TIME-LAPSE SOIL ZYMOGRAPHY (TLZ) PROTOCOL AND DATA PROCESSING

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INTRODUCTION

Time-lapse zymography (TLZ) is an *in situ* quantitative method developed for evaluating and mapping the activity of hydrolytic enzymes on soil/root surfaces. It uses time-lapse photography to measure dynamics of fluorescence development in the membrane saturated in enzymes specific substrate mixed with fluorogenic reagents and incubated on soil/root surface. TLZ calculates the enzyme activity in each pixel of the membrane based on the highest rate of signal development with correction for losses due to product diffusion in the soil and membrane. The details of TLZ approach and calibration procedure are described in Guber et al. (2019, 2021). The developed code is an open source and can be obtained upon request from akguber@msu.edu. Although the code has been tested by its developers, no warranty, expressed or implied, is made as to the accuracy and functioning of the program modifications and related program material, nor shall the fact of distribution constitute any such warranty, and no responsibility is assumed by the developers in connection therewith.

How to cite the TLZ procedure and code:

1.   Guber, A.K., Kravchenko, A.N., Razavi, B.S., Blagodatskaya, E., Kuzyakov, Y. 2019. Calibration of 2-D soil zymography for correct analysis of enzyme distribution. European Journal of Soil Science 70(4): 715-726.

2.   Guber, A.K., Blagodatskaya, E., Juyal, A., Razavi, B.S., Kuzyakov, Y. Kravchenko, A.N., 2021. Time-lapse approach to correct deficiencies of 2D soil zymography. Soil Biology and Biochemistry 157: 108225.
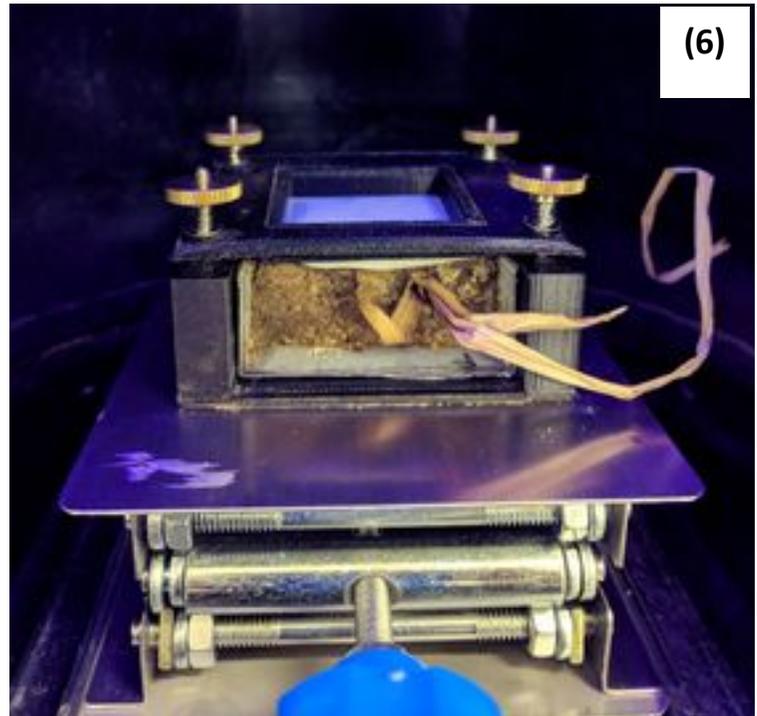
I.   Experimental setup for TLZ

Parts and software for TLZ include (Fig.1):

1. A DSLR camera compatible with Helicon remote software (Canon EOS Rebel T6 or newer models).
2. Telephoto Zoom Lens (Canon EF 75-300 mm f/4-5.6 III Telephoto Zoom Lens)
3. Fotodiox Macro Extension Tube Set for Canon EOS (EF, EF-S) Mount DSLR Cameras
4. Photocopy stand (50-70 cm height to accommodate focus distance of the telephoto lens)
5. Dimmer with LED Halo Headlight Accent Lights - UV-BlackLight + Natural White
6. Frame for rhizobox zymography
7. Helicon remote software (https://www.heliconsoft.com/heliconsoft-products/helicon-remote/)
8. TKDY ACK-E10 AC Power Adapter for Canon EOS Rebel T6 camera.
9. Hydrophilic polyamide membrane filter (HPMF) of 100 μm thickness with average pore size of 0.45 μm (Tao Yuan, China or Sigma Aldrich).

