

Video Article

# Live-cell Imaging of Fungal Cells to Investigate Modes of Entry and Subcellular Localization of Antifungal Plant Defensins

Kazi T. Islam<sup>1</sup>, Dilip M. Shah<sup>1</sup>, Kaoutar El-Mounadi<sup>2</sup>

<sup>1</sup>Donald Danforth Plant Science Center

<sup>2</sup>Department of Biology, Kutztown University of Pennsylvania

Correspondence to: Dilip M. Shah at [dshah@danforthcenter.org](mailto:dshah@danforthcenter.org)

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## Abstract

Small cysteine-rich defensins are one of the largest groups of host defense peptides present in all plants. Many plant defensins exhibit potent *in vitro* antifungal activity against a broad-spectrum of fungal pathogens and therefore have the potential to be used as antifungal agents in transgenic crops. In order to harness the full potential of plant defensins for diseases control, it is crucial to elucidate their mechanisms of action (MOA). With the advent of advanced microscopy techniques, live-cell imaging has become a powerful tool for understanding the dynamics of the antifungal MOA of plant defensins. Here, a confocal microscopy based live-cell imaging method is described using two fluorescently labeled plant defensins (MtDef4 and MtDef5) in combination with vital fluorescent dyes. This technique enables real-time visualization and analysis of the dynamic events of MtDef4 and MtDef5 internalization into fungal cells. Importantly, this assay generates a wealth of information including internalization kinetics, mode of entry and subcellular localization of these peptides. Along with other cell biological tools, these methods have provided critical insights into the dynamics and complexity of the MOA of these peptides. These tools can also be used to compare the MOA of these peptides against different fungi.

## Video Link

The video component of this article can be found at <https://www.jove.com/video/55995/>

## Introduction

Plants have evolved a sophisticated innate immune system for defense against the microbial plant pathogens<sup>1</sup>. They express numerous gene-encoded host defense peptides with putative antimicrobial activity<sup>2</sup>. Indeed, many of these peptides display antimicrobial activity *in vitro*<sup>3</sup>. Defensins comprise one of the largest groups of host defense peptides in the plant kingdom<sup>4</sup>. These cysteine-rich, cationic peptides exhibit potent growth inhibitory activity against fungal and oomycete pathogens at micromolar concentrations and represent one of the first lines of defense against these pathogens<sup>5,6</sup>. Because of their potent antifungal activity, defensins can be exploited in agrobiotechnological applications to generate disease resistant crops. Constitutive overexpression of several plant defensins has been shown to enhance disease resistance in the greenhouse and field tests of transgenic crops<sup>6</sup>. It is important to unravel the mechanisms of action (MOA) of these antifungal peptides in order to fully harness their potential as effective tools for crop protection. However, the MOA of these plant defensins are poorly understood. Current evidence suggests that they exhibit different MOA<sup>5,6,7,8</sup>. Some defensins act extracellularly on fungi and target specific cell wall/plasma membrane resident sphingolipids, disrupt membrane integrity and activate cellular toxicity pathways<sup>9,10,11</sup>. Recently, however, antifungal defensins that translocate into fungal cells have been discovered<sup>12,13,14</sup>. Some of these defensins bind to membrane-resident bioactive phospholipids, form oligomeric complexes and permeabilize plasma membranes<sup>15,16,17</sup>. Thus, some aspects of the MOA of plant defensins have been elucidated. However, the MOA of plant defensins likely involve a complex set of events which have not yet been identified and integrated into a comprehensive model. In particular, there remains a major gap in our understanding of the cellular targets of these peptides.

