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TITLE:

Quantitative Analysis of *Aspergillus nodulins* Growth Rate using Live Microscopy and Open-Source Software

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Keywords:

Aspergillus, growth rate, fungi, live cell imaging, ImageJ

Summary:

We present a label-free live imaging protocol using transmitted light microscopy techniques to capture images, analyze and quantify growth kinetics of the filamentous fungus *A. nidulans* in both submerged cultures and solid media. This protocol can be used in conjunction with fluorescence microscopy.

Abstract:

It is well established that colony growth of filamentous fungi, mostly dependent on changes in hyphae/mycelia apical growth rate, is macroscopically estimated on solidified media by comparing colony size. However, to quantitatively measure the growth rate of genetically different fungal strains or strains under different environmental/growth conditions (pH, temperature, carbon and nitrogen sources, antibiotics, etc.) is challenging. Thus, the pursuit of complementary approaches to quantify growth kinetics becomes mandatory in order to better understand fungal cell growth. Furthermore, it is well-known that filamentous fungi, including Aspergillus spp., have distinct modes of growth and differentiation under subaerial conditions on

solid media or submerged cultures. Here, we detail a quantitative microscopic method for analyzing growth kinetics of the model fungus *Aspergillus nidulans*, using live imaging in both submerged cultures and solid media. We capture images, analyze, and quantify growth rates of different fungal strains in a reproducible and reliable manner using an open source, free software for bio-images (e.g., Fiji), in a way that does not require any prior image analysis expertise from the user.

Introduction:

Filamentous fungi are of great socioeconomic and ecological importance, being both crucial as industrial/agricultural tools for enzyme and antibiotic production[1,2] and as pathogens of crop plants[3], pest insects[4] and humans[3]. Moreover, filamentous fungi such as *A. nidulans* are widely used as model organisms for fundamental research, such as studies in genetics, cell and evolutionary biology as well as for the study of hyphal extension[5]. Filamentous fungi are highly polarized organisms that elongate through the continuous supply of membrane lipids/proteins and the *de novo* synthesis of cell wall at the extending tip[6]. A central role in the hyphal tip growth and polarity maintenance is a specialized structure named 'Spitzenkorper' (SPK), a highly ordered structure consisting mostly of cytoskeletal components and the polarized distribution of the Golgi[6–8].

Environmental stimuli/signals, such water-air interface, light, CO_2 concentration, and the nutritional status are responsible for the developmental decisions made by these molds [9]. In submerged (liquid) cultures the differentiation of *A. nidulans* is repressed and growth occurs by hyphal tip elongation[6]. During vegetative growth, asexual spores (conidia) germinate by apical extension, forming an undifferentiated network of interconnected hyphal cells, the mycelium, which may continue to grow indefinitely as long as nutrients and space are available. On the other hand, on solid media hyphal tips elongate and after a defined period of vegetative growth (developmental competence), asexual reproduction is initiated and aerial conidiophore stalks extend from specialized foot cells of the mycelium[6]. These give rise to specialized developmental multicellular structures called conidiophores, which produce long chains of haploid conidia[10] that can restart growth under favorable environmental conditions.

A widely used method for measuring filamentous fungal growth is to inoculate spores on nutrient agar contained in a Petri dish and macroscopically measure the diameter of the colony a few days later[11]. The diameter/area of the colony, most dependent on changes in mycelial growth rate and less on conidiophore density[12], is then used as a value of growth. Although, measuring fungal population (colony) size growing on solid surfaces is quite adequate, it is by no means the most accurate measure of growth. Compared to population level averages (averages of fungal colony size), single cell measurements can capture the heterogeneity of a cell population and allow identification of novel sub-populations of cells, states[13], dynamics, pathways as well as the biological mechanisms by which cells respond to endogenous and environmental changes[14, 15]. Monitoring fungal cell growth and phenotype by time-lapse microscopy is arguably the most widely employed quantitative single cell observation approach.

Herein, we detail a label-free live imaging protocol using transmitted light microscopy techniques

(such as phase-contrast, differential interference contrast (DIC), and polarized microscopy) to capture images, which independently from the combined use of fluorescence microscopy can be employed to analyze and quantify polar growth of *A. nidulans* strains in both submerged cultures and solid media.

Protocol:

1. Inoculum preparation

NOTE: All steps should be performed under a laminar flow cabinet.

1.1. Streak out fungal strain of interest, from a glycerol stock (-80 °C) using a sterile inoculation loop, onto plates of minimal media (MM) supplemented with the appropriate nutritional requirements relevant to the strain examined. [MM: 10.0 g/L glucose, 20 mL/L salt solution (salt solution: 26 g/L KCl, 26 g/L $MgSO_4 \cdot 7H_2O$, 76 g/L KH_2PO_4, 2.0 mL/L chloroform) and 1 mL/L trace elements (trace elements: 40 mg/L $Na_2B_4O_7 \cdot H_2O$, 400 mg/L $CuSO_4$, 8 g/L $ZnSO_4$, 800 mg/L $MnSO_4$, 800 mg/L $FePO_4$), adjust pH to 6.8 with 1 M NaOH, add 1 % (w/v) agar and the required supplements as described by[16] and autoclave] (**Figure 1**).

NOTE: Salt solution, trace elements solution and supplements are autoclaved.

1.2. Incubate for 2-3 days at 37 °C.

1.3. Use a sterile toothpick (or an inoculation loop), transfer a small number of conidia, by gently touching a single colony, to plates of complete media (CM) [CM: 10.0 g/L glucose, 2.0 g/L peptone, 1.0 g/L yeast extract, 1.0 g/L casamino acids, 20 mL/L salt solution, 1 mL/L trace elements, 5 mL/L vitamin solution (vitamin solution: 0.1 g/L riboflavin, 0.1 g/L nicotinamide, 0.01 g/L p-amino benzoic acid, 0.05 g/L pyridoxine HCl, 1.0 mg/L biotin), adjust pH to 6.8 with 1 M NaOH, add 1 % (w/v) agar and the required supplements as described by [16] and autoclave]. Autoclave and store the vitamin solution in a dark bottle at 4 °C.

NOTE: In case fungal growth is too dense to identify and isolate individual colonies, re-streak onto a new agar plate to obtain single colonies.

1.4. Incubate for 3-4 days at 37 °C.

1.5. Obtain a conidial suspension of approximately 2 x 10^6 cells/mL by scratching 1 cm from the surface of a conidiated fungal colony grown on CM agar plates, using a sterile toothpick (**Figure S1**).

NOTE: When necessary, count conidia with a hemocytometer.

1.6. Harvest conidia of *A. nidulans* in a sterile 1.5 mL centrifuge tube with 1.0 mL of autoclaved distilled water containing 0.05% (v/v) Tween 80 for reducing the number of conidia clumps.

NOTE: Conidia can be stored for up to 2-3 weeks at 4 °C without a relevant loss of viability (A. Athanasopoulos and V. Sophianopoulou, unpublished data). However, it is recommended to filter and/or to wash conidial suspension to remove mycelial parts and nutrients, in order to prevent conidial swelling.

2. Preparation for imaging filamentous fungi growing on agar (solid) mediums

NOTE: A modified version of the 'inverted agar method[17, 18] is used.

2.1. Initially, spot 10 µL aliquots of vigorously vortexed conidial (approximately 2 x 10^4 cells/mL) at several points onto Petri dishes (Ø9 cm) 15 mL of MM with 1% (w/v) agar (**Figure 2**).

NOTE: Using the modified version of the 'inverted agar method', it is possible to image fungal samples for many hours without apparent deleterious effects on growing hyphae.

2.2. Incubate the experimental culture according to the developmental stage intended to be investigated.

2.3. Slice out a ≈0.8 mm^2 block of agar containing the colony using a sterile scalpel.

NOTE: The dimensions of the agar block to be sliced out depends on the dimensions of the equipment to be placed afterwards. In the present work, 8 well µ-slides are used (see below).