Assembling and operation

1. Connect all parts as shown in Fig. 2.
2. Install and start the Helicon remote software.
3. Switch camera to manual focus and shooting mode (M).
4. Turn the natural white lights on.
5. Place a scale grid paper or fine font print-out on the base of photocopy stand (or zymography box) and adjust the camera height and focus manually. The size of the paper sheet should be approximately 20% larger, than the rhizobox. Change the extension tube if the grid or letters are out of focus distance of the camera. Focus should be adjusted manually using the camera focusing ring and kept unchanged during TLZ.
6. Switch light source to the UV-Black Light.
7. Place a clean HPMF on the base of photocopy stand and take photo.
8. In ImageJ check the uniformity of the brightness across the image.

Figure 1. TLZ parts

(1)

(2)

(3)

(5)

(6)

Figure 2. TLZ setup

9. Adjust height of the light source to achieve the most uniform light distribution.
10. Run the calibration procedure with different light (dimmer) and camera settings (exposure time) as described in Guber et al. (2019) to achieve the best signal to noise ratio in possibly

broader range of MUF values with linear relationship between greyscale values and MUF mass in the HPMF.
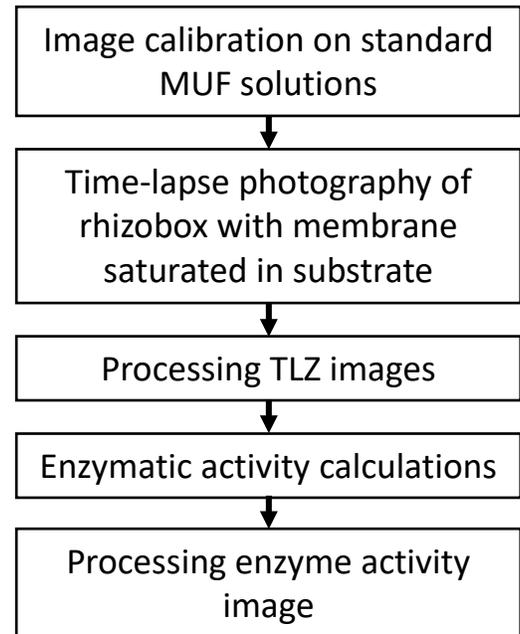
Default Camera and Helicon remote software settings for TLZ

1. Focus: Manual
2. Aperture: f/5.6
3. Exposure time: 1/125 sec
4. ISO speed rating: 800
5. Time lapse Interval: 60 sec
6. Number of shots: 40

II. TLZ procedure

Sequence of TLZ operations is shown in Fig. 3.

Figure 3. TLZ flowchart

II-1. Image calibration on standard MUF solutions

* TLZ uses UV light source and should be done in a dark room/hood.

1. Prepare 5-6 standard MUF solutions at concentrations within the range of linear increase in fluorescent intensity (e.g., 0.1, 0.25, 0.5, 1.0, and 2.0 mM).
2. Using pipette, apply 5 -10 μl of each concentration solution to HPMF in 2-3 replications.
3. Cover the membranes immediately after MUF application with a transparent film to prevent evaporation and take 3-5 photos every 5 minutes using the settings desired for TLZ.
4. Open the calibration sequence in ImageJ and convert to 8-bit (Image -> Type -> 8-bit). Alternatively, one of the RGB channel can be used (Image -> Color -> Split Channels).
5. Run *G_time.ijm* to check stability of the signal in time for different ROIs in the calibration image. Select images for MUF calibration within stable range of the signal.
6. Process the calibration images using *Calibration_Linear.ijm* macro in ImageJ software following pop-up instructions:
   a. Enter MUF mass for each calibration spot in line 10 of this macro.

b. Run *Calibration_Linear.ijm* macro

c. Select the directory with the calibration images (e.g. Calibration).

d. Open the calibration image from this directory (e.g. File -> Open -> MUF.JPG)

e. Select ROI around all MUF spots using Rectangle selection tool and click OK.

f. Select channel (All – Default, R – for Oxyred).

g. Select the background on one of the membranes using Rectangle selection tool and click OK.

