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Methods for the detection of intracellular calcium in filamentous fungi

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ABSTRACT

Calcium (Ca^{2+}), a critical secondary messenger, is also known as the molecule of life and death. The cell responds to a minute change in Ca^{2+} concentration and tightly maintains Ca^{2+} homeostasis. Therefore, determining the cell Ca^{2+} level is critical to understand Ca^{2+} distribution in the cell and various cell processes. Many techniques have been developed to measure Ca^{2+} in the cell. We review here different methods used to detect and measure Ca^{2+} in filamentous fungi. Ca^{2+}-sensitive fluorescent chlortetracycline hydrochloride (CTC), Ca^{2+}-selective microelectrode, Ca^{2+} isotopes, aequorins, and RGECOs are commonly used to measure the Ca^{2+} level in filamentous fungi. The use of CTC was one of the earliest methods, developed in 1988, to measure the Ca^{2+} gradient in the filamentous fungus *Neurospora crassa*. Subsequently, Ca^{2+}-specific microelectrodes were developed later in the 1990s to identify Ca^{2+} ion flux variations, and to measure Ca^{2+} concentration. Another method for quantifying Ca^{2+} is by using radio-labeled Ca^{2+} as a tracer. The usage of ^{45}Ca to measure Ca^{2+} in *Saccharomyces cerevisiae* was reported previously and the same methodology was also used to detect Ca^{2+} in *N. crassa* recently. Subsequently, genetically engineered Ca^{2+} indicators (GECIs) like aequorins and RGECOs have been developed as Ca^{2+} indicators to detect and visualize Ca^{2+} inside the cell. In this review, we summarize various methodologies used to detect and measure Ca^{2+} in filamentous fungi with their advantages and limitations.

- Chlortetracycline (CTC) fluorescence assay is used for visualizing Ca^{2+} level, whereas microelectrodes technique is used to determine Ca^{2+} flux in the cell.
- Radioactive ^{45}Ca is useful for quantification of Ca^{2+} in the cellular compartments.
- Genetically modified calcium indicators (GECIs) are used to study Ca^{2+} dynamics in the cell.

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Specifications table

Subject area:	Microbiology
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Resource availability:	Not applicable
Review question:	What are the methods for the detection of intracellular calcium in filamentous fungi?

Method details

Introduction

Calcium (Ca^{2+}), a ubiquitous intracellular messenger, is crucial for regulating almost all cell processes. They are in charge of various cell functions, such as gene transcription, cell proliferation, and differentiation [1]. The cytosolic free Ca^{2+} resting concentration ($[Ca^{2+}]_c$) is ∼100 nm, whereas Ca^{2+} concentration in the extracellular fluid is $\sim10^{-3}$ M; hence the gradient across the plasma membrane is more than 10,000-fold [2,3]. Numerous Ca^{2+} sensor proteins detect even the minute change in the Ca^{2+} levels, which further triggers downstream signaling processes [3]. Techniques to visualize or determine the intracellular concentration of Ca^{2+} are essential for understanding fungal physiology, adaptation to stress conditions, and virulence. In filamentous fungi, including *N. crassa* and *Aspergillus nidulans*, various techniques have been developed to visualize and measure Ca^{2+}.

Ca^{2+} visualization using chlortetracycline (CTC) is one of the oldest methods described for visualizing changes in intracellular Ca^{2+} levels and morphology in filamentous fungi [4]. CTC, discovered by Benjamin Minge Duggar in 1945, is the first member of the tetracycline class and produced by *Streptomyces aureofaciens* which appears golden in color [5,6]. CTC, produced by a strain of actinomycetes, is a fluorescent Ca^{2+} probe used to measure changes in intracellular Ca^{2+} levels [4,7]. CTC is also used for a variety of other applications, including determining the Ca^{2+} content in the blood by measuring the Ca^{2+}-binding platelet, also used to monitor the intracellular Ca^{2+} binding in cell systems, including sarcoplasmic reticulum [8], red blood cell [9], mitochondria [10], neutrophils [11] and human blood platelets [12–14]. CTC binds to Ca^{2+} and forms a fluorescent complex, making it possible to visualize the distribution pattern of Ca^{2+} [4]. The binding of the complex to membranes increases fluorescence intensity, which subsequently enables the imaging of Ca^{2+} buildup in vesicles and organelles [4,15].

The ion-selective microelectrode is a non-invasive technique to simultaneously measure ionic currents and fluxes in a cell [16]. This method allows us to determine the ion flux from the difference in ion concentration at two locations: one close to and one far from the cell [17]. The external diffusive gradient produced by ion transport across the plasma membrane is collected with the ion-selective microelectrode at two places [17]. The first Ca^{2+} ion-selective electrodes were made based on the Ca^{2+} ligand ETH for detection at the sub-nanomolar range [18] and it was used for Ca^{2+} detection in *N. crassa* [19]. Ca^{2+}-specific microelectrodes have been used for monitoring Ca^{2+} ions in cerebrospinal fluid and cataracts in the lens of rats [20,21]. Likewise, measurement of the effect of gravistimulation on the Ca^{2+} activity in maize roots [22]. In *N. crassa*, Ca^{2+} selective microelectrodes have also been used to measure the cytosolic free Ca^{2+} using mycelial cells.

