization of the human cells. Staining the fungal cells when the host cells are still intact, will result in the visualization of the extracellular parts of the hyphae. Staining again, with a different dye, after permeabilization of the human cells, will result in visualization of the entire fungal cell (Dalle *et al.* 2010; Wächtler *et al.* 2012; Yang *et al.* 2018). As a primary stain, Alexa Fluor 488 can be used, fused to Concanavalin A, which will stain the fungal cell wall. As a secondary stain, Calcofluor White can be used, also staining the cell wall (Fig. 3M, N and O). Using this setup, researchers have confirmed that actin filaments of the epithelial cells reorganize at the point where fungal hyphae enter by induced endocytosis (Park *et al.* 2005; Dalle *et al.* 2010; Wöllert *et al.* 2012; Maza *et al.* 2017). Another possibility is to use immunocytochemistry with antibodies targeting *C. albicans* and fused (either directly or via a secondary antibody) to a fluorescent dye (Falgier *et al.* 2011). Instead of permeabilizing the host cells and applying a second staining, detailed analysis of differential interference contrast (DIC) images is also possible. To improve relevance of mammalian cell cultures, it is possible to use them in a transwell setup. Here, one can differentiate between both apical and basolateral sides of a host cell type. Different medium and supplements can be supplied, different microbes can be added, etc (Graf *et al.* 2019).

Another interaction model of interest for fungal infections, is one where fungal cells encounter immune cells, especially phagocytes. These cells can take up the fungal cells through phagocytosis. In order to visualize this process, researchers have established a system based on differential staining of internalized versus extracellular yeast cells (Carneiro *et al.* 2014). First, yeast cells are stained with SytoxGreen, a nuclear stain. After potential uptake by the phagocytes, another stain, propidium iodide, is used to quench the SytoxGreen stain. However, only those cells that are not taken up by the immune cells will be prone to this quenching. Eventually, cells that were taken up in the phagocytes will remain green while the extracellular ones will turn red. This differential staining can be used to visualize the cells using a microscope but can also allow quantitative analysis by flow cytometry. Researchers of the lab of Prof Dr Robin May have similarly used flow cytometry to investigate interaction of GFP-tagged *Cryptococcus* species with macrophages in an automated fashion (Voelz *et al.* 2010). Similar setups have also been used, employing different stains or genetic fluorescent markers. Examples of stains that can be used for yeast cells are FITC and Calcofluor White (Keppler-Ross *et al.* 2010; Dementhon, El-Kirat-Chatel and Noël 2012). Staining the host cells, for instance using calcein, which is an indicator of metabolism and membrane integrity, or a labelled antibody can be used to allow visualization of adhesion and interaction with the fungal cells (Dementhon, El-Kirat-Chatel and Noël 2012; de Turris *et al.* 2015). To overcome the resolution barrier set by normal fluorescence microscopy, researchers from Ghent University have established correlated fluorescence-atomic force microscopy (El-Kirat-Chatel and Dufrene 2012).

Taking advantage of both worlds, this technique allows visualization of both interacting partners while still acquiring information about nanostructures of relevance to the interaction.