h. Threshold the Mask to identify the MUF spots (Image -> Adjust -> Threshold -> Apply). Click OK in the pop-up window.
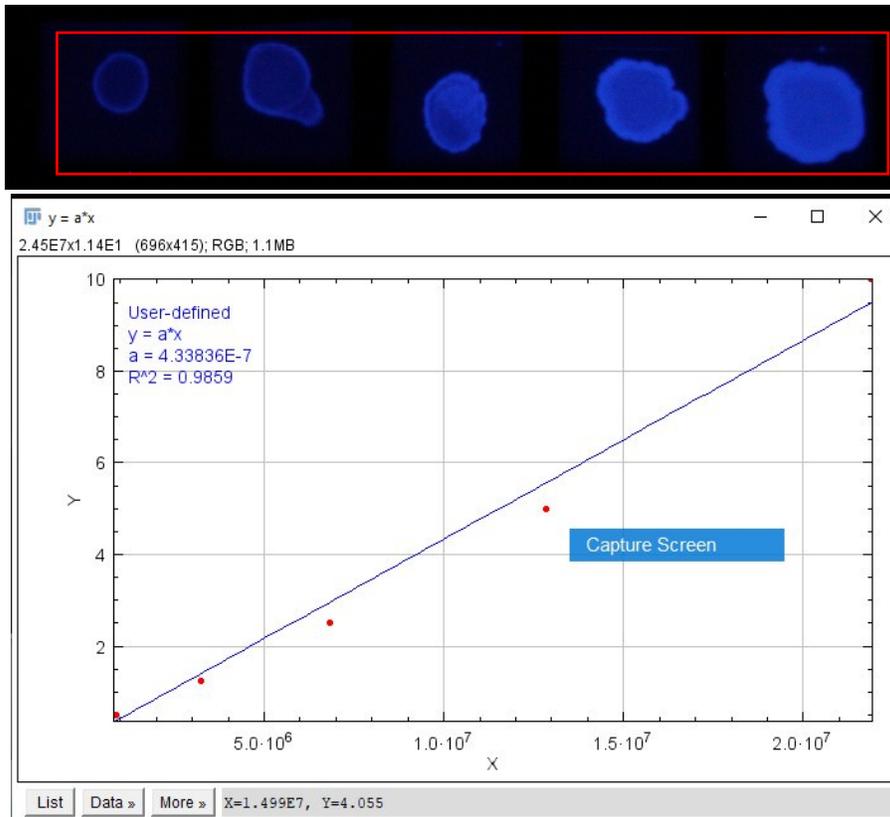
i. Enter the number of calibration spots in the image.

Figure 4. An example of MUF calibration regression. X denotes Sum(G*F), Y denotes MUF mass [nmol] in each calibration spot. The regression is close to linear for MUF mass $0 < Y < 10$ nmol. The slope of calibration line $a = 4.338 \cdot 10^{-4}$ pmol MUF pix^{-1} greyscale^{-1} (see insert).

j. Starting from the first spot, select one by one all spots using Rectangle selection tool and clicking OK after each selection. The spots must be selected in the order of MUF mass entered in line 10 of this macro.

k. Check the linearity of the calibration on the calibration plot. Save the parameter *a* for TLZ calculations (parameter *b1* in Table 1). The units for *a* are [MUF mass units/grey value/pixel].

l. The macro saves MUF mass and Sum(G*F) for each calibration spot to file Totgrey.txt, which can be used for manual calibration in Excel or any other software.

7. Use the calibration parameter *a* in the enzymatic activity calculations described below.

  * The calibration code can be used only for linear relationship between the greyscale values G and mass of the standard solution. For non-linear calibration, use a piecewise linear regression (Guber et al., 2019).

II-2. Time-lapse photography of rhizobox with membrane saturated in substrate.

* *wear gloves and use autoclaved laboratory glassware when working with enzymes.*

1. Cut HPMF of size needed for TLZ.
2. Prepare working solution of the substrate for specific enzymes.
3. Place a rhizobox on the base of the photo-table.
4. Start Helicon remote software and adjust focus manually in natural white light.
5. Set exposure time, ISO, time lapse interval and number of shots in Helicon remote software. The exposure time, ISO and focus distance must be the same as for the calibration.
6. Switch the light source to UV.
7. Saturate HPMF in the substrate solution.
8. Spray soil/root surface in the rhizobox with DI water to assure hydraulic connectivity of membrane with the soil surface.
9. Fix saturated HPMF with a clean transparent film above it in the frame for rhizobox zymography (Fig.1 (6)) and immediately place on the soil surface. Fix the frame in the rhizobox using 4 springs.