Radioactive calcium isotopes were also used as a tracer to measure Ca^{2+} levels in the cell. Naturally, calcium is a stable element, comprising six stable isotopes ^{40}Ca, ^{42}Ca, ^{43}Ca, ^{44}Ca, ^{46}Ca, and ^{48}Ca, where ^{40}Ca is the most abundant and constitutes about 97% of all natural calcium. Artificially produced calcium radioactive isotopes ranging from ^{30}Ca to ^{60}Ca, are extensively used as a radioactive tracer. To date, radioactive calcium isotopes have been widely used as Ca^{2+} tracers, for example, to measure Ca^{2+} absorption efficiency in patients,^{48}Ca as the intravenous tracer, ^{47}Ca as an oral trace [23] and ^{41}Ca as a bone tracer [24] have been used. Likewise, the localization of Ca^{2+} in different organelles has been explored widely in *S. cervisiae,* where 95 % of Ca^{2+} was sheltered in vacuoles [25], and the distribution of Ca^{2+} in various organelles using radioactive calcium isotopes has also been reported in *N. crassa* [26].

Genetically modified Ca^{2+} indicators (GECIs) are genetically modified *in vivo* Ca^{2+} sensors that detect Ca^{2+} oscillation in different cellular organelles, such as ER, mitochondria, Golgi, plasma membrane, and other Ca^{2+} stores [27–29]. GECIs are classified into three different classes, including (a) Bioluminescent sensors that are based on aequorin photoproteins, (b) Cameleon type, and (c) Single fluorescent proteins [28]. Aequorin, the first Ca^{2+}-sensitive photoprotein discovered from the jellyfish *Aequorea victoria*, is an excellent Ca^{2+} indicator [30,31]. The circumoral ring of the umbrella of *A. victoria* has specialized cells called photocytes that contain aequorin. On mechanical stimulation, the photocytes luminescent and generate a bluish-green light. Aequorin is associated with the green fluorescent protein (GFP) responsible for the bluish-green light on stimulation [30] and was first used in barnacle muscle cells [32]. Aequorin has been widely used as a Ca^{2+} indicator in a variety of systems, including mammals [33], plants [34–36], yeast [37] and filamentous fungi like *N. crassa* [30,38,39].

Single fluorescent proteins consist of the portion of CaM fused with fluorescent proteins, several members of this class such as camgaroos, pericams, G-CaMPs, case sensors, and grafted EF-hands are used for Ca^{2+} measurement [28]. G-CaMPs, which consist of the circularly permuted green fluorescent proteins (cpGFP), calmodulin (CaM) (Ca^{2+} sensory domain), and Ca^{2+}/CaM-binding M13 peptide (M13pep) [27] are single wavelength intensiometric in nature; therefore, fluorescence intensity is proportional to Ca^{2+} binding [40]. The Ca^{2+} sensory domain is present at the N/C-terminal of the indicator. M13 peptide of the myosin light chain is fused at the other end of the indicators. M13 brings conformational changes in the Ca^{2+} indicator upon Ca^{2+} binding with the CaM fragment [27]. M13 peptide also prevents the interaction of this sensory complex with the other CaM interacting proteins present within the

cellular vicinity [27]. GCaMP3 was introduced as a template in the error-prone PCR technique, which resulted in the generation of a large-size mutant library. These mutants were screened on the basis of the change in Ca^{2+}-dependent green fluorescent [41]. Subsequently, they were termed as GECOs for 'genetically encoded Ca^{2+} indicators for optical imaging' [41].

The methods used to monitor intracellular Ca^{2+} are the focus of this review. We describe each methodology with advantages and limitations.

Chlortetracycline hydrochloride (CTC)

CTC is a fluorescent Ca^{2+} probe that measures changes in intracellular Ca^{2+} levels [7]. A unique labeling and Ca^{2+} detection method for studying changes in intracellular Ca^{2+} levels and morphology in *N. crassa* [4]. During the extension process, *N. crassa* maintains a high Ca^{2+} gradient directed toward the hyphal tip, and the Ca^{2+} is stored in the storage vesicles located right beyond the apex [4,42]. Ca^{2+} channels, triggered by IP_3, are responsible for both the production and the maintenance of the high Ca^{2+} gradient at the tip of the cell [43]. However, the membrane density and Mg^{2+} affect the CTC fluorescence [44] yet the membrane-associated Ca^{2+} emits greater fluorescence than Mg^{2+} [45].