With recent advances in microscopy technologies and the development of new fluorescent probes, live-cell imaging techniques are now frequently used to study the MOA of antimicrobial peptides (AMPs). These techniques complement widely used methods such as immunolocalization, electron microscopy, atomic force microscopy or X-ray tomography<sup>18</sup>, which have been employed mostly to analyze the effects of antifungal peptides on the morphology and growth of fungal cells including the study of cell wall integrity, alterations in cell growth/branching patterns, as well as plasma membrane permeabilization and killing. Nevertheless, these studies have been limited to imaging cells at a certain time point after treatment with the peptides instead of performing time-lapse imaging on the same cells to monitor their dynamic changes in response to defensin challenge. In recent years, use of fluorescently labeled peptides in conjunction with live cell imaging using confocal microscopy has enabled real-time visualization of the dynamics of AMP-microbe interactions. Both naturally purified and chemically synthesized antifungal peptides can be tagged with fluorescent labels (e.g., DyLight, rhodamine, BODIPY, or Alexa Fluor based dyes) and directly observed during their interaction with cells by time-lapse live-cell imaging. The use of these labeled peptides has significantly increased

our understanding of the different aspects of their MOA including mode of entry, subcellular localization, intracellular trafficking, and sites of antifungal action within living fungal cells<sup>18</sup>.

Recently, several studies have shown that various antifungal peptides including plant defensins are internalized by living fungal cells<sup>12,14,19,20</sup>. The MOA of these defensins likely involve interaction with intracellular targets. We have recently reported the antifungal action of a plant defensin MtDef4 in two ascomycete fungi, *Neurospora crassa* and *Fusarium graminearum*. MtDef4 was shown to use different pathways for fungal cell entry and subcellular localization in these fungi<sup>14</sup>. This study used chemically synthesized tetramethyl rhodamine (TAMRA)-labeled MtDef4 in combination with vital fluorescent dyes (the membrane selective dye, FM4-64; membrane-permeant dye, SYTOX Green; the cell death reporter dye, propidium iodide) and metabolic inhibitors. These analyses demonstrated the kinetics of the internalization of MtDef4, its mechanisms of intracellular transportation and its subcellular targets<sup>14</sup>.

Here, a protocol for live-cell imaging using confocal microscopy is presented. The protocol utilizes fluorescently labeled peptides in combination with vital fluorescent dyes to study plant defensin-fungal interactions, in particular, the pathways of translocation and the intracellular targets of defensins in fungal cells.

## Protocol

### 1. Labeling of Defensins with Fluorophores

1. Select a fluorophore that has minimum effect on the antimicrobial properties as well as the uptake and localization of defensin inside the living cell.  
NOTE: Selecting optimal fluorophore depends on specific experimental objectives. The spectral and chemical properties, photostability, size and charge of the fluorophore should also be considered.
2. Label defensin with the selected fluorophore using an appropriate peptide labeling kit available from commercial vendors. Alternatively, chemically synthesize fluorophore-labeled defensins. In this study, label MtDef5 defensin with DyLight550 using a commercial labeling kit according to manufacturer's protocol and tetramethyl rhodamine (TAMRA)-labeled MtDef4 defensin was chemically synthesized commercially.  
NOTE: It is recommended that chemically synthesized fluorophore tagged peptides (especially for short peptides of 50 amino acids or less) be used since the fluorophore can be attached to the N- or C-terminal residue of the peptide to ensure minimum effect on its antimicrobial activity. Synthesis of long peptides with more than three disulfide bonds is challenging. In this case, it is recommended that peptide be labeled with an optimal fluorophore using a suitable commercially available peptide labeling kit.
3. Test *in vitro* antimicrobial activity of the labeled defensin and determine the minimal inhibitory concentration (MIC) against the fungi to be investigated.  
NOTE: In this study, the MIC of DyLight550-MtDef5 and TMR-MtDef4 was determined against *Neurospora crassa* and *Fusarium graminearum*.

### 2. Fungal Cultures and Growth Medium

1. Transfer conidia from a *N. crassa* stock culture to slant tube containing Vogel's agar medium<sup>21</sup> and incubate at room temperature under light for 5 days.
2. Culture *F. graminearum* PH-1 strain on plates containing complete medium (CM)<sup>22</sup> at 28 °C for 5 days. For production of conidia, inoculate 4 (10 mm diameter) plugs of 5 day old culture of *F. graminearum* PH-1 into 50 mL of carboxymethyl cellulose (CMC) medium (15 g carboxymethyl cellulose, 1 g Yeast extract, 0.5 g MgSO<sub>4</sub>·7 H<sub>2</sub>O, 1 g NH<sub>4</sub>NO<sub>3</sub>, and 1 g KH<sub>2</sub>PO<sub>4</sub>) and culture for 4-7 days at 28 °C in a rotary shaker at 180 rpm.