2.4. Invert and place the agar block into a well of an µ-slide or similar 8 chambered coverglass with coverslip suitable for live imaging.

NOTE: In case transmitted light microscopy will be used in combination with fluorescence (labeling) microscopy, the agar block can be inverted onto a droplet of liquid medium containing the live cell staining dye, just before imaging.

3. Preparation for imaging filamentous fungi growing on liquid medium

3.1. Transfer 10 µL aliquots of a vigorously vortexed conidial suspension (approximately 2 x 10^4 cells/mL) in the wells of an 8 well µ-slide containing 200 µL of (liquid) MM with the appropriate supplements (see above).

3.2. Incubate for the desired time at the desired temperature (**Figure 3**).

NOTE: If transmitted light microscopy will be used in combination with fluorescence (labeling) microscopy, liquid cultures have the great advantage that fluorescent dyes can be added at any desired time point during the experiment[19].

4. Capture images

NOTE: The choice of microscope depends upon the available equipment. In any case the microscope setup should include an inverted stage, an environmental chamber or at least a room with precise air temperature control.

4.1. Preheat the thermostated microscope chamber at 37 °C (unless otherwise indicated or as suitable for the used fungal species) to stabilize the temperature before starting. This chamber allows temperature modulation of the microscope optics and sample stage during time-lapse experiments. Be aware that optical aberrations[20] are introduced when normal immersion (designed for use at 23 °C) oils are used at 37 °C or above.

NOTE: On a tight budget, incubation chambers can be made out of cardboard and insulating packing material[21] or by using a 3D printer[22].

4.2. Turn on the microscope, the scanner power, the laser power and computer, and load the imaging software. Place the µ-slide (prepared previously) in the microscope stage and focus.

4.3. Find fields of view that contain isolated/not overlapping cells (or at least not overcrowded), in order to facilitate growth measurements during image analysis. Capture at least 50 growing cells per samples to allow robust statistical analysis.

4.4. Select the desired transmitted light microscopy approach. Reduce the exposure time or laser power and pixel dwell time and/or increase pinhole diameters, in order to minimize photobleaching of fungal cells, as described elsewhere [23, 24].

4.5. Set microscope to acquire images at desired time intervals and start time series acquisition.

NOTE: To correct for focal drift over time (especially for long experiments), due to thermal drift, diverse cell sizes, and cell motion use an autofocus strategy if is available in your microscope software.

5. Image Analysis

NOTE: This section describes the key steps of processing time-lapse microscopy images for measuring growth rate of *A. nidulans*. Opening, visualization and processing of images is accomplished with the open source ImageJ/Fiji software[25].

5.1. Import the images to Fiji using **Plugins | Bio-Formats | Bio-Formats Importer** from the Fiji menu with default settings (**Figure 4A**).

NOTE: Check whether the Bio-Formats Importer properly recognize the image calibration. The picture dimension shown in the upper information field image window, must be equal to the original picture dimensions (**Figure 4B**). Press **Shift + P** to display and change image properties in

ImageJ/Fiji software.

5.2. Where needed, use histogram matching [26] for illumination correction between different frames (**Image | Adjust | Bleach Correction | Histogram Matching**) (**Figure 4C**).

5.3. Where needed, use SIFT-algorithm (Pl**ugins | Registration | Linear Stack Alignment with SIFT**) for aligning or matching image stacks (**Figure 4D**). Selecting "**Translation**" from the expected transformation menu should be enough to correct any x-y drift.

NOTE: Other plugins can be also used to align a stack of image slices, such as Image Stabilizer (https://imagej.net/Image_Stabilizer) or StackReg (http://bigwww.epfl.ch/thevenaz/stackreg/).

5.4. Select hyphae that grow parallel to coverslip, avoiding those that are tilted. Be sure to select hyphae that propagate by polar extension and avoid hyphae presenting lateral and/or apical branching.

5.5. Use MTrackJ (**Plugins | MTrackJ**) plugin to track growing hyphal tips (**Figure 4E**) [27]. To add a track, select the **Add** button in the toolbar and place the first point at a hyphal tip using the left click of the mouse. The time series will automatically move to the next frame. To complete the tracking process, double click the mouse on the final point (or press the Esc key) (**Figure 4F**). Move to another point of interest (i.e., growing hyphal tip) in the initial time frame and restart the procedure by measuring growth rate of another hypha.

NOTE: To install MTrackJ follow the instructions presented at https://imagescience.org/meijering/software/mtrackj/

5.6. Click the **Measure** button in the MTrackJ dialog (**Figure 4G**) to open the output table. Save track measurements (**File | Save As**) to the desired file format (e.g., csv), analyze and plot them (**Figure 4H**).

NOTE: By selecting the **Movie** button, a movie is produced showing the image and track progression.

Representative Results:

Following this protocol, we captured and analyzed various images corresponding to different growth/developmental stages of the filamentous fungus *A. nidulans*. The data presented in this study were processed and analyzed using the Fiji software. Measurements were saved as csv files, statistically analyzed and prepared as graphs using commercial statistical software and/or Python programming language using software libraries like pandas, numpy, statsmodels, matplotlib and seaborn. More details can be found in the cited original publications.

In order to interpret the response of populations, it is important to determine heterogeneity of key parameters within the populations and to efficiently identify physiologically distinct subpopulations[28]. **Figure 5** shows growth of the *azhA*Δ *ngnA*Δ mutant strain, bearing deletions in

two genes, the products of which are implicated in the detoxification and assimilation of the toxic phytoproduct L-azetidine-2- carboxylic acid[29], in comparison with the WT strain. Measuring their growth rate in submerged liquid cultures, we detect a statistically significant lower growth rate of the double mutant compared to the WT strain (t(715) = 20.61, p = <0,0001) (**Figure 5A-B**). This difference in growth was not detectable measuring only the colony area of these strains (**Figure 5C-D**), emphasizing that microscopic single cell analysis is more effective in determining small differences in growth rates that are difficult to detect with macroscopic observation of the colony.

Single cell long-term live imaging is of significant value in the effort to obtain spatial and temporal information of cellular proteins dynamics. Long-term live cell imaging (**Figure 6, Video 1**) of conidial germination co-expressing the GFP-labeled core eisosomal protein PilA and the mRFP histone H1 shows that PilA[30] forms static structures with low mobility at fungal plasma membrane[31, 32]. Moreover, **Figure 7** shows that fluorescent dyes can be used for live imaging of strains grown on agar medium, in order to study dynamics of organelles and cell structures. Here, FM4-64[33] was used to visualize mitochondrial network and the vacuolar system in *A. nidulans.*

Figure 1: Schematic representation of the inoculum preparation procedure.

Figure 2: Schematic representation of the procedure followed for imaging filamentous fungi growing on agar medium.

Figure 3: Schematic representation of the procedure followed for imaging filamentous fungi growing in liquid medium.

Figure 4: Schematic representation of image analysis procedure. Steps for processing time-lapse microscopy images and measuring growth rate of *A. nidulans*.

Figure 5: Comparing traditional population measurements with single cell measurements. (**A**) Representative confocal microscopy images of *azhA*Δ *ngnA*Δ double deletion and WT strains, grown in submerged liquid cultures containing urea as sole nitrogen source at 25 ˚C. All strains were incubated for a total of 18 h, at 25 ˚C and frames were captured at 15 min intervals. Images of 2048 × 2048 pixels were collected with a pixel size of 115.02 x 115.02 nm using a TCS SP8 MP (Leica, Germany) microscope equipped with HC Plan APO 63x, N.A. 1.40 oil immersion objective. (**B**) Measurements of (**A**) are plotted in box-and-whiskers plots. Statistical significance was analyzed via t Test and is depicted with asterisks (***), indicating $p < 0.001$. (**C**) Growth at 25 ˚C of WT and double deletion strain on solid MM. Colonies were photographed at different time intervals (18 h, 36 h and 72 h), spatially calibrated and measured manually with a ruler in ImageJ program. (**D**) Measurements of the area of colonies presented in (**C**) are plotted as box-and-whiskers plots. Differences of the area of colonies were not statistically significant between the different time points of the double mutant compared to the WT strain (18h: t(8) = 0.69, p = 0.50, 36h: t(6) = 0.27, p = 0.5068, 72h: t(6) = 0.39, p = 0.70).