Assay for determining the intracellular distribution of Ca^{2+} using CTC

To visualize intracellular Ca^{2+} distribution and quantify the Ca^{2+} in the *N. crassa* hyphal tip, CTC (Sigma-Aldrich C4881-5G), dissolved in 0.1% dimethyl sulfoxide (DMSO), is used at working concentration of 100 μM. The conidia of *N. crassa* strains are inoculated onto a glass slide with Vogel's agar medium containing 100 μM CTC, and incubated at 30°C for 12 h in dark [46]. CTC fluorescence is then observed using an Inverted fluorescence microspore under a bright field and a DAPI filter system with an exposure time of 300−400 ms [47]. CTC fluorescence is affected by membrane density, and therefore, membrane fluorescence is determined using N-phenyl-1-naphthylamine (NPN) is as a control [44].

Advantages. There are several advantages of using CTC as a Ca^{2+}-sensitive fluorescent probe for imaging intracellular Ca^{2+} dynamics in living cells. CTC allows the study of the dynamics of intracellular Ca^{2+} in real-time without affecting the growth of fungi. It has a high sensitivity for detecting intracellular Ca^{2+}, which allows visualizing small changes in Ca^{2+} levels. CTC has a high specificity for Ca^{2+}, and membrane-bound Ca^{2+} gives higher fluorescence than Mg^{2+} [44,45]. It is non-toxic, cost-effective, easy to use, and used in many cell types and organisms, including neurons, cardiomyocytes, and plants, making it versatile for different applications.

Limitations. While CTC has many advantages, yet there are several limitations in its use. It is sensitive to light and can be photobleached over time, limiting the duration of imaging experiments. CTC's fluorescence intensity may be affected by other fluorescent molecules in the cell, and its sensitivity to pH change can lead to interference and inaccurate results. Some tetracycline derivatives may have similar properties to CTC and can interfere with the binding of Ca^{2+} with CTC. Therefore, it may not be the best option for some specific applications, where other indicators have higher sensitivity or better specificity. Hence, it is necessary to consider these limitations and to use appropriate controls and methods to minimize their impact on the accuracy of the imaging results.

Calcium ionic flux measurement by microelectrodes

The Ca^{2+}-selective microelectrodes are complex systems that involve a combination of surface ion exchange and ion diffusion stages. The simple hyphal morphology of *N. crassa* makes it easy to determine the current density of ions with an ion-selective probe [48,49]. The fabrication of Ca^{2+} ion selective probe (Calcium Ionophore I–Cocktail A, catalog number 21048) is done at Bio Currents Facility (Marine Biological Laboratory, Woods Hole, MA, USA) [50]. Ca^{2+} selective probe and a separate reference system must be submerged in the solution to measure the electrode potential, and a millivolt measuring system is connected to both. When the system is in equilibrium, the charge of the reference interface balances out the electrons that the Ca^{2+} selective electrode membrane has added or withdrawn from the solution. As a result, the initial stable reference voltage deviates positively or negatively, which is recorded by the external measuring device (Fig. 1) [16,51,52].

Culture preparation: *N. crassa* strains are grown in Vogel's medium [46] for 2 days in the dark and 1 day under the light for conidiation. The conidia are harvested in Eppendorf tubes using 1 ml of sterile water. Conidia are then transferred onto strips of dialysis tube with Vogel's medium in a Petri dish, and the strain is grown at 28°C overnight. The dialysis tube is cut with a razor blade, attached to the bottom of the plate with double-sided sticky tape, and then covered. The culture is flooded with 3 ml of buffer solution: KCl (10 mM), $CaCl_2$ (1 mM), $MgCl_2$ (1 mM), sucrose (133 mM), and MES (10 mM), and the pH is adjusted to 5.8 with KOH. The mycelia are incubated in the buffer solution, maintaining the pH at 5.5 stable throughout the experiment. Large trunk hyphae *in situ* are used for the study.

Microelectrical measurement of hyphal properties and ion flux: A distance from the first spike site, the hyphae are spiked with a double-barrel micropipette and a single-barrel micropipette, KCl (3 M) is put into both micropipettes [16]. A salt bridge containing KCl (3 M) in 2% agar and an Ag/AgCl electrode is connected to an electrometer and another to the Ca^{2+} -selective probe amplifier completing the circuit. The double-barrel micropipettes are then connected to the electrometer. A data acquisition board is used to control an operational amplifier configured for a voltage clamp. Currents are applied for 160 s and interrupted by 0 current rests (such as -2.5 nA, +2.5 nA, 0 nA, -5.0 nA, +5.0 nA, and 0 nA). The clamped voltage (V_0) is sampled during the last 10 ms of