### 3. Preparation of Conidial Suspension

1. ***F. graminearum* conidial suspension**
  1. Vortex the *F. graminearum* liquid culture and collect conidia by filtering 1.5 mL fungal culture through two layers of filtration material (such as Miracloth) into a 2 mL microcentrifuge tube. Centrifuge the conidial suspension at 13,226 x g speed in a microcentrifuge for 2 min.
  2. Discard the supernatant, and add 1 mL of sterile water to wash the pellet.
  3. Centrifuge the conidial suspension at 13,226 x g speed in a microcentrifuge for 2 min. Discard the supernatant and resuspend the pellet in 1 mL of 2X SFM (Synthetic Fungal Media)<sup>23</sup>.
  4. Count the conidia using a hemocytometer under a light microscope.
  5. Adjust the conidial suspension to 10<sup>5</sup> conidia/mL with 2X SFM.
2. ***N. crassa* conidial suspension**
  1. Transfer a small amount of growing culture (5 day old) of *N. crassa* using an inoculation loop to a microcentrifuge tube containing 2 mL of Vogel's liquid medium.
  2. Vortex the conidial suspension and filter through filtration material into a new microcentrifuge tube.
  3. Centrifuge the conidial suspension at maximum speed in a microcentrifuge for 2 min. Discard the supernatant and resuspend the conidial pellet in 1 mL of Vogel's liquid medium.
  4. Count the conidia using a hemocytometer under a light microscope.
  5. Adjust the conidial suspension to 10<sup>5</sup> conidia/mL with Vogel's liquid medium.

## 4. Sample Preparation and Confocal Microscopy

### 1. Subcellular localization of MtDef4 defensin into fungal cells

1. Pipette 50  $\mu$ L of each conidial suspension ( $10^5$  conidia/mL) into the 10 mm microwell of 35 mm glass bottom microwell dishes. For the observation of conidia, directly proceed to the next step, or for germlings preparation, pipette 50  $\mu$ L of *N. crassa* and *F. graminearum* conidia into the 35 mm culture dishes and let germinate for 3-6 h at room temperature.
2. Add 50  $\mu$ L of fluorescently labeled defensin at the MIC to each microwell dish and incubate for 2.5 h. The MIC of the fluorophore-labeled defensins used here (step 1.3) is 3  $\mu$ M for both *N. crassa* and *F. graminearum* and the MIC of TMR-MtDef4 for *N. crassa* and *F. graminearum* was 1  $\mu$ M and 12  $\mu$ M, respectively.
3. Add 2  $\mu$ L of membrane-selective dye FM4-64 (final concentration: 5  $\mu$ M) in culture dish and incubate for 30 min and mount immediately on the confocal microscope for imaging.
4. Select the White Light laser (WLL). Use the two laser sources 488 nm and 550 nm to excite TMR-MtDef4 and FM4-64 dye, respectively. Detect fluorescence of TMR-MtDef4 at 580-700 nm and detect FM4-64 dye at 690-800 nm.  
NOTE: Perform confocal microscopy in the dark room.