Figure 6: Long-term live cell imaging of conidial germination co-expressing proteins of interest fused with genetically encoded fluorescent tags. (**A**) Maximum intensity projections (MIP) of 4 slices generated in the z plane, of a strain co-expressing PilA-GFP and H1-mRFP. Conidia were spotted on agar medium and incubated for approximately 1 h at 30 ˚C in order to help adhesion of the cells to the agar. The agar block containing conidia was sliced out, placed into wells of a µ-slide and incubated for a total of 18 h, at 30 ˚C. Frames were captured at 25 min intervals, using a TCS SP8 MP (Leica, Germany) microscope equipped with HC Plan APO 63x, N.A. 1.40 oil immersion objective (with a pixel size of 92.26 x 92.26 nm). Images were obtained by exciting GFP at 488-nm wavelength and detecting fluorescence in the spectral band ranging from 495 to 558 nm, and by exciting mRFP at 561-nm wavelength and detecting fluorescence in the spectral band ranging from 580 to 660 nm. (**B**) Measurements (velocity of germination and hyphal tip extension) of (**A**) are plotted as line graph.

Figure 7: FM4-64 labeling of hyphae growing on solid medium. Conidia of a WT strain were cultivated on 1% agar MM Petri dishes for 16 h at 30 °C. The growing germlings were incubated for 20 min with 10 µL of MM containing 10 µM FM4-64. Following incubation, an agar block containing stained germlings was sliced out, placed into 8 µ-slide followed by a 47 min chase at 30 °C. Frames were captured every 7 min at 30 ˚C using a TCS SP8 MP microscope equipped with HC Plan APO 63x, N.A. 1.40 oil immersion objective (with a pixel size of 184.52 x 184.52 nm). Images were obtained by exciting FM4-64 at 514-nm wavelength and detecting fluorescence in the spectral band ranging from 596 to 682 nm. The FM4-64 fluorescence signal is shown as inverted image.

Figure 8: Early stages of conidiophore formation. (**A**) Conidia of WT strain were cultured on agar medium for 136 h at 30 °C, sliced out and placed into wells of a µ-slide. Frames were captured every 20 min. Images of 2048 × 2048 pixels were collected with a pixel size of 245.2 x 245.2 nm using a TCS SP8 MP (Leica, Germany) microscope, equipped with HC Plan APO 63x, N.A. 1.40 oil immersion objective. Images show the formation of conidiophore vesicles (black arrow) as well as the formation (brown arrow) and maturation of metulae (blue arrow). (**B**) Measurements (velocity of germination and unipolar hyphal tip extension) of (A) are plotted as line graph.

Video 1: Long-term live cell imaging of conidial germination co-expressing PilA- GFP and H1-mRFP.

Video 2: Early stages of conidiophore formation in *A. nidulans.*

Figure S1: Preparation of conidial suspension. By scraping with a sterile toothpick an approximately 1 cm^2 surface of a conidiated plate, a suspension of approximately 2 x 10^6 conidia/ml is obtained.

Discussion:

Monitoring fungal cell growth and phenotype by time-lapse microscopy is a powerful approach to assess cellular behavior in real-time and quantitatively and accurately determine whether a particular drug treatment and/or genetic intervention results in detectable cell growth or

phenotypic differences over time.

In this study, a reliable live-cell imaging methodology was described to measure and quantitatively analyze fungal development, including the dynamics of germ tube and hyphal tip growth in *A. nidulans*. Monitoring morphological changes over time, using transmitted light microscopy techniques, can be highly helpful to identify cells that are stressed, dying or dead. A detailed knowledge of such dynamic processes at the single-cell level allows to assess heterogeneity within a mixed population of cells and in a long-time perspective, permits the identification of pathways and detailed mechanisms by which cells respond to endogenous and environmental signals.

One advantage of this method is that it is based on a label free imaging approach (which nevertheless can be easily combined with fluorescence microscopy) that uses inherent light refraction properties of cells to create image contrast without introducing dyes/labels, which may confound the results. While fluorescent markers can be used to tag cellular compartments and proteins and significantly facilitate the segmentation and tracking of cells, transmitted light microscopy circumvents the need for genetically engineering cells, enabling researchers to avoid the cost and the time-consuming dye/label optimization and also to avoid probable phototoxicity effects coming from fluorescence imaging[13, 34].

This protocol is suitable to study growth kinetics of fungi that form hyphae or pseudohyphae. Furthermore, this approach is highly suitable for imaging different cell structures of different strains and under different developmental stages. In **Figure 8** and **Video 2**, we present the initial stages of *A. nidulans* conidiophore (i.e., structures bearing asexual spore) development. It must be noted, however, that, this method is not the most suitable for imaging conidiophore formation, since carbon/nitrogen starvation and air exposure, both necessary for the development of conidiophores, cannot be standardized by our approach[35].

In addition, this protocol is poorly suited to track fungal biofilms, which are subject to vertical extension forming a dense heterogeneous, surface-associated colonies comprised of filamentous hyphae, pseudohyphal cells, yeast-form cells, and various forms of extracellular matrix [36]. Another limitation of the technique is that although label free microscopic analysis represents a precise method for the qualitative and quantitative analysis of germination/differentiation dynamics, manual evaluation of such data is time-consuming especially when too many strains or/and conditions are examined. Thus, data from high-throughput time-lapse imaging microscopy should be analyzed by suitable computational software enabling automated or semi-automated image analysis of hyphal development and conidial germination[37–39].

In summary, herein we present a protocol for analyzing fungal growth kinetics in a reproducible and reliable manner without the need of any prior image analysis experience from the user. This protocol allows objective and accurate quantification of fungal growth and differentiation, and provides a complementary imaging approach to study fungal life cycles and fungal pathogenicity[37].

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The authors have nothing to disclose.