10. Start time lapse shooting. *Time interval between the start of TLZ and membrane removal from the substrate must be as short as possible, because the first image is used for the background removal in the image processing procedure.*
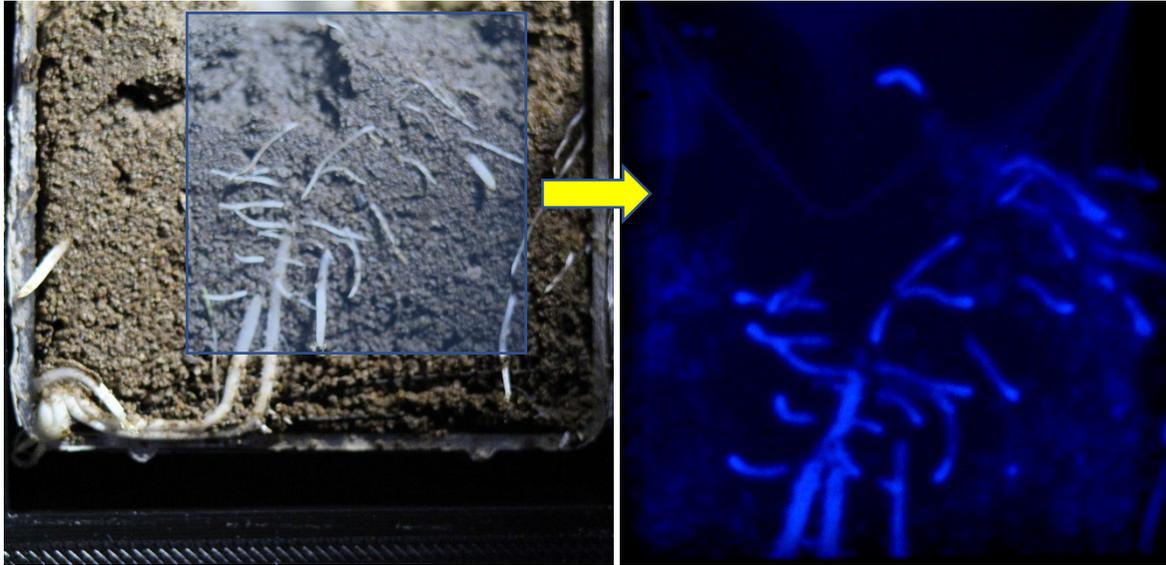

Figure 4. Soil rhizobox with young corn roots (left), and zymography image taken at 40th minute of phosphatase zymography (right).

III. Processing TLZ image sequences.

III-1. *The first step of data processing is converting the TLZ images (RGB) to 8-bit format, removing the noise and background. To do that, run TLZ-process.ijm macro in ImageJ software and follow the pop-up instructions:*

1. Select directory with TLZ image sequence. All images will be open automatically. *Check that the directory contains only zymography images taken during single TLZ session.*

2. Select ROI using a rectangle or oval tool to crop TLZ images for reduction of the area to be processed.

3. Select channel (All - Default, R - for Oxyred).

4. The macro converts RGB images into 8-bit format (without rescaling), subtracts the first TLZ image from the whole stack, and removes the image noise using a 3D median filter (radius = 3 pixels). The user can change the filter and its settings in line 65 of the macro. *Filtering may*

take several minutes. Please wait until message "Save processed images to MUF directory"

pops-up.

5. Save MUF_filt sequence in TIFF format to new directory for activity calculations (e.g. MUF_filt: File -> Save as -> Image Sequence -> MUF_filt).

6. You may view the Greyscale time dynamics in different regions of the zymogram using *G_time.ijm* macro.

III-2. *The third step of data processing is processing the enzyme activity image in individual pixels based on the linear parts of the Greyscale time dynamics and the correction factor for MUF diffusion losses. MATLAB code "Activity_time.m" uses the sequences of images obtained in* ***III-1*** *with the parameters specified in ASCII file Par.dat and time moments, when the images were taken, specified in ASCII file Time.dat. Input files Time.dat and Par.dat must be in the same directory with MUF_filt00XX.tif files.*

Table 1. Format of Par.dat file:

Line	Parameter	value	Comment
1	b1	3.31E-04	MUF calibration parameters (Guber et al., 2019):
2	a2	-0.19501	$\begin{cases} \overline{M}_{MUF} = b_1 \cdot \overline{G}; & 0 < \overline{G} < G* \\ \overline{M}_{MUF} = a_2 + b_2 \cdot \overline{G}; & G* < \overline{G} \\ b_2 = b_1 - a_2/G* \end{cases}$
3	b2	2.547E-03	
4	G*	88	
5	Npoints	> 2	Number of datapoints to calculate the derivative dMUF/dTime
6	R2min	0.8	Min acceptable R^2 for the derivative calculation.
7	Resol, um	17	Pixel size in TLZ image
8	TimeStep, min	1	Time interval used in TLZ. If TimeStep=0, enter time moments for TLZ in file Time.dat
9*	Gmin	1	Min greyscale value in the pixel to run the activity calculations. Activity = 0 for G < G_{min}
10*	SlopeMin	0.1	Min slope of G_time in MUF_filt00XX.tif to run the activity calculations. Activity = 0 for dG/dTime < G_{min}
11*	SigmaGmin	1.0	Min standard deviation in G_time to run the activity calculations. Activity = 0 for StDev(G_time) < SigmaGmin

* Use the parameters Gmin, SlopeMin, and SigmaGmin to remove image pixels with high oscillations of *G*-values, or insignificant changes of brightness on images during TLZ (noise) and to reduce the computational time. To estimate the parameter values, run *G_time.ijm* macro for different regions of MUF_filt00XX.tif sequence.

Time.dat is required only for irregular time intervals during TLZ (TimeStep=0). Time moments entered in an increasing order separated by Space.

1. Enter the Directory containing the MUF_filt00XX.tif sequence in Line 24 of the Matlab code *Activity_time.m.* Check for presence of *Par.dat and Time.dat (if required)* files in this directory.
2. Check the parameter values in *Par.dat* and time data in *Time.dat* files.
3. Run *Activity_time.m*.
4. The code generates three plots: Time series of Number of active pixels (Fig. 1), calculated Average $\pm$ 1σ activities (Fig. 2), and Average activities after correction for MUF losses (Fig. 3). The time denotes time moments, when the derivatives dMUF/dTime were calculated at $R^2 >$ R2min in image pixels. These plots are for information purpose only, to provide an idea about the time when the highest activity was detected, number of active pixels, and distributions of corrected activities over time of peak activities.
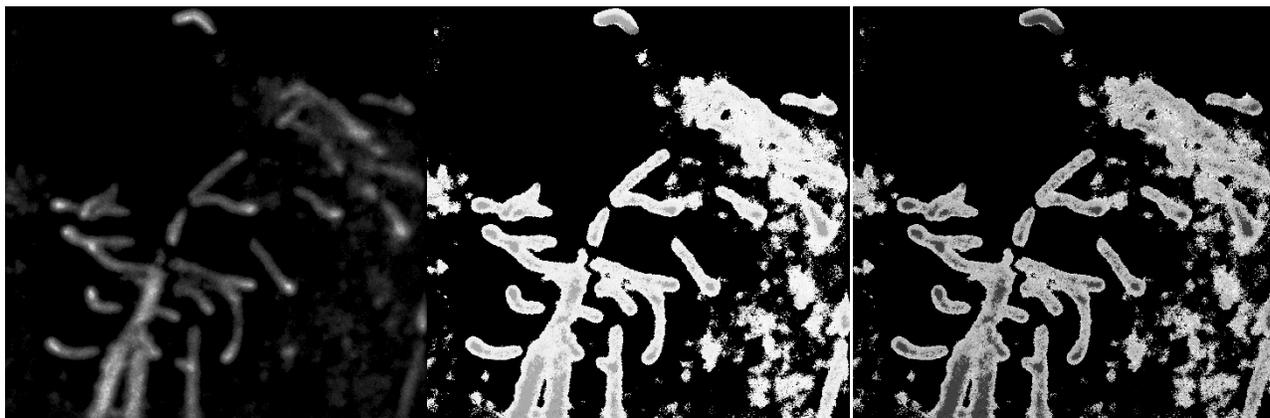

Figure 5. Examples of Text Image files: Activity.res (left), Fraction.res (center) and Time_der.res (right).

5. Results of the activity calculations saved to four Text Image files:
   - Activity.res – enzyme activities in pixels without correction for MUF losses.
   - Fraction.res – the activity correction factor for each pixel.

- Time_der.res – maximum values of derivative dMUF/dTime in image pixels.
- Time_der.res – the time moments when the derivatives were the highest in image pixels.

