Macro Instructions

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1 Introduction

These instructions describe the use of an ImageJ macro for quantifying *Zymoseptoria tritici* symptoms on wheat leaves as presented in Stewart *et al*, (2016). It is based on the method as published in Stewart & McDonald (2014) but has additional features designed to make it easier to use and uses images acquired with a flatbed scanner instead of a camera. The main improvement of the scanner macro is the addition of a QR code reader function that reads the sample name from the image. QR codes are generated using a LaTeX script wrapped in a Shell script which accepts a .txt file containing sample names as input. This is currently only compatible with mac, it will not run on a pc.

Various pop-up boxes and windows prompt the user to enter the different settings to use within the macro. This was done to eliminate the need to scroll through hundreds of lines of code searching for the handful of values that potentially need tweaking. Once a set of values has been defined, they can be used later without having to re-enter the settings every time the macro is run. This is still a fair way off a graphical user interface, a slick GUI is on the wish list if anyone feels like a coding challenge. An additional feature that measures the grey value of the pycnidia was also added. The grey value can be used as a proxy for melanisation.

This is a batch process macro and will analyse multiple pictures within a folder. There is no upper limit to the number of images that can be processed. The user defined settings will be applied to all pictures. The macro will work on either .jpg or .tif files however, high resolution .tif files are recommended. All other files will be ignored.

2   Preliminaries

1. Download ImageJ (imagej.nih.gov) and save it to your Applications/Programs folder.

2. Download the files provided in the supplementary material from Stewart *et al*, (2016).

3. Open the folder containing ImageJ, it should contain a folder named Plugins.

4. Copy the files Threshold_Colour_Zymo.java, RGBtoLab_.java, RGBtoYUV_.java, qr_decoder_1.0-2.jar & Wait_For_User_New.java to the Plugins folder.

5. Save the macro *ZymoMacro_Scanner__v2.txt* somewhere on your computer.

6. Open ImageJ64.

7. Run the macro [Plugins → Macros → Run...]. Select the macro file. The macro will automatically compile the plugins. ImageJ must be re-started for the plugins to take effect. If any of the required .java files are missing, an error message will appear and ImageJ will abort.

8. For each .java file you should now have a corresponding .class file within the Plugins folder.

9. Download MacTex (tug.org/mactex/) and follow the installation instructions to install the software to your Applications folder.

10. You should now have a folder named 'TeX' in your Applications folder. Download the files pst-barcode.tex, pst-barcode.sty & pst-barcode.pro from www.ctan.org/tex-archive/graphics/pstricks/contrib/pst-barcode and copy them to the TeX folder.

NB: The above steps only need to be done once. After you have compiled the plugins they will be available automatically when you next start ImageJ.

3   Generate QR codes

1. Make a .txt file containing your sample names. Names may contain any alphanumeric characters and # $ % & _ { }. Other symbols may not render correctly in the QR code. Each name should be on a new line, the file should

look something like:

Plant#1_isolate1.1_cultivarA_...
Plant#2_isolate1.2_cultivarB_...
.
.
.
Plant#n_isolaten.n_cultivarZ_...

2. Make a new folder somewhere on your computer. Save the file containing your sample names to the folder. Save QRscript.txt to the same folder.

3. Open Terminal (Applications ➡ Utilities ➡ Terminal).

4. Set the working directory to your folder. To change the working directory type 'cd *ized /Path/to/your/folder/*' into the Terminal window.

5. Run the shell script by typing 'sh QRscript.txt' into the Terminal window. The script will prompt you to enter various inputs.

6. After running the script, you should have a .pdf file containing the pages on which to mount your leaves.
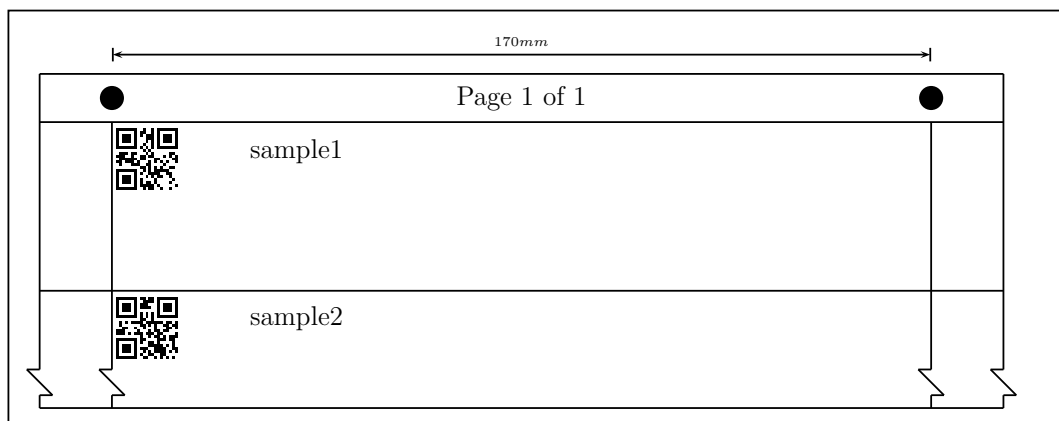

Figure 1: QRsctipt.sh output

4 Make Scans

1. Print the output pages from Section 3 on plane white paper. **IMPORTANT**: When printing, in the 'page size' tab, check the box that says 'Actual size'. Your page should look like the example in Figure 1. The distance between the center of the 2 black circles at the top of the page should be 170 mm. If this is not correct the macro will not run correctly.

