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Raman analysis of Maize protoplast

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Experiment details

Samples from the Wurtzel lab (prepared by Maria Shumskaya). Samples included;

1. Non-transformed protoplasts. Those are cells from maize leaves that are not carrying any GFP constructs. They have a small amount of carotenoids in them anyway. This small amount of carotenoids is present in all other samples as background. You can use this tube first to check how you can prepare the sample, play with it, also use as a negative control (nothing overexpressed).
2. PSY1-T-GFP. Efficiency of transformation: low (1 cell in 200-300 cells is transformed and has GFP in plastids; the rest is just non-transformed empty protoplasts). Expectations: high carotenoid concentration in plastids.
3. PSY1-P-GFP. Efficiency of transformation: low (1 cell in 200-300 cells is transformed and has GFP in plastids). Expectations: high carotenoid concentration in fibrils (analyze only cells with fibrils in plastids, not all of them have fibrils, some are just with GFP in stroma. You do not need to analyze cells of that type).
4. PSY2-GFP. Efficiency of transformation: high (1 cell in 10 cells is transformed and has GFP in plastids) . Expectations: high carotenoid concentration in plastoglobuli (dots).
5. PSY2-RFP. Efficiency of transformation: very low. I'm sending it just in case GFP is a problem for your laser.
6. LHCP-GFP. Efficiency of transformation: low (1 cell in 200-300 cells is transformed and has GFP in plastids). This protein is a thylakoid protein from a photosystem. Expectations: carotenoid content should be the same as if the cells were not transformed with anything. It is an additional negative control.

Samples were prepared by centrifuging protoplast at 500 x g and concentrated by removing all but 100 ul of the supernatant. 25 ul of concentrated sample was loaded to microscope well-frame and covered with a #1 coverslip (18 x 18 mm).

It was not easy to pick out transformed cells on the Raman microscope. We spent a lot of time searching for the appropriate cells to visualize. In the end we only imaged PSY1-T-GFP, PSY1-P-GFP and PSY2-GFP and about 16 cells total worth analyzing.

Raman Imaging - WiTec Alpha300 R

Laser excitation – 532 nm, 1 mW laser power

20 X objective (NA = 0.45), theoretical resolution 0.8-1um.

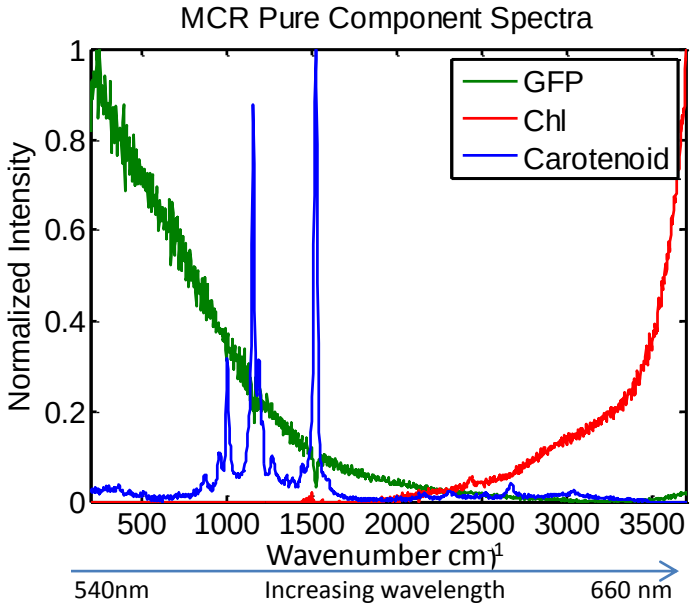
For most images, we had a step size of 500 nm (pixel size).

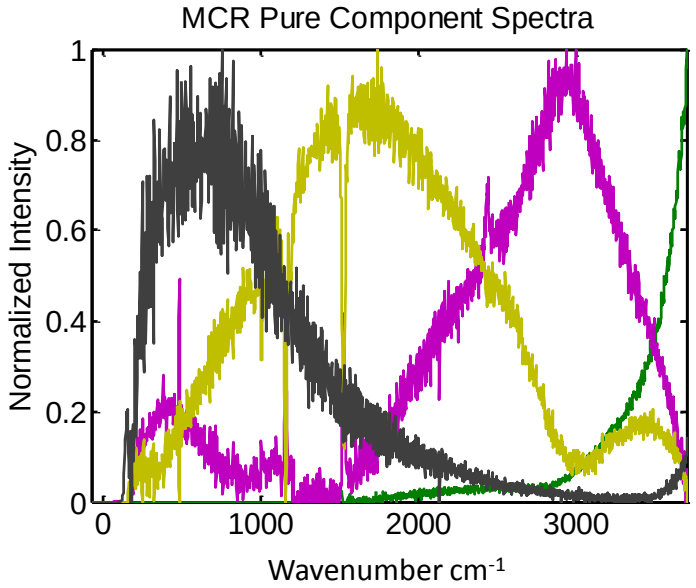
Analysis

Spectral images were converted to a format that could be read by our in-house analysis routines. Regions devoid of protoplasts were masked out and multivariate algorithms were applies to mathematically model the spectral variance.