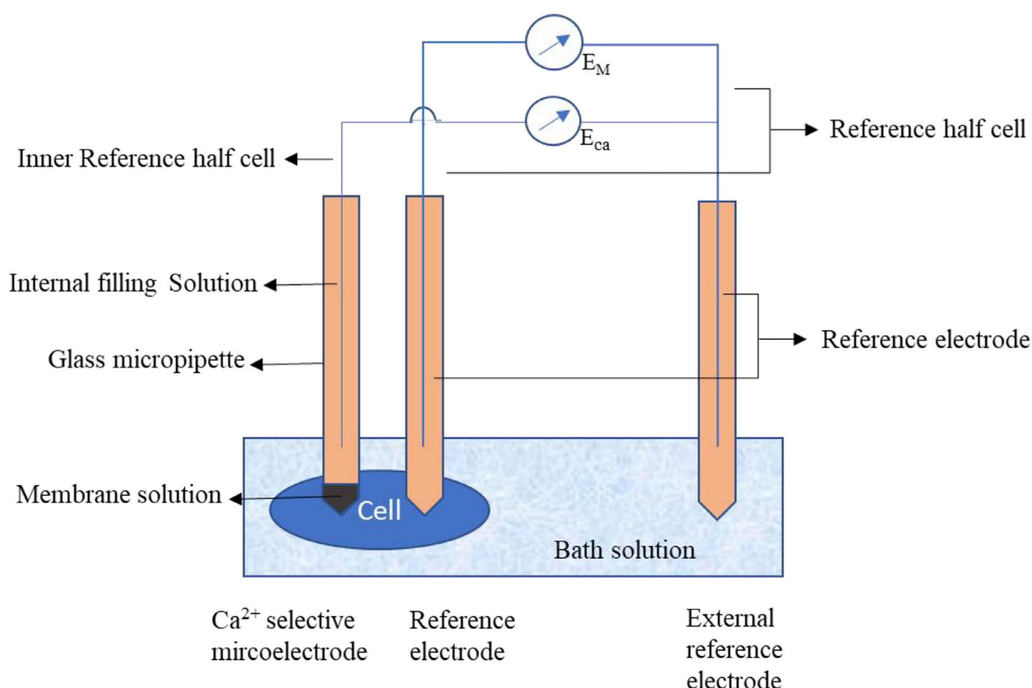

Fig. 1. Schematic representation of a Ca^{2+} membrane microelectrode cell assembly for intracellular Ca^{2+} measurement [52].

voltage clamp and the clamping current. The hyphal voltage (V_d) (measured simultaneously with V_0 at a distance, d, further along the hypha) is then obtained by connecting the single barrel microelectrode to an electrometer. The slope of the relationship between V_d and V_0 was used to compute the fading voltage of the distance d. Ca^{2+} ion fluxes are calculated based on the concentration difference between hyphal cells by considering their cylindrical geometry. The effect of the hyphal voltage on fluxes is measured by the following formula

$$J = \left(\frac{D}{r}\right)\left[\frac{C_2 - C_1}{\ln\left(\frac{r_2}{r_1}\right)}\right]$$

Where J is the net ion (nmol cm^{-2} s^{-1}), D is the diffusion coefficient (value of D for Ca^{2+}, 0.4×10^{-5} cm^2 s^{-1}), r is the hyphal radius, C_1 and C_2 are the concentrations at the two excursion points, and r_2 and r_1 are the distances between the two excursion points at the hyphal centre [16,53].

Advantages. Ca^{2+} selective microelectrodes can measure Ca^{2+} activities of the cytoplasm without disturbing the organelles as their tip size prevents the rupture of organelles. Even though the majority of intracellular Ca^{2+} measurement findings have previously been reported as concentrations, some researchers using Ca^{2+} selective electrodes have opted to convert Ca^{2+} ion concentrations to activities and constantly record the Ca^{2+} changes inside cells as long as the impalement of the cell is adequately maintained. One of the advantages is that there is no upper limit to their sensitivity range, and measurements are still viable at Ca^{2+} concentrations of 10 nM or less even. As many changes occur in intracellular activities quite slowly (on a second-to-minute timescale), the response time is compatible with recording such changes.

Limitations. Many ion-sensitive microelectrodes are not highly selective when measuring only one type of ion. This can lead to interference from other ions, which makes it difficult to ascertain the change in activity regarding the desired ion. Ion-sensitive microelectrodes are sensitive to electrical interference and must be well-shielded to maximize their performance. Ion-sensitive microelectrodes are passive devices that do not alter the cells under test. However, they require the impalement of the cell membrane to function accurately.

Calcium isotope ^{45}Ca

Radioactive isotopes are used as tracers based on the fundamental postulations that it replaces the native element inside the system, and then the added isotopes are absorbed and metabolized in the same proportion [54]. In *N. crassa*, a radioactive ^{45}Ca isotope was successfully used to quantify Ca^{2+} in various cell types and organelles (Fig. 2) [26].

Measurement of cellular calcium uptake (Mycelium mass): Vogel's medium (2% agar) was used to culture *N. crassa* strains [46]. The conidia are collected, diluted to 10^6 conidia/ml, and added to 6 ml of Vogel's medium, which also contains varying amounts of nonradioactive calcium (3 to 3000 μM) and radioactive isotope ^{45}Ca, which has a specific activity of >10 Ci/g (Perkin Elmer Life Sciences). The mixture is then incubated at 30°C for 25 h. 20 mM nonradioactive $CaCl_2$ is then added to the medium before the mycelium mass is promptly harvested using 25 mm acetate Plus filters. Then, ∼6 ml of growing medium containing 20 mM of nonradioactive $CaCl_2$ is used to gently wash the filter paper three times. The mycelium's radioactivity is then calculated using a liquid scintillation counter (Fig. 2(A)) [26].