Apart from working with monolayers of mammalian cells, researchers can also investigate the interaction between microbes and host cells using *ex vivo* or *in vitro* 3D model systems, as such adding information and complexity to the interaction model. Different niches where fungal infections occur, can be mimicked. An example of how *ex vivo* systems can prove effective in studying a fungal infection is given by Wendland *et al*. They obtained a piece of the intestinal epithelium of female pigs and inoculated them with a *C. albicans* suspension. The surface layer where interaction had taken place, was scraped off and visualized using staining with Calcofluor White (Wendland *et al*. 2006). Similarly, ear skin from pigs was used to investigate effectivity of treatment against a mixed *C. albicans*—*Staphylococcus aureus* infection (Nair *et al*. 2016). In a model of teeth infections, premolar teeth extracted from humans were infected with *C. albicans*. After treatment, fungal viability was investigated using fluorescein diacetate (Gowri *et al*. 2016). Other human or animal materials that were already used for *ex vivo* investigation of *Candida* infection are saliva, tongues, oral or vaginal mucosa, corneas, blood, skin, etc. (Shin *et al*. 2013). Alternatively, it would also be possible to use organoid models. Organoids are self-organizing, 3D cultures of human or animal cells (Kim, Koo and Knoblich 2020). They are typically generated from pluripotent or adult stem cells and form mini organs mimicking the actual organ system in the host. A great advantage organoid-based infection models have, is the option to investigate patient-specific conditions, as the organoids can be generated from patient stem cells. Different conditions can be applied inside and outside of the organoid and also separate microbiomes can be established. As far as we know, no organoid systems have been used for *Candida* infections. On the contrary, various infections with bacteria have been investigated, such as *Samonella*, *Helicobacter*, etc. as reviewed by Bartfeld (Bartfeld 2016). Finally, to take one more step towards a fully relevant model system, it is possible to use organ-on-chip systems. In these devices, it is possible to integrate various tissue and cell types and interactions, flow of fluids, such as blood, and mechanical aspects into one compact system. In such a model for infection, one can assess evolution of the pathogen, the response of the host as well as heterogeneity of both (Tang *et al*. 2020). In this respect, many bacterial as well as viral infections have been investigated, while fungal infections are severally underrepresented (Bein *et al*. 2018). Maurer and coworkers used an intestine-on-chip model to show how *Lactobacillus rhamnosus* reduces tissue damage and translocation by *C. albicans* in the gut (Maurer *et al*. 2019). The whole process can be beautifully visualized by differential fluorescent tagging of all interaction partners.

Taken together, it can be acknowledged that many relevant *ex vivo* and *in vitro* systems exist that can replace animal models. Although the complexity of these systems will always be lower compared to an actual animal, the option to integrate human components and even personalized parts into these systems holds great promise for advancing infection research. As mentioned earlier, important aspects to keep in mind when visualizing thicker organic samples using fluorescence microscopy, are autofluorescence, transparency of the tissue and scattering of light. In some cases, it is more desirable to use a specific imaging setup such as intravital microscopy or appropriate fluorophores, such as infrared markers.

Microbe-microbe interactions

Apart from host-microbe interactions, fluorescently labeling microbes can also aid in elucidating relevance and mechanisms of microbe-microbe interactions. As microbes inhabit almost all niches in our body, it is fair to state that the interaction between pathogens and the rest of the microbiome is likely of utmost importance in determination of disease outcome. Recently, the Wheeler lab described how *C. albicans* and the bacterium *Pseudomonas aeruginosa*, enhance virulence compared to single-species infections, using the zebrafish larvae as host system (Bergeron *et al*. 2017). The main site of infection of *P. aeruginosa* is the lungs. Using zebrafish as a model system, the infection route showing the highest degree of homology is the swimbladder. By labelling the bacterium with a red fluorophore, dTomato, and the fungus with a far-red FP, iRFP670, these researchers were able to visualize how both species synergistically enhance virulence of one another, as well as mortality. Using a transgenic fish line with EGFP-labelled neutrophils allowed for concomitant visualization of immune cell recruitment upon mixed species infection. Multispecies interactions can also be studied using *C. elegans* as a model system. By using a *C. albicans* strain that constitutively expresses GFP, researchers visually showed that the pathogenic bacterium *Acinetobacter baumannii* reduces filamentation of the fungus and concomitant killing of the host organism to a large extent (Peleg *et al*. 2008). Similar research shows the same negative relation is true for *C. albicans* and the opportunistic intestinal pathogen *Enterococcus faecalis* (Cruz *et al*. 2013). In the latter case, an mCherry-expressing *C. albicans* strain and GFP-expressing *E. faecalis* strain were used, as shown in Fig. 3C and D.