III-3. *The third step of TLZ processing is calculating the enzyme activity using the results of Activity_time.m procedure. Specifically, text image file Activity.res is multiplied by Fraction.res to generate the activity image Act_cor-BW.tif corrected for MUF losses. The user has an option to remove pixels with late detection time (less reliable activity calculations).*

1. Run *Activity_process.ijm*
2. Threshold "Mask" image, which is a 2D map of time moments when the activities in the pixels were the highest. Image -> Adjust -> Threshold. Select the lower thresholds t_{min} >1 min and upper thresholds t_{max} based on time when you believe the appearance of new active pixels was mostly associated with MUF diffusion within the membrane (Guber et al., 2021). There is no rule of thumb for choosing the upper threshold. For β-glucosidase TLZ in ~ 200 soil samples t_{max} occurred more frequently within the range 28-32 min. Click OK in "Run Threshold for MASK" pop-up window.
3. The macro creates an 8-bit image with corrected activities and RGB image with a color scale for presentation. The lookup table and parameters of the scale window can be changed in lines 56-57 of the macro. The name of 8-bit activity image is SourceDirName-BW.tif and RGB activity image - SourceDirName-RGB.ipg.
4. The 8-bit activity image SourceDirName-BW.tif is intended to be used for further analysis of spatial distribution, while the RBG image for presentation.
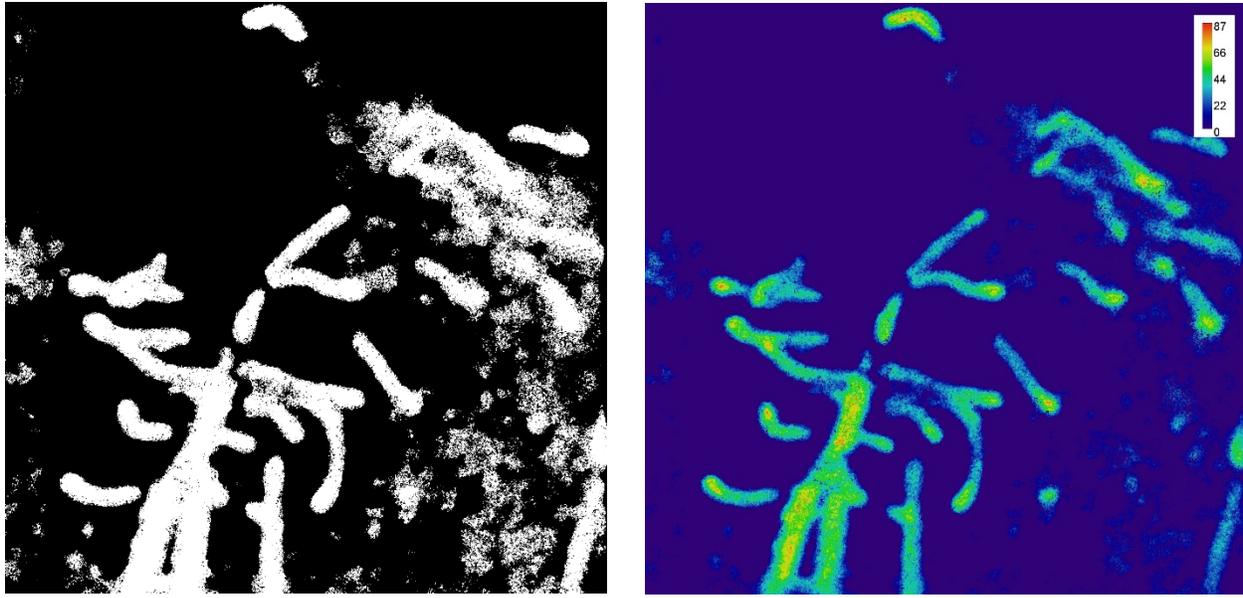5. Close all active windows before processing next TLZ sequence: File -> Close All.

Figure 6. Results of phosphatase activity calculations in 32-bit format (left) and RGB (right). The activity units are pmol mm^{-2} min^{-1}

III-4. Description of the G_time.ijm macro

G_time.ijm macro plots time series of G-values in the center of selected region in TLZ sequence.

1. Open a TLZ sequence.

2. Open *G_time.ijm* macro.

3. For irregular time intervals enter time moments when the photos were taken in line 14 and comment line 20 in the macro.