Measurement of calcium uptake in conidia: In 2 ml of Vogel's medium with 1.6 Ci of ^{45}Ca, wild-type strains are produced. The cultures are incubated at 30°C for 3 days in the dark and then at room temperature for 6 days under the light. Conidia are collected from the liquid's surface in minute quantities, suspended in sterile autoclaved water, and counted using a hemocytometer. A liquid scintillation counter is used to monitor radioactivity and determine the quantity of calcium/conidium (Fig. 2(B)).

Measurement of calcium uptake in cell fractions: The concentration of Ca^{2+} in different cell organelles in *N. crassa* was determined by cell fractionation [55,56] using ^{45}Ca isotope [26]. The strains were cultured in Vogel's medium (1 or 2 L), which contains 50 Ci of ^{45}Ca and 10% conidia/ml of inoculum. The cultures are allowed to grow with vigorous aeration for 15 h at 25°C. The mycelia are harvested by filtering using cheesecloth and adding cold sorbitol (1 M) and Tris buffer (10 mM, pH 7.5). They were vigorously mixed with glass beads in Bead Beater, and unbroken cells were removed by centrifuging the homogenate at 1000 x g for 10 min. The supernatant is centrifuged at 15000 x g for 30 min, resulting in a pellet of dense organelles. A step gradient consisting of 7 ml 50% sucrose, 2 ml 40% sucrose, and 2 ml 30% sucrose, all in Tris buffer (10 mM, pH 7.5), is laid on top of the suspended pellet in sorbitol (1 M) and Tris buffer (10 mM, pH 7.5). After centrifugation at 20000 x g for 1 h, the suspension forms a thick layer of mitochondria on top of the 40% sucrose, with the dense vacuoles remaining in the pellet. A microsomal fraction is produced after centrifuging an aliquot of the supernatant at 250000 x g for 30 min. After the fractions have been well separated, each fraction is suspended in Tris buffer (10 mM, pH 7.5) in separate tubes. The radioactivity of each fraction is then determined using a liquid scintillation counter (Fig. 2(C)) [26].

Advantages. The advantages of measuring Ca^{2+} absorption by this technique are that Ca^{2+} concentration can be estimated with accuracy it has high precision. It can be applied to a wide range of species, including humans to fungi [23,26,57,58]. Additionally, this methodology does not require extensive sample preparation required for the microelectrode-based method.

Limitations. The drawback of this method is the preparation of radioactive isotopes, thus making it expensive. It is also time-consuming as it is not a rapid method to detect Ca^{2+}, and the long half-life of isotopes like ^{45}Ca, which is 162 days, emitting beta rays, this undesirable irradiation can be hazardous.

Genetically modified calcium indicators (GECIs)

Aequorin

Aequorin, a 22 kDa photoprotein that generates a green fluorescent protein, is composed of apoaequorin (the apoprotein), coelenterazine (the luciferin), and bound oxygen [30,59]. It exhibits a strong affinity for free Ca^{2+}, converting aequorin upon binding to Ca^{2+} into apoaequorin, carbon dioxide, and coelenteramide. The reaction's energy is released as blue light (λ_{max} 469 nm) (Fig. 3) [60,61]. It can be employed as a Ca^{2+} reporter because luminescence quantity depends on the amount of free Ca^{2+} present [30]. A chemiluminometer or a photon imaging detector can be used to detect luminescence [62,63]. The native aequorin gene (aeqA and aeqD gene) generates very low luminescence signals for detecting Ca^{2+}. The *aeqS*, a synthetic codon-optimized apoaequorin gene, was created for expression in *N. crassa* to solve codon bias, leading to lower apoaequorin expression [30,38]. This method was further optimized using the codons of the aequorin gene and constructed the recombinant aequorin to accurately determine Ca^{2+} concentration throughout a wide dynamic range in *N. crassa* [30,38].

Strain generation: The *N. crassa* strains *band* mutant strain (*bd A*) (FGSC1858) and 74 A (FGSC262) are routinely grown on Vogel's medium [46]. The *bd A* strain is transformed with pNCAEQ1 plasmid and 74 A is transformed with pNCAEQ3 plasmid, using hygromycin B for selection. Homokaryotic strain 73a is generated for further expression analysis [30,38].

Aequorin expression analysis: For protein extraction, conidia are inoculated in Vogel's medium at 30°C for 18 h, 180 rpm; thereafter the mycelia are harvested, frozen in liquid nitrogen, powdered, and transferred to Eppendorf tubes. Then, 0.2 g of mycelia is suspended in 1 ml extraction buffer (10 mM EGTA, Tris-Cl (50 mM, pH 7.4), 500 mM NaCl, 10 mM β mercaptoethanol, and water). Samples are vortexed for 30 s, then pelleted at 13,000 x g for 5 min, and stored at -70°C. The Bradford protein assay [65] (Bio-Rad Microscience) is used to quantify the total protein concentration in the supernatant, and in vitro protein concentration is then carried out to determine the amount of aequorin present. Then, ∼200 mg aliquot of total soluble protein is mixed with 0.25 mM coelenterazine in methanol to give a final concentration of 2.5 mM, and thereafter it is incubated at 30°C for 4 h in the dark. The in vitro aequorin constitution mixture containing 20 mg of total soluble protein diluted 1:50 in buffer (200 mM Tris-Cl, 0.5 mm EDTA, pH 7.0), which was then integrated for 20 s following the addition of an equal volume of 100 mM $CaCl_2$, and 100 ml of this

