



## Video article

## Live-cell imaging of rapid calcium dynamics using fluorescent, genetically-encoded GCaMP probes with *Aspergillus fumigatus*

Alberto Muñoz<sup>1,2</sup>, Margherita Bertuzzi<sup>2</sup>, Constanze Seidel, Darren Thomson, Elaine M. Bignell<sup>\*,1</sup>, Nick D. Read

Manchester Fungal Infection Group, Division of Infection, Immunity and Respiratory Medicine, University of Manchester, CTF Building, 46 Grafton Street, Manchester M13 9NT, UK



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## ABSTRACT

Calcium signalling plays a fundamental role in fungal intracellular signalling. Previous approaches (fluorescent dyes, bioluminescent aequorin, genetically encoded cameleon probes) with imaging rapid subcellular changes in cytosolic free calcium ( $[Ca^{2+}]_c$ ) in fungal cells have produced inconsistent results. Recent data obtained with new fluorescent, genetically encoded GCaMP probes, that are very bright, have resolved this problem. Here, exposing conidia or conidial germlings to high external  $Ca^{2+}$ , as an example of an external stressor, induced very dramatic, rapid and dynamic  $[Ca^{2+}]_c$  changes with localized  $[Ca^{2+}]_c$  transients and waves. Considerable heterogeneity in the timing of  $Ca^{2+}$  responses of different spores/germlings within the cell population was observed.

## 1. Introduction

Filamentous fungi need to respond rapidly to the diverse environmental signals that they encounter in their naturally heterogeneous microhabitats. Fast responding intracellular signalling pathways are necessary to quickly activate and regulate downstream elements to elicit appropriate responses to these environmental stimuli. Intracellular  $Ca^{2+}$  signalling possesses the necessary properties to fulfil the role for such rapid signalling (Berridge et al., 2000, Kudla et al., 2010) but its dynamics have been little analysed in fungi at the single-cell level because of the lack of routine means to easily image its often very rapid and dynamic changes.

Calcium signalling and homeostasis are essential for hyphal growth, differentiation and virulence of filamentous fungi (Juvvadi et al., 2014), including calcium signalling-dependent multi-drug resistance (Li et al., 2019). Indeed, in *Aspergillus nidulans*, transient  $Ca^{2+}$  pulses have been demonstrated to coordinate actin assembly and hence stepwise hyphal extension (Takeshita et al., 2017). The low resting level (50–100 nM) of cytosolic free  $Ca^{2+}$  ( $[Ca^{2+}]_c$ ) is maintained by  $Ca^{2+}$ -pumps and -antiporters, and cytoplasmic  $Ca^{2+}$ -buffering. However,  $[Ca^{2+}]_c$  becomes an intracellular signal when its concentration is transiently increased and this is typically very rapid and commonly localised within a cell

(Berridge et al., 2000, Kudla et al., 2010).

Previous  $[Ca^{2+}]_c$  imaging with  $Ca^{2+}$ -sensitive dyes produced very variable results with cells often not taking up dye or sequestering dye within organelles (Hickey et al., 2004). We developed an easy, routine method for  $[Ca^{2+}]_c$  measurement in filamentous fungi (Nelson et al., 2004), including *Aspergillus fumigatus* (Munoz et al., 2015), using 96-well plate luminometry with genetically encoded aequorin as a  $Ca^{2+}$ -reporter. This bioluminescent probe is well suited for average quantitative measurements of  $Ca^{2+}$ -signatures in cell populations but not for the imaging/measurement of  $[Ca^{2+}]_c$  dynamics at the single-cell level because its light output is too low and it is biochemically consumed during  $[Ca^{2+}]_c$  measurement (Munoz et al., 2015). A newer approach has been to use genetically encoded ratiometric cameleon probes (Kim et al., 2012, Kim et al., 2015) but issues with low expression of this reporter in some fungi (e.g. *A. fumigatus*, *Neurospora crassa*, unpubl. results) has limited its widespread exploitation. In the current study we report the use of a newer class of genetically encoded reporters called GCaMPs (Akerboom et al., 2012, Chen et al., 2013) which have various superior properties for  $[Ca^{2+}]_c$  imaging in filamentous fungi. GCaMP reporters are composed of a circularly permuted (cp) GFP in between the calmodulin (CaM)-interacting region of chicken myosin light chain kinase (M13) at the N-terminus and a vertebrate CaM at the C-terminus.

\* Corresponding author.

E-mail address: [E.Bignell@exeter.ac.uk](mailto:E.Bignell@exeter.ac.uk) (E.M. Bignell).