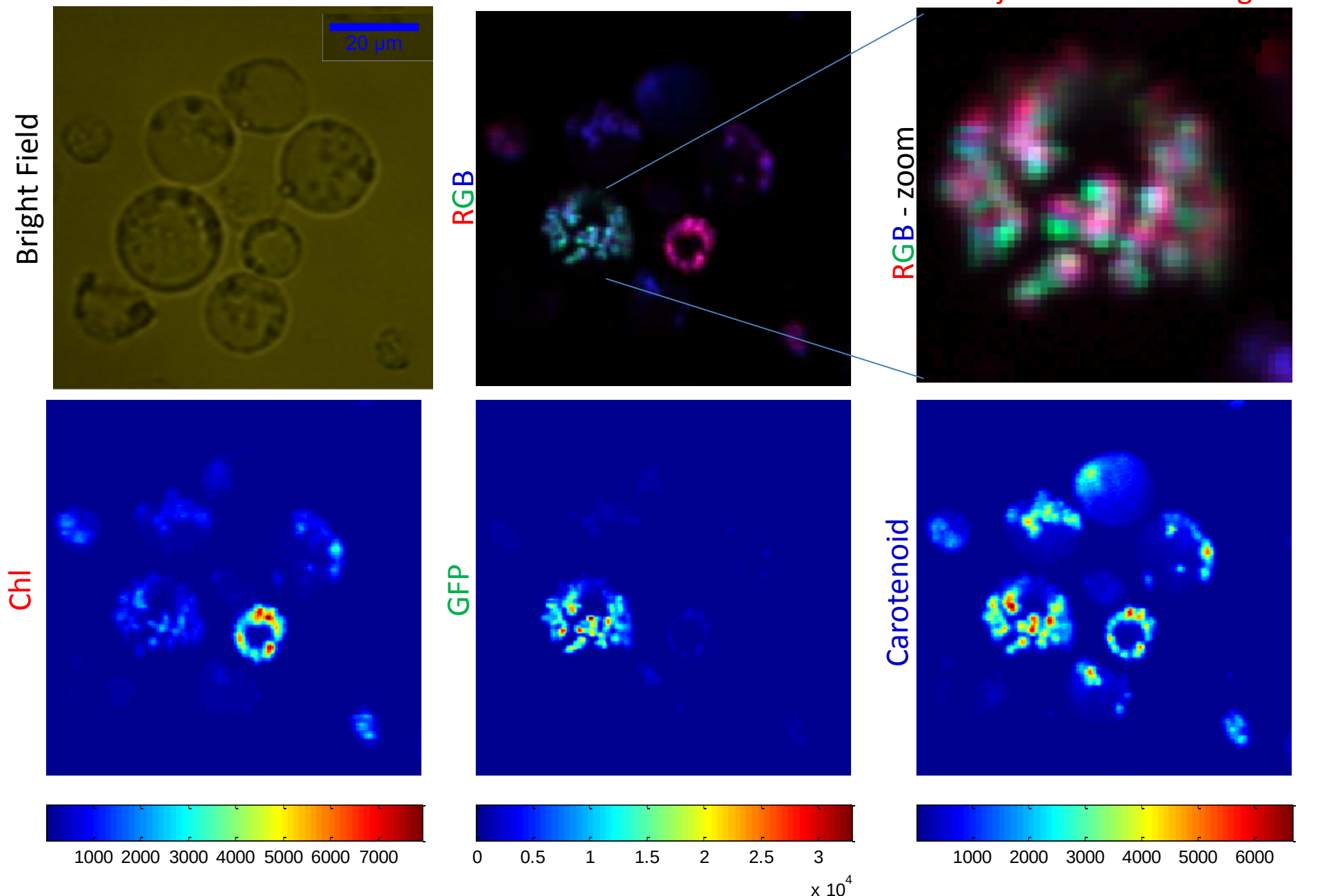
PSY2-GFY, efficiency of transformation ~10%

6 spectral components (plus an offset) were required to explain the spectral variance of the images. 3 are explained as GFP, Chl and carotenoid. The other 3 are minor (in percent contribution) and might arise from autofluorescence among other spectral congestion.