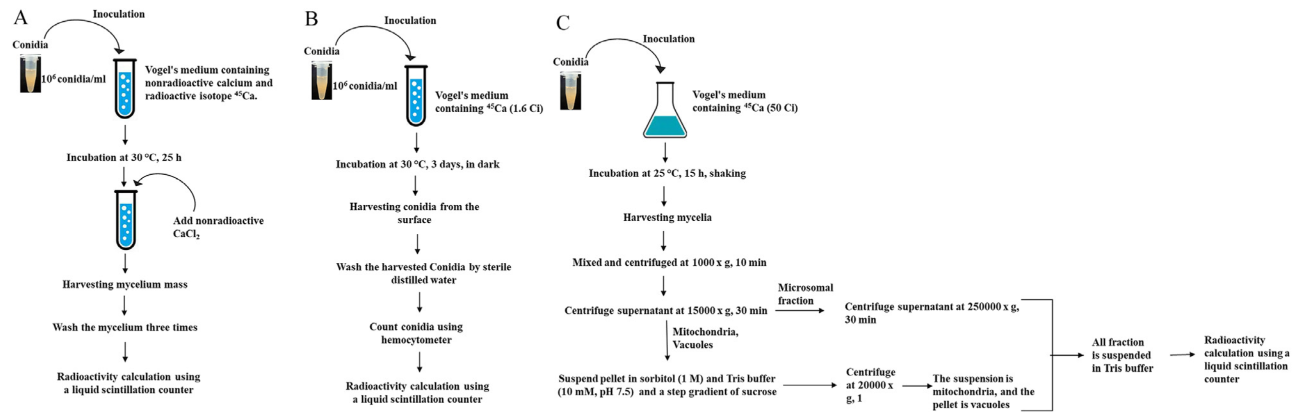

Fig. 2. Schematic detailing the procedures for measuring calcium uptake in *N. crassa* cells using the radioactive isotope ^{45}Ca [26]. (A) Accumulation of calcium in mycelium. (B) Estimation of calcium concentration in conidia. (C) Quantification of calcium concentrations in cellular fractions.

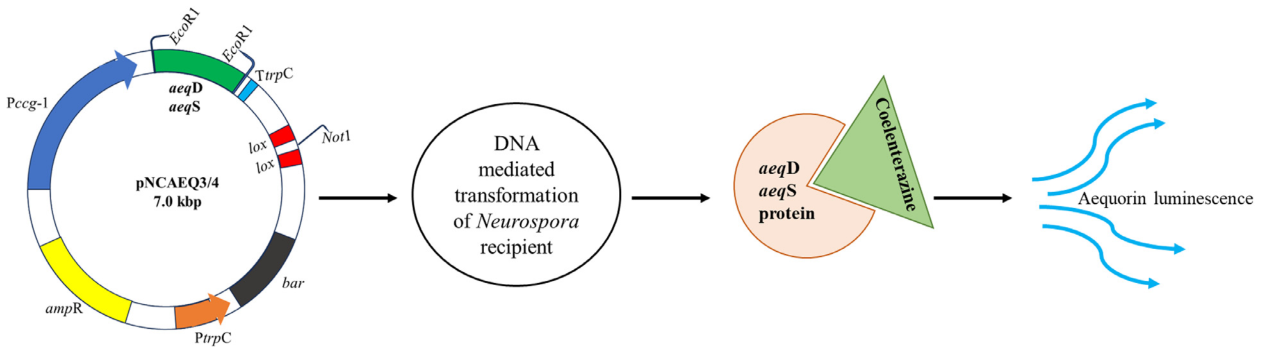

Fig. 3. Schematic representation of Ca^{2+} concentrations detection in *N. crassa* cells by aequorin. The plasmid is transformed in *N. crassa* and the protein radiates blue luminescence, that is measured by a photomultiplier tube [30].

was used to detect aequorin luminescence. Aequorin light emission can be detected using a specially made tube luminometer with a photomultiplier tube (air-cooled type 9829A, Thorn EMI) or a plate luminometer (Berthold MicroLumat LB96P) (Fig. 3) [30,66]. Aequorin concentration is determined using the total luminescence detected by making a standard curve using it, and the values are then converted into µg aequorin g^{-1} total protein.

Calcium levels calibration: The relative light units (RLU) used to measure the light emission are normalized by translating them to Ca^{2+} concentrations using the empirically obtained equation shown below, where k = luminescence (in RLU) s^{-1}/total luminescence (in RLU) [30,67].

$$pCa = 0.332588(-\log k) + 5.5593$$

The RLU values are multiplied by 1.24 to account for the 24% quenching of aequorin luminescence caused by ethanol [30].