4. For regular 1-minute time interval no changes in macro are needed.

5. Select a ROI using either a rectangle or oval tool.

6. Run the macro.

7. The StDev of G-values is printed in "Log" window.

8. The time series of G-values is plotted in "Plot G" window.

9. In the "Plot G" window click Data -> Add Fit to get the parameters of linear regression.

10. Parameter *b* of the linear regression and maximum G-value can be used to estimate the parameters SlopeMin and Gmin for Matlab procedure *Activity_time.m*, when a region with

the lowest activity is selected. StDev of G-value printed in "Log" window can be used to estimate the parameter SigmaGmin for the same procedure.
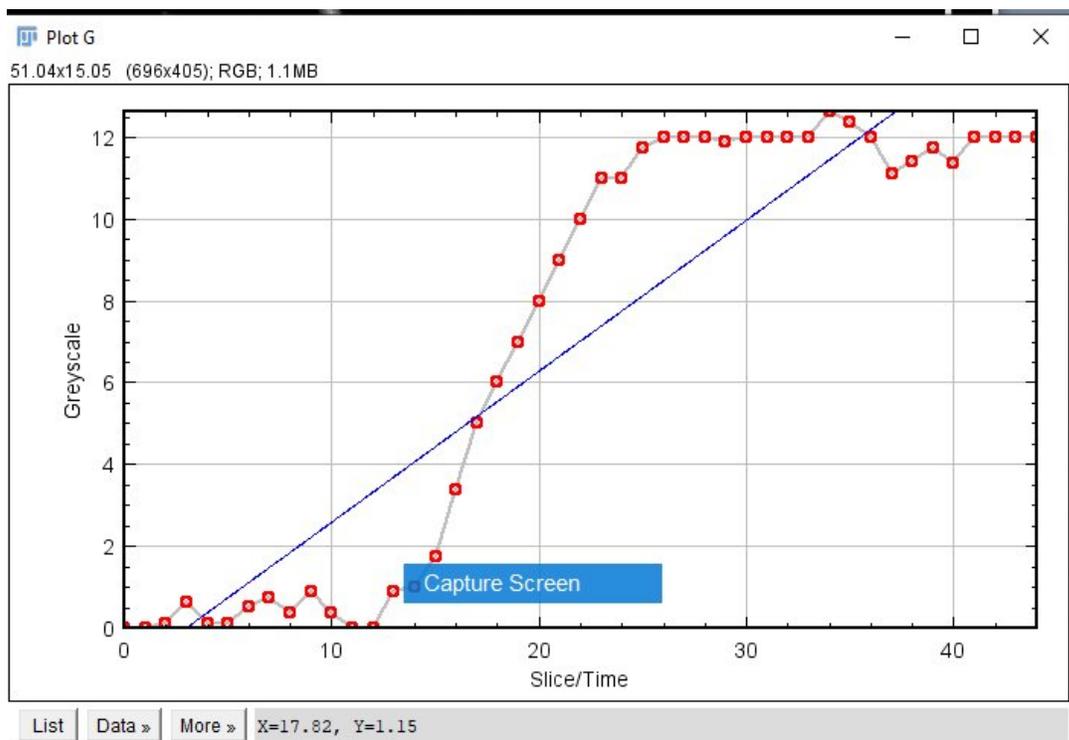

Figure 7. An example of *G-values* time series plot. *G-values* increased linearly within time interval 13-22 minutes.

Examples

Examples of calibration and TLZ data processing are provided in directories "Calibration" and "TLZ" for β-Glucosidase and acid-Phosphatase.

IV-1. Calibration on standard MUF solutions for b-Glucosidase and Phosphatase TLZ.

For calibration, 5 µl of 0.1, 0.25, 0.5, 1.0, and 2.0 mM MUF solutions were applied to the membrane (Fig. 4 top) and photographed (…\TLZ\Calibration\MUF.jpg). The image was processed using *Calibration_Linear.ijm* macro. Results: Sum(G*F) for each MUF mass are saved in file "Totgrey.txt" and plotted (Fig. 4 bottom). The calibration regression was close to linear with the slope a = 4.338 10^{-4} pmol MUF pix^{-1} greyscale^{-1}.

IV-2. TLZ for β-Glucosidase

Directory "…\TLZ\beta-glucosidase\RAW" contains sequence of images taken at 1-minute interval during 45-minute β-Glucosidase TLZ on the surface a cylindrical soil core. This sequence was processed using macro *TLZ-process.ijm* and resulting MUF_filtXXXX.tif files were saved to "…\TLZ\beta-glucosidase\MUF_filt" directory in 8-bit format along with the selected region "…\TLZ\beta-glucosidase\RAW\Selection.roi". The last file can be used for alignment of calculated enzyme activity with a soil surface image if was taken.