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Figure 1

Click here to access/download;Figure;Figure 1 Revised.png

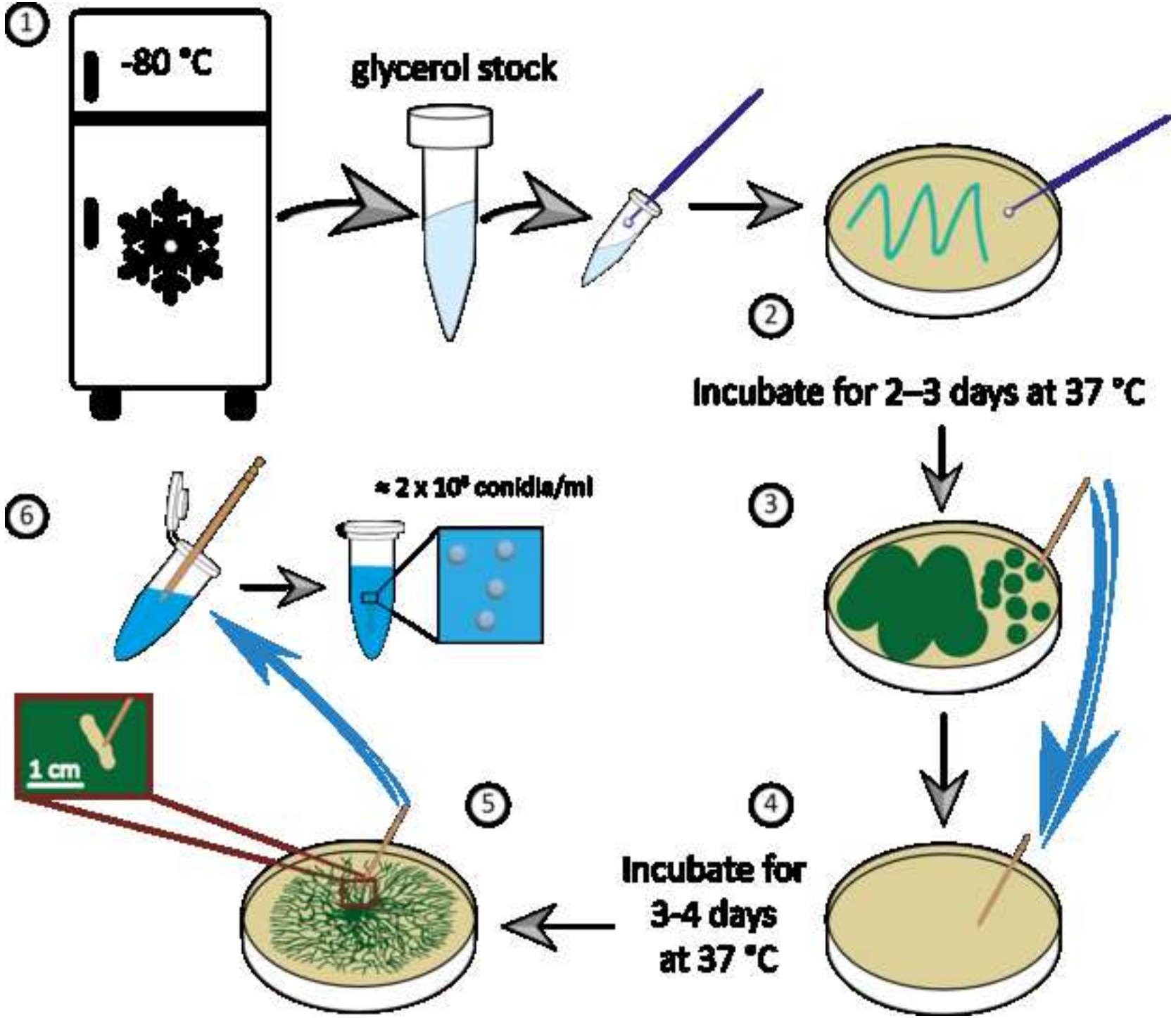


Figure 2

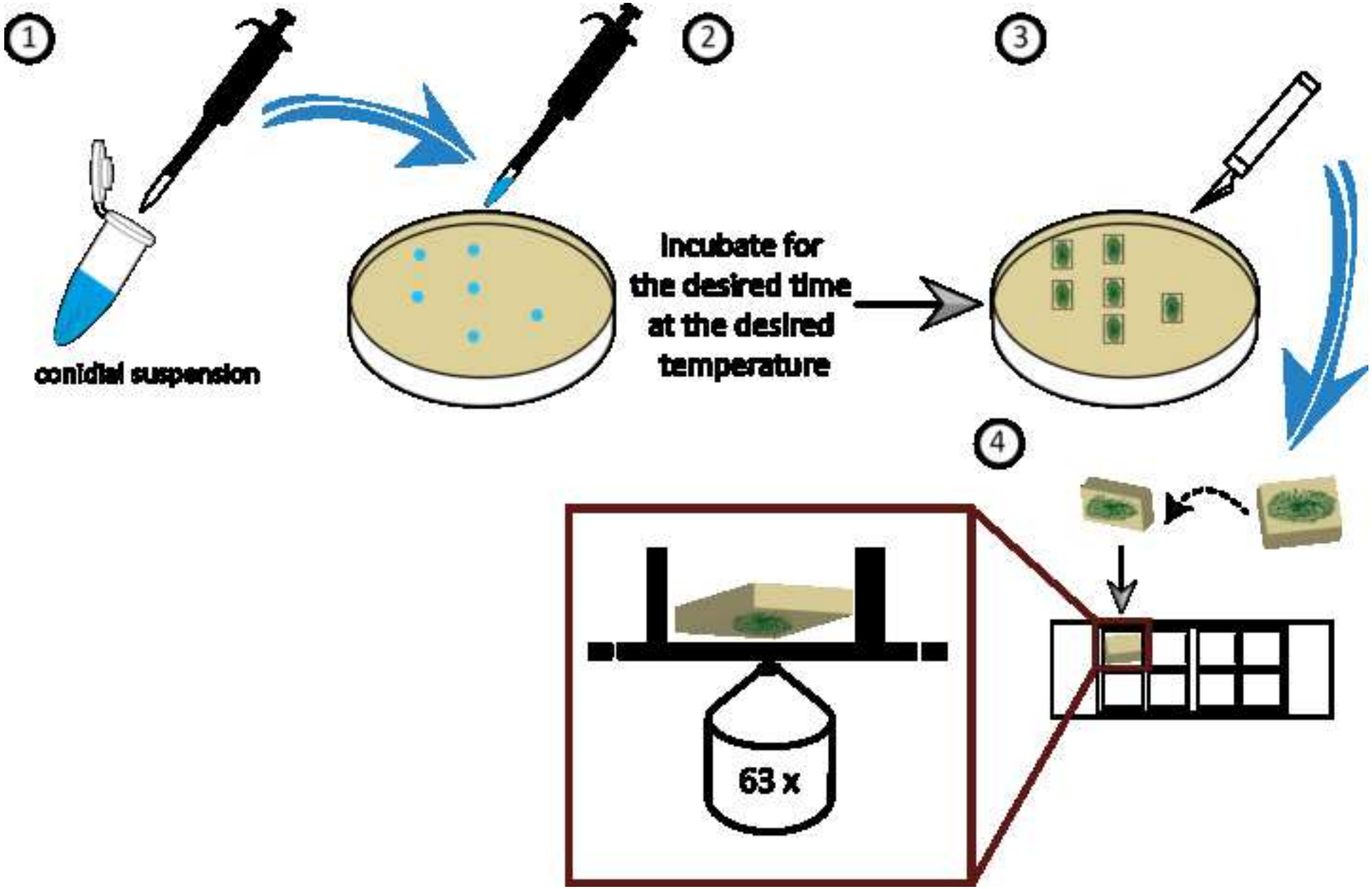


Figure 3

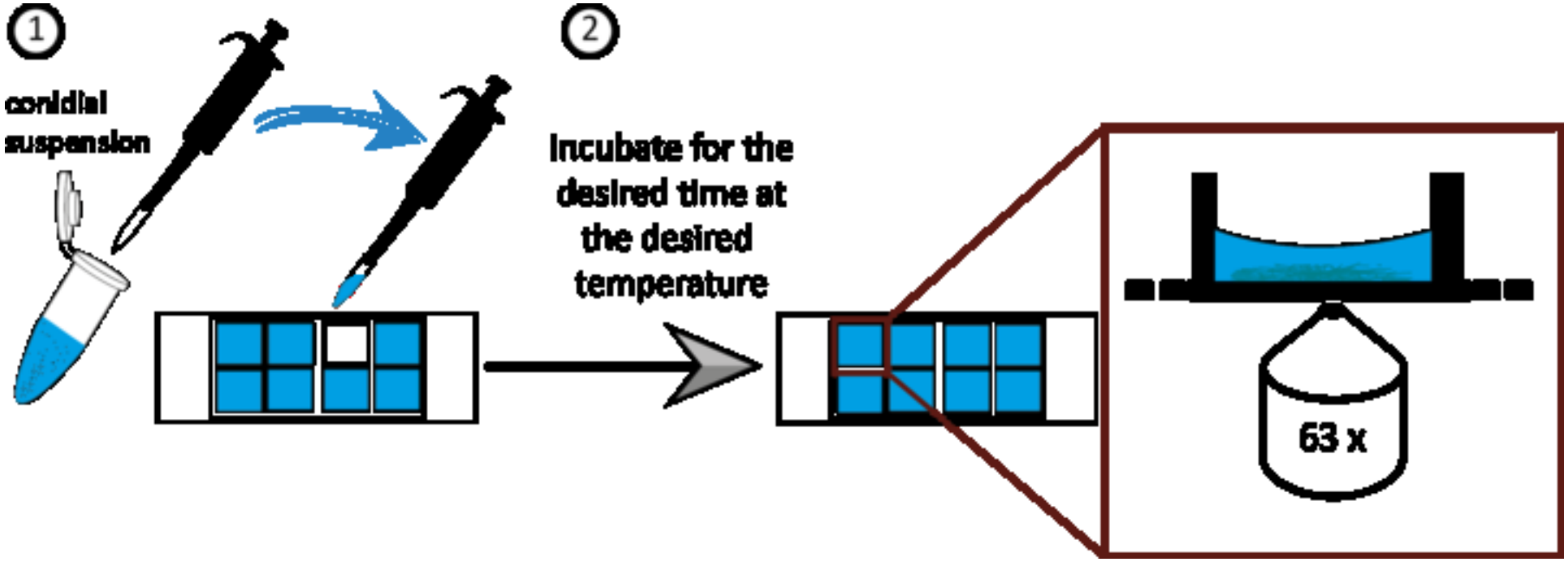


Figure 4

Click here to access/download;Figure;Figure 4 Revised.png

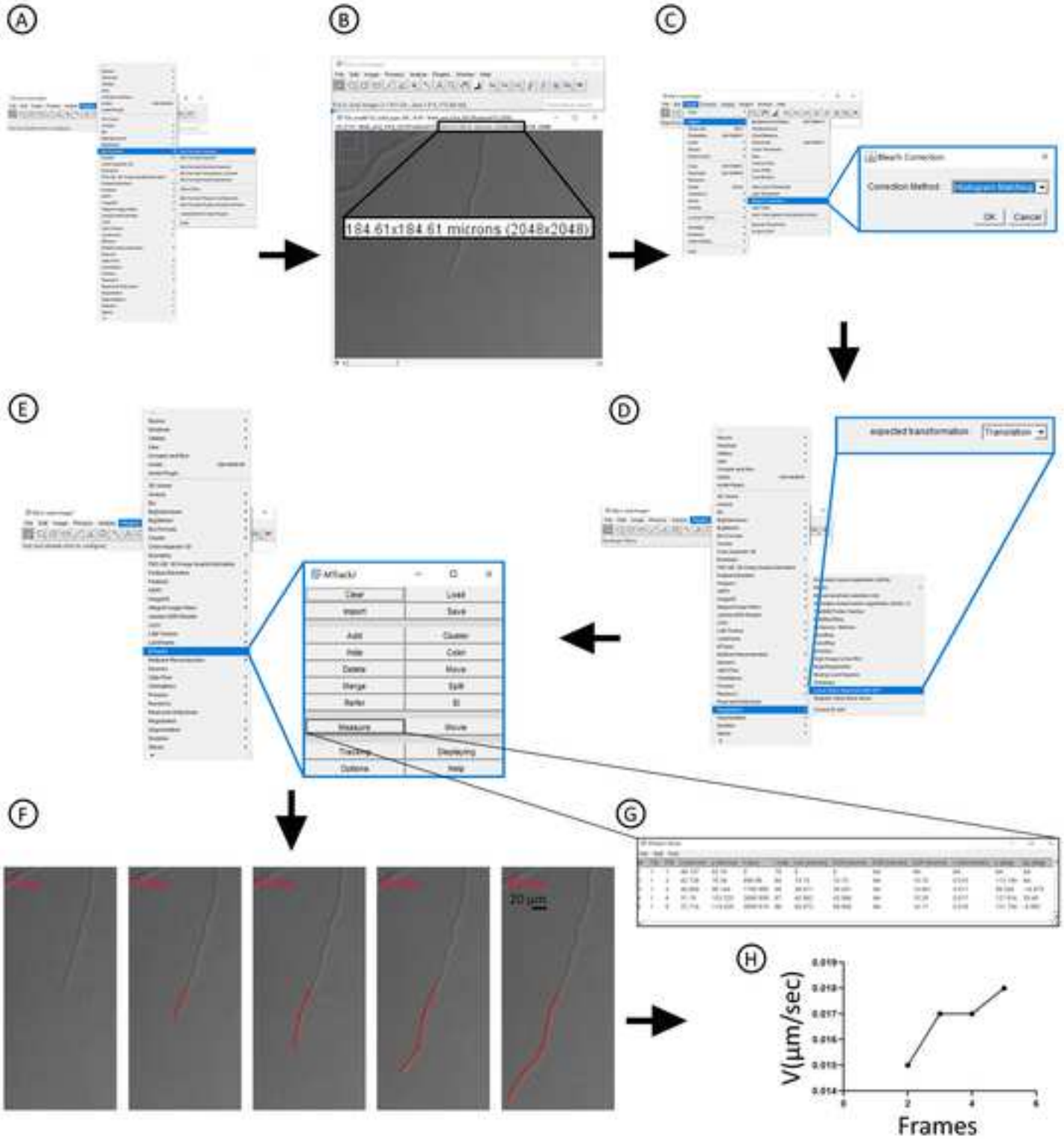


Figure 5

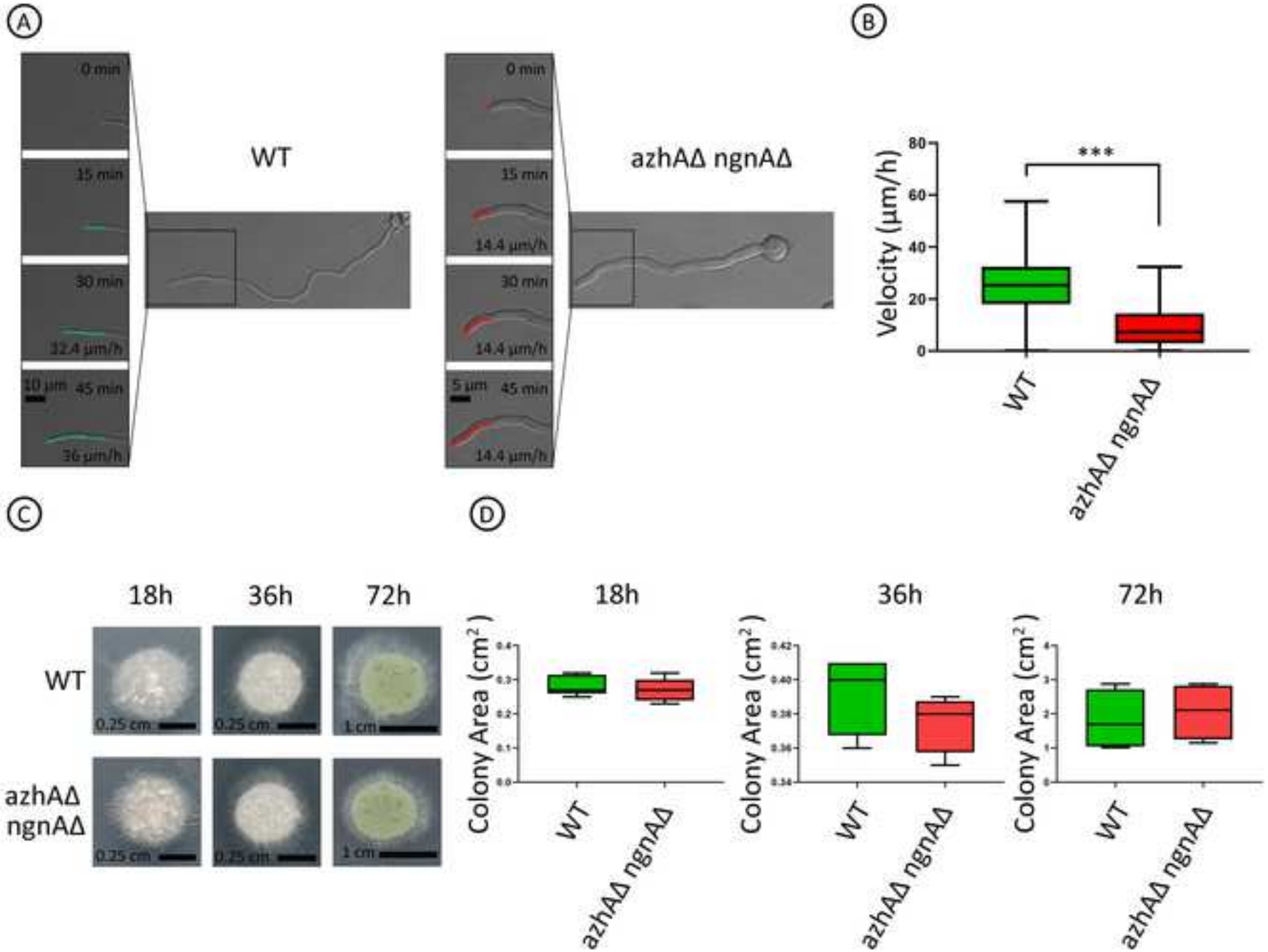


Figure 6

Click here to access/download;Figure;Figure 6 Revised .png

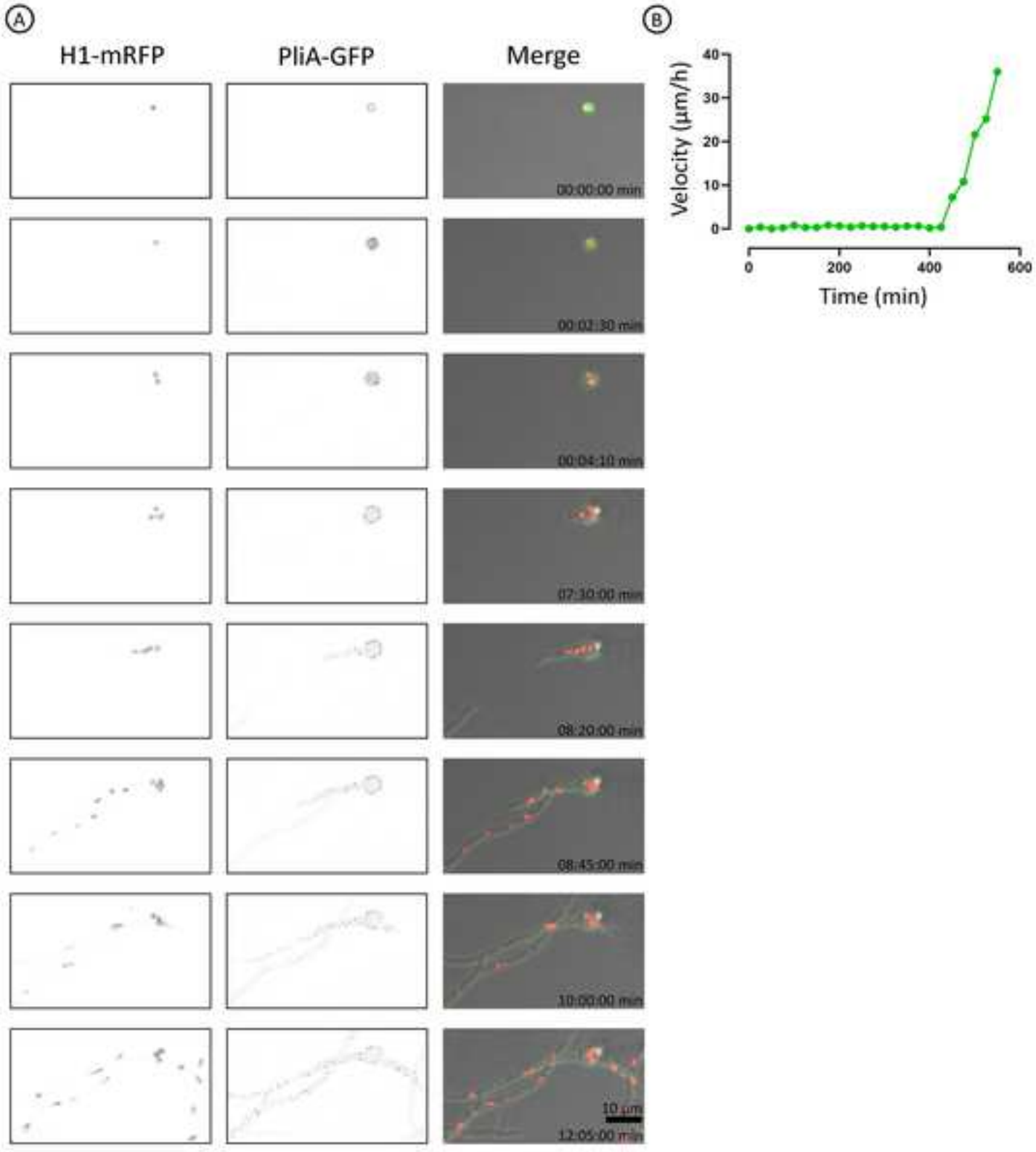


Figure 7

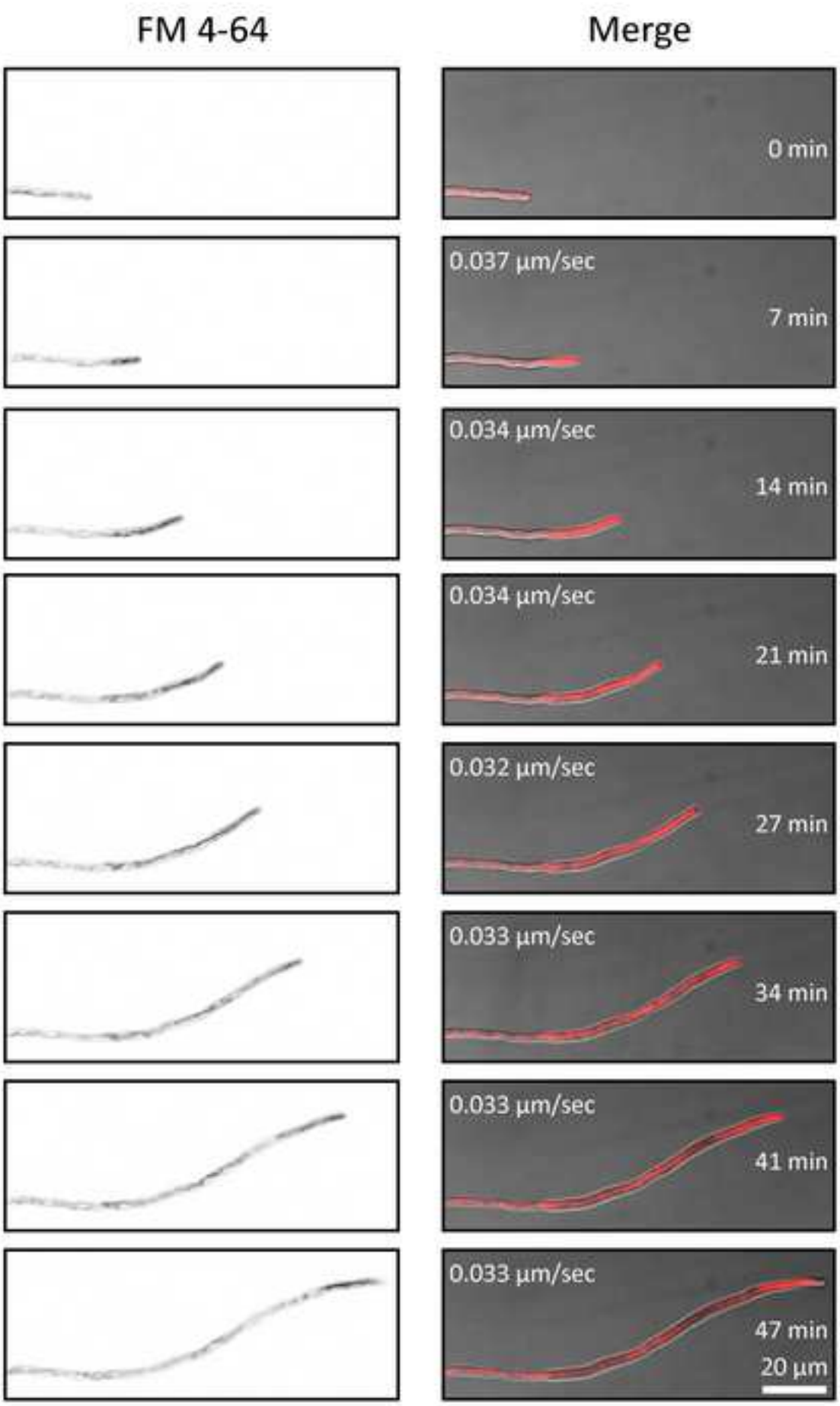
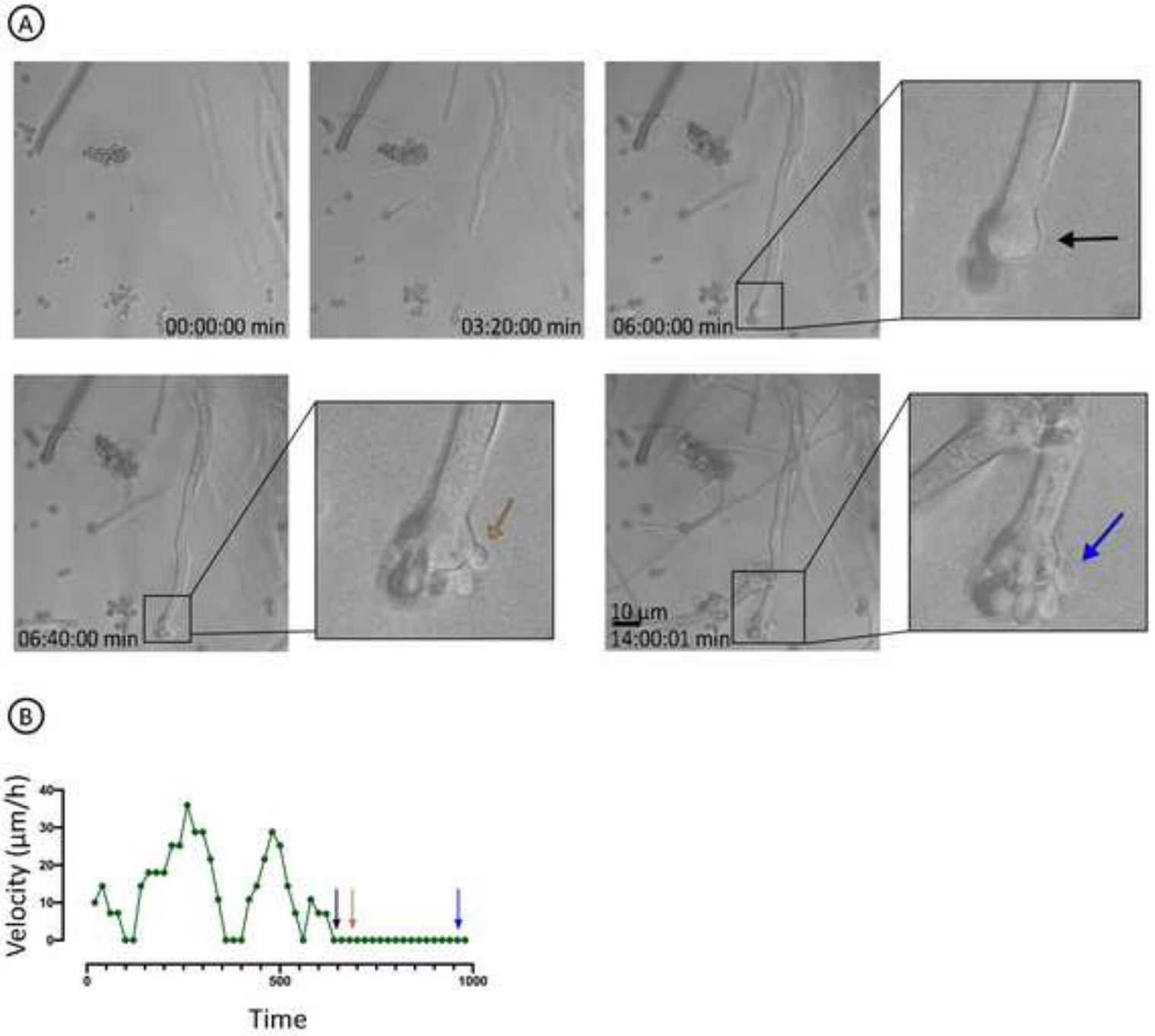


Figure 8



Response to editor and reviewers:

We are deeply grateful to the editor and the 4 reviewers for taking the time to provide quite valuable comments and suggestions for our manuscript entitled "Quantitative analysis of *Aspergillus nidulans* growth rate using live microscopy and a free & open source software." The manuscript has been revised according to the suggestions and comments of the editor and reviewers. We have addressed the editor and reviewers' comments point by point, as seen below. Moreover, we have done thorough English editing and corrected the grammatical mistakes in the revised manuscript.

Response to Editorial and production comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
   Response: The manuscript has been carefully revised.

2. Please provide an email address for each author.