Advantages. Aequorin has a very strong selectivity for free Ca^{2+}, and measures Ca^{2+} across a dynamic range. It is kept within the cell compartment that it has been intended for, is relatively stable within the cytoplasm, and is non-cytotoxic. In addition, unlike autofluorescence, autoluminescence is not found in most natural systems; hence detecting luminescent light is advantageous [30,63].

Limitations. There are a few limitations of using aequorin for Ca^{2+} detection, including enough light emitted for detection. Other limitations include the free Ca^{2+} level could be very low, the requirement of a very high spatiotemporal resolution, inefficient introduction methods, or the system under study might be too compact. The use of fluorescent dyes in systems other than filamentous fungi could potentially complement the use of aequorin [30,38].

Red fluorescent indicators (R-GECOs)

Members of this class give different chromatic signals according to which the nomenclature has been done [40]. GECOs providing green signal is termed as G-GECOs (green fluorescents genetically encoded Ca^{2+} indicator). G-GECOs have various types based on their dissociation constants like G-GECO1 (750 nM), G-GECO1.1 (620 nM), and G-GECO1.2 (1150 nM). Additionally, they offer nearly two times as much fluorescent light (2300 to 2600%) as standard G-CaMPs, while Ca^{2+} is bound [41]. Red fluorescent indicators (R-GECOs) were generated by replacing the cpGFP (circularly permuted GFP) of GECO1.1 with cp version of mApple (Red fluorescent protein, isolated from Discosoma species) [41,68]. R-GECO construct has been widely used to measure the Ca^{2+} oscillations at different concentrations in *A. nidulans* [64].

Strain generation: Amplify RGECO sequence from pPD60 RGECO.1 plasmid (R-GECO-KpnI-Fw and R-GECO-XbaI-r) and cloned in pJET1.2 plasmid using KpnI and XbaI restriction enzymes. Digest R-GECO ORF clone in the pCMB17apx plasmid with similar restriction enzymes to yield pNT76 plasmid for protein expression. Amplify ORF of *chsB* from genomic DNA using gene-specific primers and clone in the pSH44 plasmid (N-mCherry tagged), to yield pNT77 plasmid. Transform generated plasmids into the *A. nidulans* TH02A3 recipient strain and screen microscopically to confirm fluorescence (Fig. 4) [64].

Visualization and concentration determination: *A. nidulans* strains are generally grown in eight-well glass bottom slides with minimum media and kept overnight at 28°C. Visualize properly grown cells under a fluorescence microscope (Axiophot microscope; Plan-apochromatic 63×1.4 Oil objective lens). Capture images using ZEISS AxioCam MRM CCD camera [64]. The concentration of Ca^{2+} can be calculated by using the given equation, where k_d is the effective dissociation constant, F is Fluorescence intensity, F_{min} is minimum fluorescence intensity, and F_{max} is maximum fluorescence intensity [69].

$$\left[Ca^{2+}\right] = k_d\left(\frac{F - F_{min}}{F\max - F}\right)$$

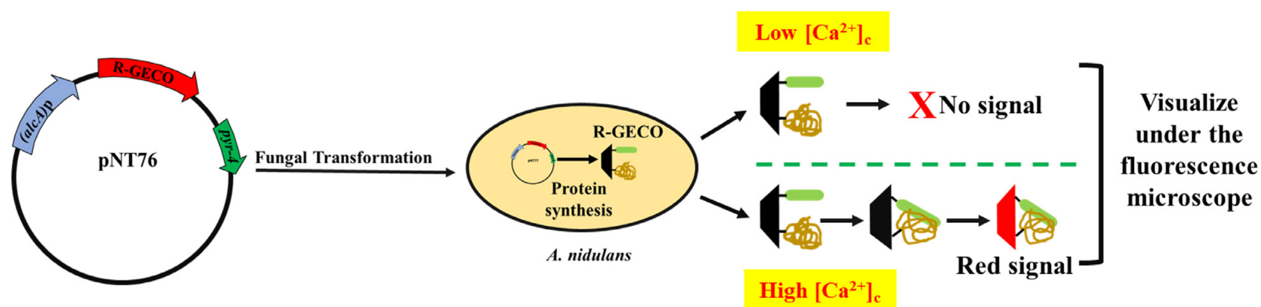

Fig. 4. Schematic representation of the R-GECO containing plasmid (pNT76) followed by transformation into fungi. Activation of R-GECO signaling upon changing the cytosolic calcium concentration $[Ca^{2+}]_c$ [64].

Table 1
Reported range of calcium sensitivity using various methodologies of calcium detection.