<sup>1</sup> Present address: Medical Research Council Centre for Medical Mycology, University of Exeter, Geoffrey Pope Building, Stocker Road, Exeter, EX4 4QD, UK.

<sup>2</sup> Authors contributed equally to this study.

Binding of  $\text{Ca}^{2+}$  causes the M13 and CaM domains to interact and the resulting conformational change leads to an increase in cpGFP fluorescence (Akerboom et al., 2012, Chen et al., 2013).

## 2. Results and discussion

The aims of this study were to:

- (1) Demonstrate, with a temporal resolution  $< 1$  sec, the very rapid and dynamic subcellular changes in  $[\text{Ca}^{2+}]_c$  that can be readily imaged in conidial germlings of *A. fumigatus*.
- (2) Determine the subcellular influence of high  $\text{Ca}^{2+}$  stress (200 mM  $\text{CaCl}_2$ ) on immediate exposure and prolonged treatment of this high  $\text{Ca}^{2+}$  stress to conidial germlings.
- (3) Determine whether ungerminated conidia undergo  $\text{Ca}^{2+}$ -signalling and can respond to high  $\text{Ca}^{2+}$  stress.

Previously we have demonstrated that *A. fumigatus* responds to exposure to the following range of physiologically significant stressors: hypo-osmotic and hyper-osmotic shock, oxidative stress, Caspofungin antifungal treatment, and high external  $\text{Ca}^{2+}$  (200 mM  $\text{CaCl}_2$ ) stress (Juvvadi et al., 2015, Muñoz et al., 2015). It was previously demonstrated that treatment with 200 mM  $\text{CaCl}_2$  results in transcriptional changes that stabilise 30 min following exposure (Loss et al., 2017). Recently we have also provided evidence that external  $\text{Ca}^{2+}$  increased as a result of cell injury can act as a ‘danger’ or ‘alarm’ signal that activates a putative innate immune system involved in cell regeneration in filamentous fungi (Medina-Castellanos et al., 2018).

We decided to treat conidia and conidial germlings of *A. fumigatus* with high external  $\text{Ca}^{2+}$  (200 mM  $\text{CaCl}_2$ ) as an example of an external stressor, and because using it produced a highly reproducible  $\text{Ca}^{2+}$  signature at the cell population level in 96-multiwell plates (Nelson et al., 2004, Muñoz et al., 2015). We tested and compared strains expressing either GCaMP5 (Akerboom et al., 2012) or its derivative GCaMP6, an improved and more sensitive version which was generated via mutagenesis at the interface between cpGFP and CaM (Chen et al., 2013) (Mo\*\*\*vie 1–3). Notably, while GCaMP6 was used only once before, its first use having been in the filamentous fungus *Trichoderma atroviride* to evaluate the regeneration process of hyphae in response to danger signals (Medina-Castellanos et al., 2018), the present study reports for the first time the use of GCaMP5 in filamentous fungi. The fluorescence from strains expressing GCaMP6 was significantly brighter than that of GCaMP5 and could be even readily observed with the naked eye through the microscope eyepieces following addition of 200 mM  $\text{CaCl}_2$  (data not shown). Nevertheless, excellent results were still obtainable with the GCaMP5 strain using widefield fluorescence optics and a cooled CCD camera (Mo\*\*\*vie 1).

The initial experiments were performed with a GCaMP5-expressing strain that had germinated in liquid AMM in a microfluidic chamber and had been treated with high external  $\text{Ca}^{2+}$  (200 mM  $\text{CaCl}_2$ ) applied by continuous perfusion. Mo\*\*\*vie 1 shows a representative example of the results obtained with a group of ten conidial germlings; similar variation in the results were obtained with other conidia germlings of similar lengths.

Prior to stimulation with high  $\text{Ca}^{2+}$ , the  $[\text{Ca}^{2+}]_c$ -dependent fluorescence of the germlings shown in Mo\*\*\*vie 1 was low with no obvious subcellular increases in  $[\text{Ca}^{2+}]_c$  above resting level in any of the germlings. Upon contact with 200 mM  $\text{CaCl}_2$  applied by controlled perfusion through the cell culture chamber, the cells unexpectedly responded at markedly different times. A variety of changes and dynamics in  $[\text{Ca}^{2+}]_c$  were observed. Nine out of the ten cells responded to the  $\text{Ca}^{2+}$  stress and a period of 162 sec passed between the first and the ninth cell reacting with transient  $[\text{Ca}^{2+}]_c$  increases even though all of the germlings will have been stimulated at the same time (see also the graph in Suppl. Fig 3A). Transient  $[\text{Ca}^{2+}]_c$  elevations were observed five times in one of the cells, three in five of the cells, two in two of the cells and one in one of