Multiple microbial species often colocalize in biofilms. These are 3D-structures of microbial communities, where several micro-organisms can survive in the presence of an extracellular matrix. Whether pathogens, such as *C. albicans*, benefit from these interactions can be studied using fluorescence imaging. To label the different partners in a biofilm, one can use different methods. Apart from expression of a genetic label and using a chemical dye, another option is immunofluorescence, in which microbe-specific antibodies tagged with a fluorescent dye, such as FITC, are used. Further, FISH or fluorescence *in situ* hybridization makes use of fluorescent probes that specifically target a certain region in the species-specific DNA or RNA. Using combinations of several of these labels allows for visualization of integrative processes including multiple organisms. Diaz *et al*. showed that *C. albicans* and the bacterium *Streptococcus oralis* act synergistically in an *in vitro* model of the oral mucosa (Diaz *et al*. 2012). *Candida* cells were labelled by an antibody fused to FITC, *S. oralis* was labelled using a *Streptococcus*-specific FISH probe and the epithelial cells were stained using the Hoechst 33258 nuclear stain. Using the same tools, the researchers also showed the important role of glucosyltransferase in the biofilm-related interaction between *Streptococcus gordonii* and *C. albicans* (Ricker, Vickerman and Dongari-Bagtzoglou 2014). Apart from 2D images, also 3D models of biofilm structures can be generated. This is especially interesting for analysis of biofilm architecture and location-specific composition.

WHAT IS POSSIBLE?—DIAGNOSIS

One of the main hurdles in efficient treatment of fungal infections, is accurate and rapid detection of the infecting pathogen. Many of the procedures used today are still very time-consuming and not adequately sensitive or specific, which in

turn leads to incorrect or inefficient treatment of the infected patient. The golden standard in clinical diagnostics of invasive candidiasis remains blood culture. Disadvantages are the low sensitivity and the long time needed to detect growth, on average 37 h (Fernandez *et al.* 2009). Furthermore, after the incubation period, the infecting species still needs to be identified, causing again a delay in the treatment procedure ranging from a few hours to several days (Ibáñez-Martínez, Ruiz-Gaitán and Pemán-García 2017). Standard identification procedures are based on carbohydrate assimilation or fermentation reactions, growth on chromogenic culture media, such as CHROMagar, induction and observation of hyphae formation, etc. Lately, the use of matrix assisted laser desorption/ionization—time of flight (MALDI-ToF) mass spectrometry after cultivation has gained popularity to identify fungi in a clinical setting and even to test drug susceptibility (Peng *et al.* 2019). These protocols typically take several days to be completed (El-Kholy *et al.* 2015). The most primitive way of detecting fungal cells in an infection using fluorescence, is by staining and macroscopic or microscopic evaluation. Using an aniline blue dye in the medium, researchers were able to differentiate between several *Candida* species and other fungi based on macroscopic observation of fluorescence of colonies under UV light irradiation (Goldschmidt *et al.* 1991). A major disadvantage, again, is the time required for growth of the *Candida* cells on this modified medium. In this respect, microscopic observation of fluorescent cells is a better alternative. Calcofluor White staining has been used most often. Slight differences in fluorescence pattern between different *Candida* species can be observed, with *C. albicans* showing the most distinctive pattern, i.e. hyphae and pseudohyphae (Safavieh *et al.* 2017; Yao *et al.* 2019). Other fluorescent whiteners, such as blankophor, can be used as well (Hamer, Moore and Denning 2006). Finally, since *Candida* species are known to be highly autofluorescent, this could also be used as a direct readout, without the need of staining (Graus, Neumann and Timlin 2017).

FISH

More recently, the use of alternative fluorescence techniques, has pushed the limits of these standard practices in terms of timing (from days to hours), sensitivity and specificity. FISH is used to detect the ribosomal RNA (rRNA, such as 18S or 26S rRNA) of a certain species by attachment of a fluorophore, such as FITC, to the sequence-specific probe, followed by fluorescence detection. In standard FISH, the probe backbone consists of a basic DNA phosphate ring structure. However, the intensity of these positive signals is often very low, due to electrostatic repulsion between the probe and target sequence. By replacing the DNA backbone by a non-charged polyamide backbone, the hybridization can be tighter and of higher specificity, causing higher intensity signals (Oliveira, Almeida and Azevedo 2020). Peptide nucleic acid fluorescent in situ hybridization or PNA FISH thus represents a promising alternative to the golden standard detection techniques (Rigby *et al.* 2002). Several studies have confirmed that this technique can indeed point out that an alternative therapy strategy is needed. Alexander *et al.* and Forrest *et al.* both show that PNA FISH was 100% sensitive and specific in the rapid identification of *C. albicans* in blood samples (Alexander *et al.* 2006; Forrest *et al.* 2006). In case *C. albicans* was detected, caspofungin treatment was replaced by fluconazole therapy, as this is the drug of choice for these infections. Due to this fast and reliable identification of the species type, the usage of caspofungin could be significantly reduced, along with the accompanying costs per patient. Since the initial description of PNA FISH in *C. albicans* in 2002, several adaptations were made. A second-generation two-color assay detects *C. albicans* as green fluorescence (FITC) and *C. glabrata* as red (Texas Red) allowing for simultaneous detection of both species. The commercially available Yeast Traffic Light system (AdvanDx, Woburn, MA), can be used to detect *C. albicans* and *C. parapsilosis* as green, *C. glabrata* and *C. krusei* as red and *C. tropicalis* as yellow (Fig. 4) (Radic *et al.* 2016).