2. Mount the leaves onto the pages using white opaque tape. Do not obscure the QR codes or the black reference circles.

3. Scan the pages using a flatbed scanner with a picture resolution of 1200 dpi. Other resolutions will not work correctly with the macro.

5 Run the Macro

Open ImageJ64, this is the 64 bit version if ImageJ and should be present within the ImageJ folder. Change the available memory to at least 2500 MB (Edit ➙ Options ➙ Memory & Threads...). You will have to re-start ImageJ64 after changing the memory settings.
Run the macro [Plugins ➙ Macros ➙ Run...]. Select the macro file.

- If you did not previously run the macro or would like to enter new settings go to section **3.1**.

- If you would like to use saved settings go to section **3.2**.

Grey boxes denote ▢ Window Titles ▢ of the various pop-up windows.

5.1 Use New Settings

1. ▢ Settings ▢

   Select 'Use new settings' from the drop down dialog and click OK.

2. ▢ Choose a sample picture to define settings ▢

Chose a picture you would like to use to define the settings. A picture containing leaves that are part green and part necrotic is ideal. Select picture and click Open.

3. Leaf Area

Use the threshold sliders to select the leaf area. The aim is to highlight the leaf in red without highlighting any of the background. Highlighting small spots of dirt, lines, text etc. in the background is not a problem, these will later be removed automatically. After selecting the leaf, click OK. (Do not click the buttons in the threshold window).

4. Threshold

Use the colour threshold window to select the NECROTIC part of the leaf. This is easiest using the CIELab colour space. To change colour space, check the CIELab button at the bottom of the window (it can take a few seconds to change). Move the L*, a* & b* sliders until the green part of the leaf turns white. As a starting point, moving the a* (middle panel) sliders to 120 and 255 is recommended with further fine adjustments made as needed. You can switch between the thresholded and original image using the Original and Filtered buttons. Once you have selected the necrotic part of the leaf, click USE THESE SETTINGS in the threshold window, a results table will appear. Click OK in the dialogue box.

5. Macro Settings

Enter the settings to use for detecting pycnidia, default settings are given. Unfortunately there is no way to preview these settings, it is a case of trial and error to optimise the settings for your images. 'Maxima' alters the sensitivity of pycnidia detection. Increase the value to reduce the sensitivity, increments of 5 are suggested. Reducing the sensitivity reduces background noise but also reduces the ability to detect pycnidia. If you have leaves with individual lesions and would like to know how many pycnidia are present in each lesion select 'Yes' from the drop down menu and decide the size of the lesions you want to count pycnidia in. Lesions greater than 10 mm are recommended. Click OK.

6. Choose a Directory to save macro settings

The settings will be saved with a time stamp in the file name so you can use them again in the future. Chose a location to save the settings and click Choose.

7. Choose a folder containing your pictures

Choose a folder containing the pictures you want to analyse. Click Choose. The macro will now begin to run. Progress information is printed in the Log window. When the macro is finished, 'Macro is finished' is printed in the Log window.

5.2 Use Saved Settings

1. Settings

   Select 'Use saved settings' from the drop down dialog and click OK.

2. Choose a file containing saved macro settings

   Select a file containing saved macro settings. Click Open.

3. Choose a folder containing your pictures

   Select a folder containing the pictures you want to analyse. Click Choose.

The macro will now begin to run. Progress information is printed in the Log window. When the macro is finished, 'Macro is finished' is printed in the Log window.

6 Macro Output

The macro will create 2 folders within the folder containing the pictures. The Overlay folder contains .png files of the analysed pictures. The leaf is outlined in blue, necrotic areas in yellow and pycnidia in red. What ever has been measured/counted will be outlined. The Results folder contains the results. For each picture four .xls tables are produced:

- *filename*-results**Area** contains the leaf area
- *filename*-results**PycCountSize** contains the size of each pycnidium
- *filename*-results**Necrosis** contains the size of each necrotic area
- *filename*-results**Grey** contains the grey value for each pycnidium

These files are useful if you want to look at the distribution of measurements within a leaf. The Summary file is the summary of the results generated automatically by ImageJ. The Output file is a custom results table and contains the most useful information. For each picture it gives the total leaf area, total necrotic area, % leaf area covered by lesions (PLACL), number of pycnidia, mean pycnidia size, pycnidia per cm^2 and pycnidia grey value. The Log file contains a record of the settings used, pictures analysed and time taken.

7   References

Stewart, E.L., Hagerty, C.H., Mikaberidze, A., Mundt, C. C., Zhong, Z. & McDonald, B.A. (2016) An improved method for measuring quantitative resistance to the wheat pathogen *Zymoseptoria tritici* using high throughput automated image analysis. Phytopathology.

Stewart, E.L. & McDonald, B.A. (2014) Measuring Quantitative Virulence in the Wheat Pathogen *Zymoseptoria tritici* Using High-Throughput Automated Image Analysis. Phytopathology. Vol 104, No. 9, 985-992.