the cells. The  $[\text{Ca}^{2+}]_c$  typically increased in either a specific subcellular region or throughout a whole cell, although sometimes  $[\text{Ca}^{2+}]_c$  was observed to increase in two subcellular regions at more-or-less the same time. Waves of  $[\text{Ca}^{2+}]_c$  developed from the  $[\text{Ca}^{2+}]_c$  foci and moved down the lengths of germ tubes. As reported previously in *Magnaporthe oryzae*, *Fusarium oxysporum*, and *Fusarium graminearum* (Kim et al., 2012), there was no discernible repetitive pattern in  $[\text{Ca}^{2+}]_c$  waves along individual fungal hyphae. Furthermore, no co-ordination of the timing of the initiation of  $[\text{Ca}^{2+}]_c$  waves was observed between the groups of germlings analysed (Mo\*\*\*vie 1 and 2). However, it was clear that long germlings produced stronger  $[\text{Ca}^{2+}]_c$  signals than short ones (Mo\*\*\*vie 1 and 2).

The velocities of the  $[\text{Ca}^{2+}]_c$  waves were of a similar magnitude in germ tubes and more mature hyphae. Quantitative measurements of 12  $[\text{Ca}^{2+}]_c$  waves (two in germ tubes and 10 in more mature hyphae) in GCaMP5-expressing cells at  $\sim 25^\circ\text{C}$  were combined and found to have an average wave velocity of  $4.1 \pm 2.1 \mu\text{m}/\text{sec}$ , which is within the range reported for animal cells (typically up to about  $30 \mu\text{m}/\text{sec}$ ) (Jaffe, 1993). Intracellular  $[\text{Ca}^{2+}]_c$  waves in animal cells are generated by  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$ -release (Zhao et al., 2001).

We next analysed the influence of prolonged high  $\text{Ca}^{2+}$  stress (200 mM  $\text{CaCl}_2$ ) over 20 min on conidial germlings at the end of which an extra 200 mM  $\text{CaCl}_2$  treatment was applied. The group of longer germlings and hyphae that were analysed (Mo\*\*\*vie 2) were more advanced in growth than in Mo\*\*\*vie 1. Playing back the movie at a faster frame rate (23.5 times faster) in this movie highlighted that at this later stage of morphogenesis,  $[\text{Ca}^{2+}]_c$  waves more commonly arose from the tips than from subapical regions. Bidirectional  $[\text{Ca}^{2+}]_c$  waves were common when they originated from localised subapical foci. Again, considerable heterogeneity in the timing of  $[\text{Ca}^{2+}]_c$  responses of the 25 different germlings within the cell population was observed. Capturing images at 1.8 sec intervals to generate this movie induced significant photobleaching of the GCaMP6. If required, the photobleaching could have been expeditiously decreased by reducing the excitation intensity or frequency of image capture. The gain setting on the cooled CCD was increased on the detector after  $\sim 13$  min to adjust for this in order that the fluorescence signal roughly equated to that at the beginning of the movie. Precise quantification of the  $[\text{Ca}^{2+}]_c$  would require correction for photobleaching (Carbo et al., 2017). The perfusion of an extra 200 mM  $\text{CaCl}_2$  resulted in a global increase in  $[\text{Ca}^{2+}]_c$  throughout all fungal hyphae, as result of  $\text{Ca}^{2+}$  uptake from the external medium (AMM containing 200 mM  $\text{CaCl}_2$ ). Whether this was  $\text{Ca}^{2+}$ -channel dependent or cell stress dependent would require further analysis.

We finally analysed the effects of high  $\text{Ca}^{2+}$  stress (200 mM  $\text{CaCl}_2$ ) on a subpopulation of 45 ungerminated, swollen and unpolarised spores of the GCaMP6-expressing strain that had been incubated for 16 h at  $25^\circ\text{C}$  and had undergone isotropic growth prior to the formation of germ tubes (d’Enfert, 1997). Almost all the spores (44/45) responded to the  $\text{Ca}^{2+}$  stress but they exhibited great heterogeneity in the timing and intensity of their  $[\text{Ca}^{2+}]_c$ -responses (see also the graph in Suppl. Fig. 3B). Furthermore, 71% of the spores exhibited multiple transient  $[\text{Ca}^{2+}]_c$  increases. Over a period of 9 min exposure to 200 mM  $\text{CaCl}_2$  perfused through the slide culture chamber, 12 (27%) exhibited one transient increase in  $[\text{Ca}^{2+}]_c$ , 26 (58%) spores exhibited two to four transient  $[\text{Ca}^{2+}]_c$  increases whilst six (13%) exhibited five or more.