   Response: The email addresses of all authors have been added to the manuscript.

3. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep, and note in the protocol section. Please use Calibri 12 points.
   Response: The manuscript has been formatted accordingly.

4. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary.
   Response: The manuscript has been formatted according to JoVE Instructions for Authors.

5. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
   Response: All commercial products have been moved in the Table of Materials and Reagents.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."
   Response: The text in the protocol section has been corrected and has been

used exclusively the imperative mood.

7. The Protocol should contain only action items that direct the reader to do something.
   Response: The text in the protocol section has been changed accordingly.

8. Only one note should follow one step.
   Response: The text in the protocol section has been changed accordingly (please see also below).

9. In the JoVE Protocol format, “Notes” should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Other details can be moved to the discussion section.
   Response: The number of “Notes” has been reduced significantly.

10. Please revise the protocol text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.).
    Response: The manuscript has been carefully revised and personal pronouns have been avoided.

11. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed?
    Response: After the comments and suggestions of the editor and the reviewers, we are confident that our protocol contains all the necessary information.

12. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
    Response: The essential steps of the protocol for the video include the protocol text from step 2 to step 5. More precisely, the essential steps go from “***2.Preparation for imaging filamentous fungi growing on agar (solid) medium…”*** to ***“…NOTE: By selecting “Movie” button, a movie is produced showing the image and track progression***.” The corresponding pages in the manuscript have been yellow highlighted.

13. Please ensure the results are described in the context of the presented technique. e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.
    Response: We have revised our manuscript accordingly.

14. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."
    Response: We have not used or modify an already published figure. All figures and videos are new.

15. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
    a) Critical steps within the protocol
    b) Any modifications and troubleshooting of the technique
    c) Any limitations of the technique
    d) The significance with respect to existing methods
    e) Any future applications of the technique
    Response: We have modified the Discussion in order to cover the above prerequisites.

Response to reviewer's 1 comments:

1. Minor grammatical, typo and wording corrections (I can offer the copy-edited manuscript with comments & correction, but this is apparently ot desired by JoVE?)
    Response: We thank the reviewer for pointing out grammatical and typo errors. We have performed thorough English editing and corrected the grammatical mistakes in the revised manuscript.

2. Please specify which strain/isolate of A. nidulans that was used in this study, e.g. by adding ATCC or FGSC number. (e.g. in line 82)
    Response: The FGSC number of our wt strain is FGSC A1149. Although, we have used various strains in this work, we have included all strains with their genotypes and references (see also below). Moreover, the sentence in line 82 was modified as follows:" …can be employed to analyze and quantify polar growth of *A. nidulans* strains in both submerged cultures and solid media".

3. In connection to (2), please provide the TABEL OF MATERIALS (lines 356-357)
    Response: All strains with their genotypes and references have been added in the Table of Materials and Reagents.

4. Please check whether first letter capitalization of the vitamin names is correct. (lines 105-106)
    Response: Checked and corrected.

5. Add cells/mL to mentioned cell suspension, e.g. lines 115, 131 and 157
   Response: We have added “cells/ml” in the corresponding sections.