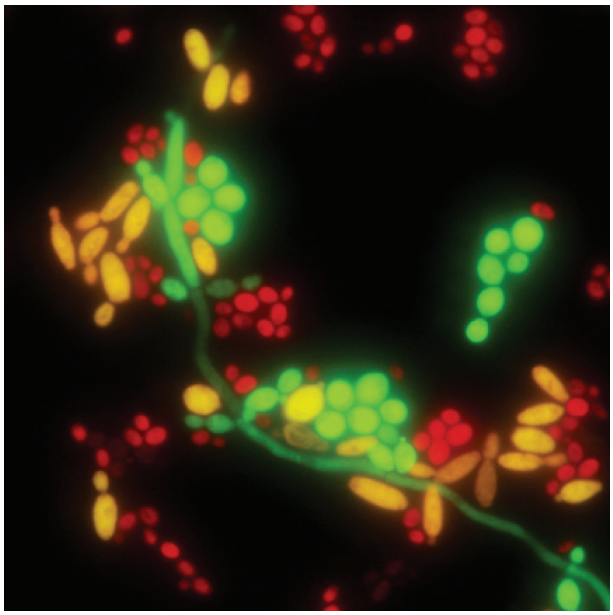

Figure 4. Identification of *Candida* species using PNA-FISH. The commercially available Yeast Traffic Light system (AdvanDx, OpGen, Woburn, MA), can be used to detect *C. albicans* and *C. parapsilosis* as green, *C. glabrata* and *C. krusei* as red and *C. tropicalis* as yellow.

qRT-PCR

Other diagnostic tools for fungal infections, are based on the detection and amplification of nucleic acids. Although these tests are not widely used in the clinic yet, they offer a substantial set of advantages over the currently-used culture-based diagnostics. The amplification of the target nucleic acid sequence can happen in an isothermal or non-isothermal fashion, with an example of the latter being PCR (Safavieh *et al.* 2017). The readout of the amplified nucleic acid sequence is often based on fluorescent labeling. A recently developed diagnostic tool that uses fluorescence as a readout, is quantitative, real-time PCR or qRT-PCR, where pathogenic DNA or RNA is amplified and fluorescently detected. One of the major advantages of direct pathogen detection in patient samples is the high sensitivity and the absence of a culturing step. Whereas normal PCR suffers from high levels of false positives, qRT-PCR is much more reliable (Bretagne and Costa 2005). A fully automated platform of extraction and detection could significantly decrease the processing time needed to obtain results. Several systems for the rapid identification of *C. albicans* by real-time PCR have been generated, with some of them allowing for simultaneous probing of several fungal as well as bacterial species in multiplex systems (Horváth *et al.* 2013). Most of the systems make use of the difference in melting temperatures of sequence-specific probes, targeting for instance ITS regions, or free dyes, such as SYBR Green, or combinations of both (Horváth *et al.* 2013).