The parameter file "…\TLZ\beta-glucosidase\MUF_filt\Par.dat" was created in an ASCI editor as input for *Activity_time.m* MATLAB procedure. The calibration parameter *b1* was estimated in section *IV-1*. Parameters *a2* and *b2* are used for piecewise calibration function that includes oversaturating MUF values (Table 1) and will be ignored in this example by setting G* to a much larger value, than was obtained in MUF_filtXXXX.tif files. Since 45-minute TLZ generated 45 datapoints in each zymography pixel, we choose 10-minute time sliding window for the derivative dMUF/dT calculations (Npoints 10). Larger window removes oscillations from MUF time series. However, too large windows potentially reduce *dMUF/dT* values. Proper value of the Npoints can be obtained from linear parts of *G-values* time series using *G_time.ijm* macro (Fig. 7). Parameter "TimeStep" was set equal to 1 minute, which was the TLZ time interval. File "Time.dat" was not required in the activity calculations since the time interval did not change during TLZ. Parameters "Gmin", "SlopeMin" and "SigmaGmin" were estimated from *G-values* time series to remove pixels with low and/or not reliable *G-values* and reduce the processing time.

Running *Activity_time.m* MATLAB procedure, with the path to MUF_filtXXXX.tif files ("…\TLZ\beta-glucosidase\MUF_filt") specified in line 24 of *Activity_time.m* and parameter values specified in "Par.dat" file, generates and saves "*Activity.res*", "*Fraction.res*", "*R2.res*" *and "Time_der.res"* text image files. Three plots with statistics of the activity and number of active pixels will be saved along with the text image files to "…\TLZ\beta-glucosidase\MUF_filt" directory. There are: Fig-1.jpg, Fig-2.jpg and Fig-3.jpg.

Finally, running *Activity_process.ijm* with the results of *Activity_time.m,* one generates "*beta-glucosidase-BW.tif*" and *"beta-glucosidase-RGB.jpg"* files with corrected activities in 32-bit and RGB formats, respectively. The first file is aimed to be used for further statistical analysis of activity distribution and/or association with soil, roots or pores, depending on the goal of soil zymography. The second file can be used for presentations.

IV-3. TLZ for acid Phosphatase

Directory "…\TLZ\ Phosphatase\RAW" contains sequence of images taken at 5-minute interval during a 45-minute acid Phosphatase TLZ on the surface rhizobox. This sequence was processed using macro *TLZ-process.ijm* and resulting MUF_filtXXXX.tif files were saved to "…\TLZ\Phosphatase\MUF_filt" directory in 8-bit format along with the selected region "…\TLZ\Phosphatase\RAW\Selection.roi". The last file can be used for alignment of calculated enzyme activity image with photo of soil surface if it was taken.

The parameter file "…\TLZ\Phosphatase\MUF_filt\Par.dat" was created in an ASCI editor as input for *Activity_time.m* MATLAB procedure. The calibration parameter *b1* was estimated in section *IV-1*. Parameters *a2* and *b2* are used for piecewise calibration function that includes oversaturating MUF values (Table 1) and will be ignored in this example by setting G* to a much larger value, than was obtained in MUF_filtXXXX.tif files. Since 45-minute TLZ generated 9 datapoints in each zymography pixel, we choose 25-minute time sliding window for the derivative dMUF/dT calculations (Npoints 5). Parameter "TimeStep" was set equal to 5 minutes, which was the TLZ time interval. File "Time.dat" was not required in the activity calculations since the time interval did not change during TLZ. Parameters "Gmin", "SlopeMin" and "SigmaGmin" were estimated from *G-values* time series to remove pixels with low and/or not reliable *G-values* and reduce the processing time.

Running *Activity_time.m* MATLAB procedure, with the path to MUF_filtXXXX.tif files ("…\TLZ\Phosphatase\MUF_filt") specified in line 24 of *Activity_time.m* and parameter values specified in "Par.dat" file, generates and saves "*Activity.res*", *"Fraction.res", "R2.res" and "Time_der.res"* text image files. Three plots with statistics of the activity and number of active pixels will be saved along with the text image files to "…\TLZ\Phosphatase\MUF_filt" directory. There are: Fig-1.jpg, Fig-2.jpg and Fig-3.jpg.

Finally, running *Activity_process.ijm* with the results of *Activity_time.m,* we generated "*Phosphatase-BW.tif"* and *"Phosphatase-RGB.jpg"* files with corrected activities in 32-bit and RGB formats, respectively.