6. I suggest to add:"..or as suitable for the used fungal species." at the end of the statement in bracket sin line 172
   Response: In agreement with this comment, we have changed the sentence accordingly.

7. Figures 5, 7 and 8: Improve figure layout by decreasing the font size of time and scale bars, and display scale bar only once in the bottom image of each set.
   Response: We agree with the reviewer; all the figures have been modified accordingly.

8. I suggest to add "unipolar" to: "(velocity of germination and unipolar hyphal tip extension)" in line 348, because I think it makes an important difference. Also during vesicle formation does the hyphal tip extend, however, in an isotropic and not polarised manner anymore.
9. Response: We agree with the reviewer; the sentence has been changed accordingly.

10. add Video 1 to the review manuscript. I could not find it.
    Response: The video has been added.

11. Rethink and weaken the argument in lines 380-385, because this only applies when working with wild type strains. Your mutants are of course genetically modified. Hence, I would suggest not to put so much emphasis on this argument. Also, application of FM dyes is not that expensive anymore if you buy generica (e.g. SynaptoGreen), and the application is also quite simple and very fast.
    Response: Although we understand the reviewer’s point of view, we are aware of using dyes is time consuming, requires advanced instrumentation, can (at least in long term experiments) influence/compromise cellular metabolism and lead to significant phototoxicity and photobleaching (Ounkomol et al. 2018). Thus, we have decided to keep the text at that point as it is

Response to reviewer’s 2 comments:

Major Concerns:

1. This method may be useful for certain people, but I don't think it will have an impact on this area, because the authors only introduced a method using an existing plugin function, not something they created by themselves.
   Response: No comment

2. The authors show the measurement of the growth rate of a single hypha. Is it possible to measure the growth rate in the same way for movies that contain

multiple hyphae? This function should be useful when the movie contains many hyphae. If it is only for one hypha, there is no difference in effort from manual calculation.
Response: Multiple hyphae in the same time frame can be measured as implied by the following sentence (lines 241-243) in the original manuscript: "Move to another point of interest (i.e. growing hyphal tip) in the initial time frame and restart the procedure by measuring growth rate of another hypha".

3. How about measurement under different growth conditions (pH, temperature, carbon and nitrogen sources etc.)?
Response: Yes, it is possible to measure the growth rate under a wide variety of different growth conditions. Here, we only describe the protocol, and show one example as a proof of concept (Biratsi et al. 2021). We also mention in the discussion section that this protocol can be applied in a wide variety of conditions.

4. If this method is useful, the authors need to show the effect and utility.
Response: In this manuscript, we have already provided an example (Biratsi et al. 2021), as a proof of concept, where the measurements performed by this method are more accurate than simple growth tests. We are, thus, confident that other researchers will find it useful and apply it under the conditions of their scientific interests.

5. How do the authors evaluate the branching?
Response: In order to properly select (and measure) hyphae that propagate by polar extension, we have included the following step in the Image analysis section: "Select hyphae that grow parallel to coverslip, avoiding those that are tilted. Be sure, that you select hyphae that propagate by polar extension and avoid hyphae presenting lateral and/or apical branching."

6. How do the authors evaluate the Z-axis misalignment by defocus? Is there any suggestion to take a time-lapse movie without Z-axis defocus?
Response: Maintaining the specimen in focus during Time-lapse microscopy of live cells is crucial, especially for long experiments. Autofocus can be achieved using automated focus control methods or deep learning approaches (Wei and Roberts 2018). We have added the following NOTE to step 5 of the Capture images section: "To correct for focal drift over time (especially for long experiments), due to thermal drift, diverse cell sizes, and cell motion use an autofocus strategy if is available in your microscope software."

Minor Concerns:

1. Fig 5B, how many hyphae did they measure? N=?
Response: The number of hyphae measured in Fig 5B is 717, please also refer to the main text (REPRESENTATIVE RESULTS section), and in particular: "Measuring their growth rate in submerged liquid cultures, we detect statistically significant lower growth rate of the double mutant compared to

the WT strain (t(715) = 20.61, p = <0,0001) (Figure 5A-B).

Response to reviewer's 3 comments:

Major Concerns:

1. Results: The results section misses coherence. Add a few lines to glue the paragraphs together. For each experiment that is described, explain what the experiment is about (context, research question) and why the described procedure is well-suited for it.
   Response: In agreement with this comment, the results section has been rewritten.

Minor Concerns:

1. Overuse of 'NOTES'. Often the added information in the note is more suited to be part of the procedure step. For instance, the note in line 192 can easily be added as regular text in step 3, and the note in line 200 can easily be included in step 4. Go through the entire manuscript and adapt this where suited, so that the ratio of procedure description versus notes is well-balanced.
   Response: In agreement with this comment, the number of 'NOTES' has been significantly reduced, following publisher's instructions to the authors.

2. Discussion: It would be interesting to include a reflection on how well-suited the method would be for other filamentous fungi, and what kind of constraints or limitations could occur when applying this procedure with other fungal specie
   Response: We have added some information in the Discussion section concerning the constrains of our approach on other filamentous fungal species. More precisely we have added the following text:
   "…This protocol is suitable to study growth kinetics of fungi that form hyphae or pseudo-hyphae. Although, this protocol is poorly suited to track fungal biofilms, which are subject to vertical extension forming a dense heterogeneous, surface-associated colonies comprised of filamentous hyphae, pseudo-hyphal cells, yeast-form cells, and various forms of extracellular matrix [36]…"

3. The fungus grows in 3D, but the images are in 2D. How do you take into account the influence of the angle in which the fungus is growing on the measured length of the hyphae? In video 2, for instance, you can see that the hyphae of interest is growing quite flat in the field of view, but the second hyphae that is coming in from the left bottom clearly grows tilted, which will have an effect on the measured length and therefore the growth kinetics, as these are measured in 2D. It would be interesting to add how you select which hyphae to analyse.
   Response: We found this suggestion very constructive. We have included the criteria we use to select and analyze hyphae. We have added the following step

(step 5.4) in the Image analysis section: “Select hyphae that grow parallel to coverslip, avoiding those that are tilted. Be sure, that you select hyphae that propagate by polar extension and avoid hyphae presenting lateral and/or apical branching.”

4. Line 237: MTrackJ is not a standard plugin in ImageJ. Add one line on the fact that it needs to be installed, including a reference on how you can obtain it (link in line 245).
   Response: We have added the following NOTE at step 5.5: “To install MTrackJ follow the instructions presented at https://imagescience.org/meijering/software/mtrackj/”

5. Line 258: refer correctly to Python software
   Response: Corrected.

6. Lines 276-282 (with exception of the sentence 'In Figure 8 and Video 2…') are better suited for the Discussion.
   Response: In agreement with reviewers’ comment this text was move to the Discussion.

7. Line 356-357: Table is included
   Response: Corrected.

8. Video 1 is missing.
   Response: We have added video 1.

9. Two font types are used in the images. Preferably use one font type. Or, if the arial-like font type is used to add info to the subpart of the image, while Times new roman is used to describe an action, make sure to be consistent, i.e. use arial for 'glycerol stock' in Figure 1.
   Response: We have revised all the images and kept only one font type (Calibri).

Response to reviewer’s 4 comments:

References:

Biratsi, Ada et al. 2021. “A Highly Conserved Mechanism for the Detoxification and Assimilation of the Toxic Phytoproduct L-Azetidine-2-Carboxylic Acid in Aspergillus Nidulans.” *Scientific Reports* 11(1): 7391.

Ounkomol, Chawin et al. 2018. “Label-Free Prediction of Three-Dimensional Fluorescence Images from Transmitted Light Microscopy.” *Nature methods* 15(11): 917–20.

Wei, Ling, and Elijah Roberts. 2018. “Neural Network Control of Focal Position during Time-Lapse Microscopy of Cells.” *Scientific Reports* 8(1): 7313.

Figure S1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Figure S1 Revised.png