SYBR Green is a commonly used fluorescent dye that intercalates between the DNA double helix. The readout of amplification by sequence-specific probes can be based on FRET, with excitation of the donor probe transferring energy to the acceptor probe when bound to the adjacent nucleic acid sequences. Another option is a Taqman probe, where a specific nucleic acid sequence links together a fluorophore and a quencher molecule. Once the DNA or RNA is amplified, both molecules are separated upon which the fluorophore emits light. Researchers have established systems for specific and simultaneous detection of multiple clinically-relevant *Candida* species, such as *C. albicans*, *C. glabrata*, *C. dubliniensis*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, *C. lusitaniae*, *C. guilliermondii* and importantly *C. auris*, as well as some relevant bacterial species (Horváth *et al.* 2013; Ashrafi *et al.* 2015; Asadzadeh *et al.* 2018; Arastehfar *et al.* 2019; Nabili *et al.* 2013; Zhang *et al.* 2016). Apart from real-time detection, also DNA microarrays take advantage of a fluorescent readout. Thousands of potentially complementary sequences are attached to a surface and probed with a fluorescently labelled query sequence (Campa *et al.* 2008). Upon hybridization, the fluorescent pattern is visualized and analyzed for species specificity. Finally, another option to analyze amplification of pathogen-specific DNA or RNA, is through capillary electrophoresis-laser-induced fluorescence. Using fluorescently labelled primers, variability in length of, for instance, the ITS rRNA can be monitored during capillary electrophoresis (Obručová *et al.* 2016). A major disadvantage of using qRT-PCR or other nucleic acid-based methods for detection of pathogens, is the false-positive results that can be yielded from dead pathogens. As DNA is stable over a long period of time, a positive result in the assay does not necessarily mean that the pathogen is alive or viable (Keer and Birch 2003; Artz *et al.* 2006). In this sense, it is preferable to detect mRNA as the stability significantly less compared to DNA. Apart from fluorescence-based monitoring of *Candida* infection, obviously also other techniques can be used, such as nuclear magnetic resonance, gas-liquid chromatography, etc. (Safavieh *et al.* 2017). These are, however, not in the scope of this review.

CHALLENGES

Using fluorescence as a tool in research always imposes certain challenges. The success of simply tagging a protein-of-interest with a fluorophore depends on several factors, such as the type of fluorophore and whether it is the best option in these specific cellular conditions, the linker type and length, the effect of the tagging on protein function, the method used to monitor fluorescence, etc. No matter which research questions need to be answered or which tool is used to do so, some extent of optimization and quality control is always needed. Yet, apart from the general challenges that apply to fluorescence measurements in any given organism, certain additional obstacles are specific to working with *Candida* species. These complications can be subdivided in two categories, namely those related to the proper expression of fluorescent proteins or probes, and those related to fluorescence analysis.

Expression of FPs and probes

Candida albicans belongs to the *Saccharomycetaceae* family and more particularly to the CTG clade, which represents a subgroup in this family (McManus and Coleman 2014). Members of this clade frequently translate the CTG codon as a serine instead of a leucine, due to the recognition of the codon by an unorthodox seryl-tRNA with 5'-CAG-3' anticodon (Ohama *et al.* 1993). Similar to *C. albicans*, most of the other clinically-relevant *Candida* species, such as *C. auris*, *C. parapsilosis*, *C. tropicalis*, *C. dubliniensis*, *C. guilliermondii* and *C. lusitaniae* belong the CTG clade, meaning that they all have an aberrant codon usage. It is reasonable to state that this slows down genetic manipulation and general experimentation in these fungi to a great extent. Most of the established tools or markers that are available for *S. cerevisiae* or mammalian systems, cannot be used directly in *C. albicans* or any of the other CTG clade members. In 1998, it was shown that presence of only one CTG codon was enough to completely inhibit translation of the GFP mRNA in *C. albicans*, indicating that codon optimization is indispensable (Morschhäuser, Michel and Hacker 1998). Even in case translation is permitted, presence of a CTG codon can lead to structural and thus functional variation in the protein (Cutfield, Sullivan and Cutfield 2000; Feketová *et al.* 2010; Miranda *et al.* 2013). Codon optimization is indeed essential in many cases. Some *C. albicans* optimized fluorescent proteins have been generated, in which the CTG codon has been replaced. Apart from specific CTG adaptations to the fluorescent protein, also optimization based on codon usage or preference has been performed and proved to further enhance expression and fluorescence intensity (Cormack *et al.* 1997; Gerami-Nejad, Berman and Gale 2001; Van Genechten *et al.* 2020). Other than *C. albicans*, *C. glabrata*, *C. krusei* and *C. kefyr* do not belong to the CTG clade and are more related to *S. cerevisiae*. Translation of established technologies has shown to be more straightforward in these species (Zordan *et al.* 2013; Ho and Haynes 2015; Demuyser *et al.* 2018). However, apart from aberrant codon usage by some species, many yeast species have AT-rich and highly plastic genomes, which also tend to complicate genetic manipulation (Massey *et al.* 2003). Translocations, truncations and ploidy changes are commonly observed. These processes allow for adaptation to new niches and contexts (Selmecki, Forche and Berman 2010). One practical implication is that integration of a plasmid in the genome of *C. albicans* happens at a highly variable frequency. In order to use *Candida* overexpression strains in research and compare expression or fluorescence intensity of tagged proteins, one should thus analyze the number of plasmid integration events that happened in each of the overexpression transformants (Demuyser *et al.* 2017; Demuyser *et al.* 2020; Van Genechten *et al.* 2020). This can simply be done by qPCR analysis on genomic DNA, using primers in the promoter region of the plasmid. Additionally, overexpression strains induce artifacts such as accumulation of the FP in the ER, vacuole and other terminal membranes, leading to misinterpretation of the localization results (Moore and Murphy 2009).