In conclusion we have shown continuous, localised, rapid and dynamic changes in  $[\text{Ca}^{2+}]_c$  in subcellular regions of *A. fumigatus* germlings in response to the application of high  $\text{Ca}^{2+}$  stress using GCaMP probes that have a very good signal:noise ratio. Our results demonstrate the extraordinary otherwise-hidden heterogeneity in the responses of different but similar-looking, and apparently developmentally equivalent, spores/germlings, in the cell population. These  $[\text{Ca}^{2+}]_c$  changes can be observed in long time-courses ( $< 15$  min) under very controlled conditions with drug and other treatments applied in a continuous flow slide culture system. Short germlings, hyphae and ungerminated spores responded to both immediate and prolonged high  $\text{Ca}^{2+}$  stress. Of

particular interest is that spores are highly sensitive to their microenvironments before they produce germ tubes and this will clearly need to be taken into account in order to understand the intimate and highly dynamic interplay of rapid signalling processes that occur within the infection court to determine the establishment of the pathogen-host cell interaction. This study highlights the utility of single cell fluorescence reporters to gain a deeper understanding of these processes.

### 3. Methods

The *A. fumigatus* strains used were GCaMP5<sup>ΔakuB</sup> (*ΔakuB*<sup>KU80</sup>; *AngpdA*<sup>P</sup>-*GCaMP5*-*ptrA*) and GCaMP6<sup>ΔakuB</sup> (*ΔakuB*<sup>KU80</sup>; *AngpdA*<sup>P</sup>-*GCaMP5*-*ptrA*), which were generated by transforming the parental isolate *ΔakuB*<sup>KU80</sup> (da Silva Ferreira et al., 2006) with the plasmids pSK379-GCaMP5 and pSK739-GCaMP6 according to the protocol described in Szewczyk et al. (2006). These plasmids target the insertion of the expression cassette containing GCaMPs under the control of the *A. nidulans* *gpdA* promoter (*AngpdA*) at the 3'-flanking region of the *A. fumigatus* histone 2A locus (*his2A*) (Wagener et al., 2008). The *A. oryzae* pyrithiamine resistance marker (*ptrA*) was used for selection in fungal cells (0.5 μg/ml pyrithiamine). Transformants were verified for the presence, site of genomic integration and single copy number of GCaMP5 and GCaMP6 using PCR (using the oligonucleotides QC\_GCaMP\_1 GTCAGAGCTATAGGTCGG and QC\_GCaMP\_2 CTTGAAGTCGATGCCCTT) and Southern blotting using GCaMP- and *his2A*-specific probes (Supplementary Fig. 1). For Southern blotting analysis, genomic DNA was digested with the restriction enzyme EcoRI. Relative to the parental isolate *ΔakuB*<sup>KU80</sup>, no growth alterations due to the *AngpdA*-driven expression of the reporters GCaMP5 and GCaMP6 were observed upon growth onto solid media Aspergillus Complete media (ACM) (Supplementary Fig. 2). Plasmids and strains generated were deposited into the collection of the Manchester Fungal Infection Group.

For preparation of the experiments, strains were harvested using sterile H<sub>2</sub>O from cultures grown on solid ACM for 5 days at 37 °C (Pontecorvo et al., 1953). Spore suspensions were filtered using Miracloth (Calbiochem), centrifuged for 10 min at 4000 rpm and washed twice with sterile H<sub>2</sub>O. Spores were enumerated using a haemocytometer and resuspended to the desired concentration in sterile H<sub>2</sub>O.

The *A. fumigatus* strains GCaMP5<sup>ΔakuB</sup> and GCaMP6<sup>ΔakuB</sup> were cultured in Aspergillus Minimal media (AMM) (Pontecorvo et al., 1953). Dormant conidia were transferred to a Cellasic microfluidic culture chamber (Merck) and incubated at 25 °C. Conidia and subsequently conidial germlings were immobilized by between silicone and glass whilst being kept in focus and exposed to AMM supplemented with 200 mM CaCl<sub>2</sub> and applied by continuous perfusion at 0.5–1 psi flow rates for the duration of the time course.

### Declaration of Competing Interest

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

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fgb.2020.103470>.

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