Method	Reported range of calcium sensitivity	Reference
Calcium ionic flux measurement by Microelectrodes	10^{-7} to 10^{-8} M	[52]
Cellular calcium uptake (mycelium mass) using radioactive isotope ^{45}Ca	0.0195 μM–0.45 μM	[26]
Calcium uptake in conidia by using radioactive isotope ^{45}Ca	Each conidia contain 0.095 fmol of calcium	[26]
Calcium uptake in cell fraction by using radioactive isotope ^{45}Ca	0.63–9.47 μmol/g protein	[26]
Aequorin	5.2–6.7 pCa ($\log_{10}[Ca^{2+}]$)	[66]
Red fluorescent indicators (R-GECOs)	0 μM to 1 μM	[64]

Advantages. R-GECOs are a valuable tool for the optical imaging of the Ca^{2+} dynamics within cellular compartments having less half-life time [70]. They show specific binding towards Ca^{2+} binding proteins due to the presence of M13 peptide sequence. They can be used to mark various targets simultaneously in deeper cellular regions due to their availability in multiple variants. Most importantly, they are resistant to photobleaching and are easily accessible [71].

Limitations. R-GECOs provide weak intensity and more background noise in red fluorescence [71] and there are high chances of tissue damage while using blue fluorescent excitation. It is difficult to obtain a high spatial resolution with GECOs; hence they are preferable for brain imaging [27,72]. M13 peptide present in the GECOs may interact with other cellular proteins, leading to no specificity and activation of other cellular proteins other than CaM [27].

Conclusion

From prokaryotes to eukaryotes, Ca^{2+} signaling is an evolutionarily conserved process. Ca^{2+} signaling influences various cellular processes in fungi, such as growth, development, fertility, stress tolerance, and virulence [3]. This paper discussed different approaches to visualize and quantify Ca^{2+} in fungal cells. To critically evaluate the function of Ca^{2+} as a signaling molecule in the cells, measuring free cytosolic Ca^{2+} and detecting the changes of Ca^{2+} level is required. However, the range of Ca^{2+} sensitivity varies in various methodologies used (Table 1). Development of a thorough understanding of the stability of Ca^{2+} and by using an indicator that provides the strongest signal possible, we can minimize the possibility of alteration in the cellular functions while using these indicators. CTC has potential applications in the field of cellular and molecular biology for the study of intracellular calcium dynamics. Microscopic analysis showed enhanced fluorescence in the internal Ca^{2+} storing vesicles in the growing hyphae of *N. crassa* [4]. Several reports have provided new insights into the use of CTC as a probe for intracellular Ca^{2+} imaging and highlights the potential of CTC as a valuable tool for the study of Ca^{2+} mediated cellular processes [73][73]. When Ca^{2+} selective microelectrode is properly applied to biological systems, it provides an enormous experimental return. It is a direct effective measurement method for determining how Ca^{2+} transport functions in muscle fibers, plants, and fungal cells in their dynamic living state. In a walled, turgid cell of *N. crassa* hyphae, the voltage dependence of Ca^{2+} fluxes have been quantitatively studied [16]. A recent report describes an in vivo monitoring of Ca^{2+} ions in rat cerebral fluid with the help of improved Ca^{2+} selective microelectrode fabrication [20]. In filamentous fungi, *N. crassa*, the major fraction of Ca^{2+} is deposited in vacuoles [26]. Given the numerous roles that Ca^{2+} plays, Ca^{2+} signaling can serve as a target for developing novel antifungal medications. The radioactive tracers were first used to trace the pathway of Ca^{2+} metabolism in the human body. Radioisotopes are used to study tumor localization and have also helped to trace early tumors. The recent development of recombinant aequorin has made it easy for high-throughput screening of filamentous fungi with compromised calcium signaling. The recombinant aequorin can be used to identify drug targets in filamentous fungi [30]. The study of Ca^{2+} dynamics has become easier after the construction of GECOs. It is now possible to simultaneously image many Ca^{2+} markers in the same cell. The GECO construct has only been used in a limited number of filamentous fungi to date, but it can be made accessible for other filamentous fungi and higher eukaryotes because of its improved sensitivity and specificity [41,64]. Considering the methodology's requirement, resource availability, and cost-effectiveness, a suitable method to visualize and determine the concentration of intracellular Ca^{2+} may be chosen. We described five different techniques for quantifying and visualizing intracellular Ca^{2+}

in filamentous fungi. Each method has different ranges of selectivity and sensitivity, prompt response, the accuracy of quantification, and different cell types. It is necessary to exercise caution while selecting a particular method for a given application. In the future, more accurate methods for measuring Ca^{2+} in filamentous fungi will emerge, which will be immensely helpful for understanding fungal physiology and adaptation to different environmental conditions.

Ethics statements

The authors have complied with MethodsX's ethical standards; no human participants, animal research, or data from social media were used in this study.

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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.

CRediT authorship contribution statement

Megha Rasaily: Conceptualization, Methodology, Writing – original draft, Writing – review & editing. **Serena Ngiimei D:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing. **Rahul Kumar Thaosen:** Conceptualization, Methodology, Writing – original draft. **Surabhi Gupta:** Conceptualization, Methodology, Writing – original draft. **Sangeeta Deka:** Conceptualization, Methodology, Writing – original draft. **Ranjan Tamuli:** Conceptualization, Methodology, Writing – original draft, Visualization, Supervision, Writing – review & editing.

Data availability

No data was used for the research described in the article.

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