Fluorescence analysis

Apart from challenges in proper expression of the tagged construct, a few other peculiarities specific to one or more *Candida* species, are worth considering before starting fluorescence experimentation.

Keeping those cells from moving

When you want to monitor alterations in cellular fluorescence intensity over time, for instance upon addition of an inducer or inhibitor, time-lapse experiments are possible. Most types of microscopes allow for continuous imaging, either through a camera or programmed image acquisition. One of the main obstacles when performing time-lapse experiments, is the constant movement of cells. Cells often move around when in liquid

and show aberrant fluorescence when left too long, as the liquid dries out. There are several options to immobilize cells for a longer period of time. First, one can opt to coat the microscopy slide or dish with Concanavalin A or polylysin. However, adding a compound or nutrient to the cells often changes the focus substantially, which is detrimental in a time-lapse setup. As an alternative, one can use a microfluidic system where the cells can be immobilized by physical restraining (Demuyser *et al.* 2018). The CellASIC® ONIX2 microfluidics device from Merck uses plates with microfluidic chambers where the cells are immobilized by an elastic ceiling. Different plates are available, designed for various organisms and cell types. For persistent focus shift, another option is to use a microscope with autofocus function, in which an internally reflected infrared laser automatically corrects the focus over a longer period of time (Bathe-Peters, Annibale and Lohse 2018).

Autofluorescence

It has been shown by other research groups as well as ours, that *C. albicans* produces a yellow pigment, termed riboflavin or vitamin B_2 (Bai *et al.* 2011; Demuyser *et al.* 2020). Apart from its intense yellow color, this vitamin possesses the unique quality of fluorescence. Excitation is maximal at 450 nm with a smaller peek visible at 370 nm. The emission spectrum reaches its maximum at 530 nm. Importantly, many FPs are excited with light of the same range as riboflavin or emit light around the same maximum. As an example, the ECFP-EYFP FRET pair is excited at 434 nm and emits light at 477 and 527 nm. This indicates that extreme care should be taken when using these fluorophores in certain situations. We show that riboflavin production is augmented in *C. albicans* when PKA activity increases as well as in iron-limiting conditions (Demuyser *et al.* 2020). Using cyan and yellow FPs under these particular conditions might lead to artefacts in the signal due to riboflavin production. Proper controls should be analyzed to avoid misinterpretation of the signals. Other factors can cause autofluorescence in fungal cells as well, such as tryptophan metabolism or melanin production (Chaskes and Phillips 1974; Morris-Jones *et al.* 2005). In some cases, unique autofluorescence spectra by different *Candida* species can even be exploited in combination with hyperspectral fluorescence microscopy to diagnose the causing agent in infections (Graus, Neumann and Timlin 2017).