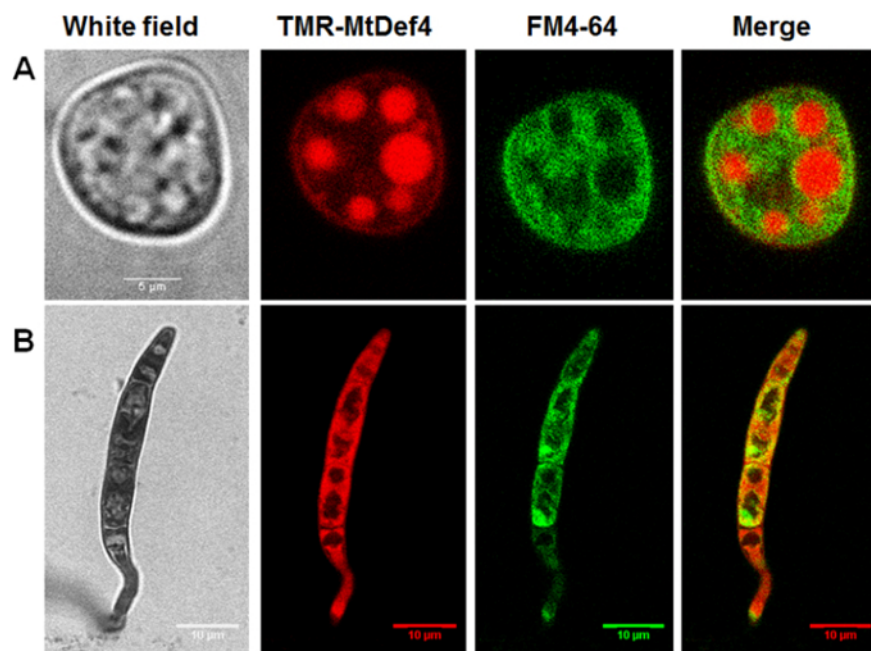
### 2. Time lapse imaging of internalization of MtDef5 defensin

1. Pipette 50  $\mu$ L of *N. crassa* and *F. graminearum* conidial suspension ( $10^5$  conidia/mL) into 10 mm microwell of 35 mm glass bottom microwell dishes. The 10 mm microwell is uncoated and has a No. 1.5 cover glass at the bottom. For the observation of conidia, directly proceed to the next step. For observation of germlings, incubate conidia for 3-6 h at room temperature before proceeding with the microscopy.
2. Turn on WLL. Select the laser line at 550 nm to excite the fluorophore-labeled defensins used here (step 1.3) and FM4-64 with 1.00% intensity and activate the corresponding detectors. The fluorophore-labeled defensins used here (step 1.3) was detected at 560 - 600 nm and the FM4-64 dye was detected at 690 - 800 nm.  
NOTE: Use low laser intensity for live cell imaging as high laser intensity can cause damage to the live fungal cells.
3. Mount the microwell dish on the microscope. Find the cells using 10 x objectives initially, then switch to 100x/1.44 OIL objective for higher magnification.  
NOTE: Set the scan mode to xyz (combination of Z-stack and time-lapse modes), Z position, Zoom, frequency of image capture, etc. before proceeding to the next step.
4. Add 50  $\mu$ L of the fluorophore-labeled defensins used here (step 1.3) of at the MIC of 3  $\mu$ M and 2  $\mu$ L of membrane-selective dye FM4-64 (final concentration 5  $\mu$ M) to each microwell dish, and start time-lapse imaging. Place small pieces of wet filter papers in the microwell dishes to prevent evaporation. The frequency of image capture was 3 min 30 s and the total period was 2 h 30 min.  
NOTE: Adding defensin and fluorophore after mounting microwell dish in the microscope can defocus the region of interest. Therefore, after adding defensin and fluorophore to the microwell, region of interest needs to be refocused which can cause delay in image acquisition.
5. Use a confocal microscope for all confocal imaging and carry out the microscopy at room temperature in a dark room.

## Representative Results

Live cell imaging was carried out to track and compare the internalization and subcellular localization of two defensins, MtDef4 and MtDef5, from *Medicago truncatula*; in fungal cells. TMR-MtDef4 was chemically synthesized while MtDef5 was labeled with Dylight550 (Dylight550-MtDef5). Conidia were incubated with either defensin in combination with the membrane selective dye FM4-64. **Figure 1** shows that TMR-MtDef4 has different trafficking pathways in *N. crassa* compared to *F. graminearum*. In *N. crassa*, the FM4-64 does not co-localize with the defensin but rather stains the membranes of vacuoles within which the defensin is sequestered. In *F. graminearum*, on the other hand, TMR-MtDef4 is not localized within any specific membrane bound organelles but is diffused in the cytoplasm (**Figure 1**).