During an infection, fungal cells experience all sorts of stresses. It is therefore relevant to study stress response in the lab as well. Application of stress, however, often leads to increased autofluorescence levels and thus a decreased signal-to-noise ratio. This does not pose a problem when organic dyes are used, since they have high excitation coefficients. Yet, the *in vivo* brightness of fluorescent proteins is often much lower and the signal can be 'lost' within the elevated autofluorescent background. As most of the autofluorescent signal originating from riboflavin is excited with blue light and emits in the green channel, it is recommended to use a blue-shifted or red-shifted FP when performing experiments that could potentially increase autofluorescence levels.

Phototoxicity

Laser light, used in fluorescence experiments, can also cause phototoxicity in living cells (Icha *et al.* 2017). Excitation light is absorbed by organic molecules, such as flavins and porphyrins, which become degraded when reacting with oxygen, resulting in the production of reactive oxygen species (ROS). These damage the cell by oxidation of DNA, proteins and fatty acids, possibly leading to growth arrest or even cell death. Similarly, excited fluorophores can react with oxygen, upon which they bleach. The ROS resulting from this photobleaching likewise cause toxicity in the cells. One option to reduce phototoxicity, is to omit riboflavin or pyridoxal from the culture medium (Icha *et al.* 2017). LoFlo medium by Formedium is an example of such medium lacking folic acid and riboflavin. Such medium has been used in research on *C. albicans* fluorescence (Van Genechten *et al.* 2020). Otherwise, antioxidants or ROS scavengers can be added to the medium to minimize cellular ROS levels (Stockley *et al.* 2017). Using a lower illumination intensity or pulsed illumination will also reduce phototoxicity effects. Alternatively, selectively illuminating the focal plane by light sheet microscopy, TIRF microscopy or 2-photon microscopy or using excitation light with a longer wavelength is also possible (Icha *et al.* 2017). The sensitivity of *C. albicans* to phototoxicity has been exploited in treatment strategies. Such photodynamic therapy entails the addition of certain photosensitizers, or non-toxic dyes, which produce ROS under illumination with a harmless visible light. Specifically targeting the dyes to pathogenic cells allow for precise eradication of the infection without much chance of triggering resistance (Dougherty 2002; Dai *et al.* 2012). In the past, the unwanted toxicity of many compounds, such as cosmetics, have even been tested using *C. albicans* as a model organism, indicating its sensitivity to phototoxicity (Knudsen 1985; Faergemann and Larkö 1988; Horikawa and Miura 1988).

CONCLUSION

In this manuscript, we offer researchers a comprehensive overview of fluorescent techniques and applications in the field of fungal pathogenesis. We summarize the available fluorescent labels, based on fluorescent proteins, bioluminescent markers and dyes, and disclose how novel fluorophores could overcome several practical limitations researchers face today. Fluorescent imaging can contribute to insights in fungal pathogenesis on several levels. Subcellularly, structures can be visualised and protein-protein interactions can be detected. Information concerning the pH, oxygen level, enzyme activity and concentration of secondary metabolites can be obtained. The use of biosensors opens great possibilities for investigation of these physiologically relevant parameters. On the cellular level, fluorescence measurements can be used to report on phenomena such as viability, loss-of-heterozygosity, phenotypic switching and drug efflux. In the context of drug resistance and organismal niche adaptability, these characteristics are important to consider. Infections are often found to be polymicrobial in nature and the interaction between these micro-organisms affects the disease outcome. Differentially imaging these microbes offers unique insights in how fungi and bacteria influence each other and the host organism. Several animal models are available to image fungal cells in their relevant context. Transparency and low background fluorescence are important characteristics of model systems in a fluorescence setup. Recent improvements, however, allow standard animal models, such as rodents, to be used in an imaging setup as well. Finally, also diagnosis of fungal infections on the organismal level is possible using fluorescence. FISH and qRT-PCR are commonly used tools to identify the presence and cause of an infection. Taken together, fluorescence imaging offers great potential to advance our knowledge on fungal metabolism, virulence, interactions and infectability. Guided by this review, scientists will be able to explore the elaborate possibilities to optimize their specific experimental setup in function of the stated research question. It is important to keep

investing time and funding in optimization of existing techniques and introduction of novel technologies as only in this manner unknown yet relevant aspects of fungal pathogenesis can be discovered.

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