Time lapse imaging of *N. crassa* cells labeled with both the fluorophore-labeled defensins used here (step 1.3) and FM4-64 shows that the defensin is able to enter fungal cells within 30 to 40 min of treatment (**Figure 2**). Upon entry into the cells, the fluorophore-labeled defensins used here (step 1.3) does not localize within any specific organelle but readily diffuses into the cytoplasm. This is in contrast to TMR-MtDef4 which enters *N. crassa* cells and remains trapped within the vesicular bodies even after 3hrs of treatment (**Figure 1**).



**Figure 1: MtDef4 has different trafficking pathways in *N. crassa* and *F. graminearum*.** TMR -MtDef4 localizes into vesicular bodies in *N. crassa* (A) but is diffused into the cytoplasm of *F. graminearum* (B). Conidia of *N. crassa* and *F. graminearum* were co-labeled with 1 μM and 12 μM TMR-MtDef4 (red), respectively, and with FM4-64 (green). Images were taken after 3 h of treatment. [Please click here to view a larger version of this figure.](#)



**Figure 2: MtDef5 is internalized by *N. crassa* and diffuses inside the cells.** DyLight550-MtDef5 is internalized into *N. crassa* cells and diffuses into the cytoplasm. *N. crassa* cells were co-labeled with 3 μM DyLight550-MtDef5 (red) and FM4-64 (green). Video was recorded for 2 h and 30 min. The delay between the addition of MtDef5 and starting image acquisition was 5 min. Scale bar = 4 μm. [Please click here to view this video.](#) (Right-click to download.)

## Discussion

In this study, a reliable live-cell imaging methodology with the use of fluorescently labeled antifungal defensins was described to study the kinetics of the internalization of these peptides into fungal cells and to determine their subcellular targets. This method is a powerful tool to visualize the dynamics of the interaction between defensins and fungal cells temporally and spatially.

Various methods have been used to study the internalization and intracellular localization of plant defensins in fungal cells. In these methods, defensin-treated cells are usually fixed and then processed for immunolocalization, electron microscopy, or X-ray tomography<sup>15,24,25</sup>. In addition, most of these techniques have been restricted to imaging cells at a specific time point rather than in real time using time-lapse imaging of the same living cells to monitor the dynamic changes taking place in response to defensin challenge. The ability to visualize, analyze and compare the dynamic events taking place in fungal cells in real time during the antifungal action of defensins makes this technique effective and exciting. In addition, it provides better understanding of how the dynamic intracellular localization of the peptide affects the morphogenesis of individual fungal cells with time.

One of the important aspects of this method is determining the subcellular targets using fluorescently labeled peptides along with vital fluorescent dyes (e.g. FM4-64;SG). Subcellular localization determines the environment in which a peptide operates, and represents an important step toward elucidating its interaction partners, function, and potential role(s) in the cellular machinery<sup>26,27</sup>.

A minor limitation of this technique is that the fluorescent peptide often exhibits reduced antifungal activity compared with the unlabeled peptide. If the peptide is labeled using a commercially available peptide labeling kit and shows complete loss of antifungal activity, a chemically synthesized peptide labeled with a small fluorophore at its N- or C-terminus is recommended.

In summary, live cell imaging of fungal cells challenged with a fluorophore-tagged defensin is an effective tool that can provide direct, high spatio-temporal resolution of the MOA of an antifungal defensin. For more precise MOA study, this technique can be combined with fluorescence lifetime imaging microscopy (FLIM)<sup>28</sup> which will allow measuring the interaction kinetics of a peptide with other peptides, or with other labeled molecules or cellular constituents in real time. This will enrich our understanding of the ways antimicrobial peptides work thereby speeding up their development as antifungal agents for use in agriculture and medicine.

## Disclosures

The authors have nothing to disclose.

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