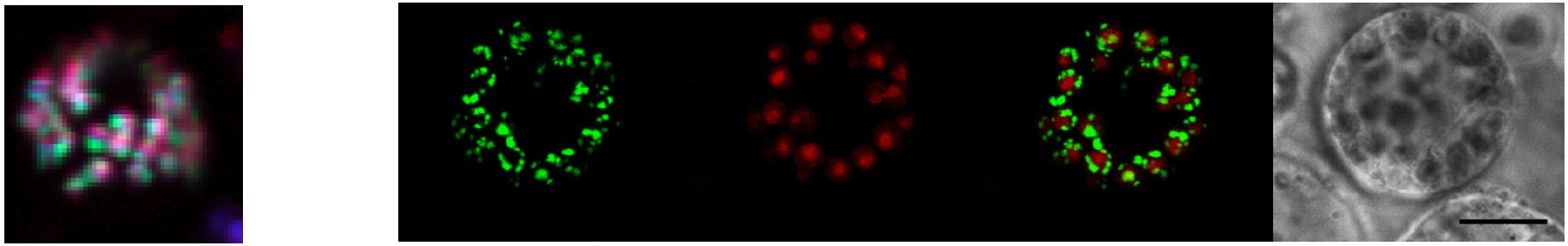


PSY2-GFY protoplasts



On the previous slide, note the one cell that has very bright GFP fluorescence. The location of the GFP signal is punctate and is consistent with images sent from Maria.

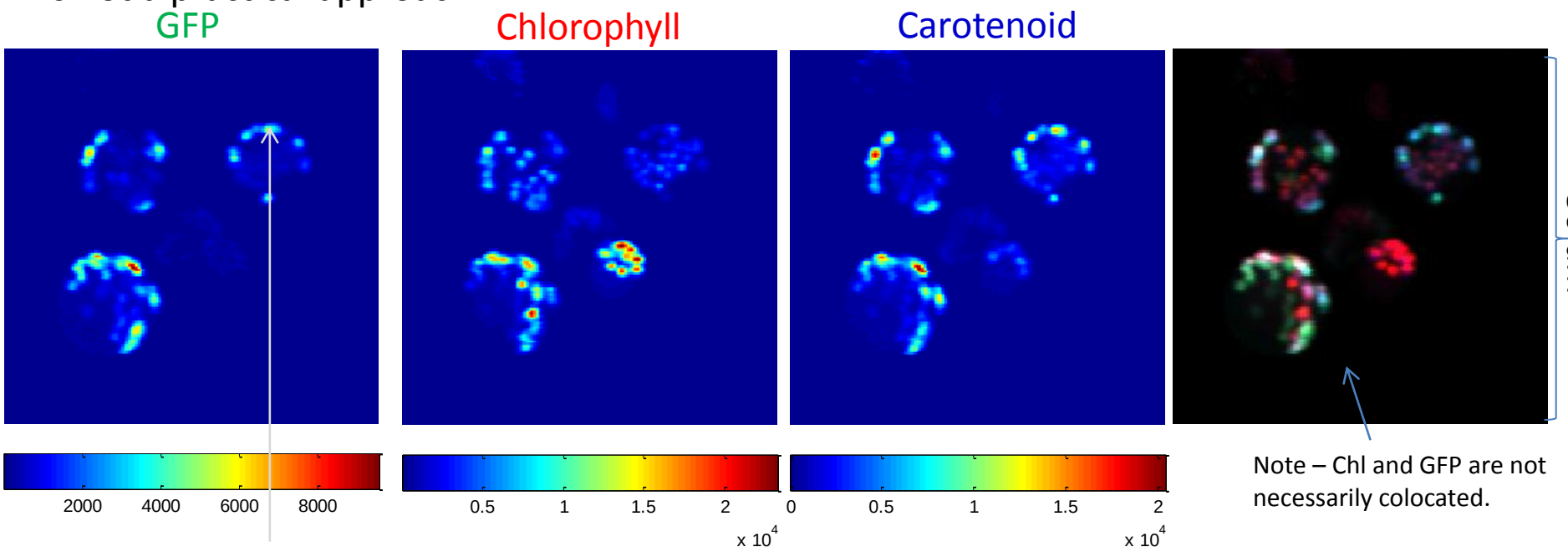
Raman GFP Chl Merge



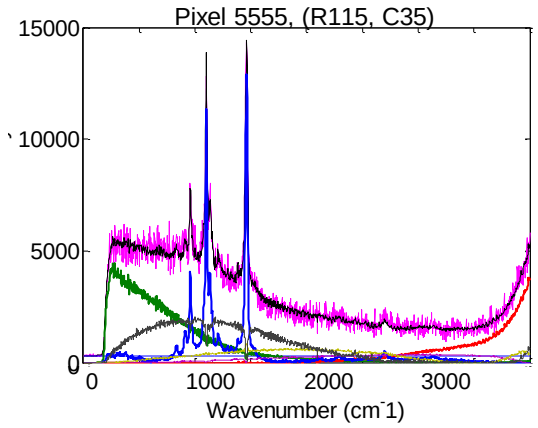
Also from the previous slide, it appears that the protoplast will obvious GFP emission is slightly increased in carotenoid, however this is based solely on empirical observation.

PSY1-P-GFP

We could not see the spindle shapes in these protoplast under bright field illumination. We search for cells by random sampling but with 1 in 300 cells estimated to be transformed, this is not a practical approach.



Note – Chl and GFP are not necessarily colocated.



This is a single pixel spectrum. Note that all of the these cells have some GFP signal, but it is about ¼ less then the previous example. Does this indicate that GFP is expressed in all cells at low levels? Does that make sense?
Alternatively, the model might not be perfect.

No obvious spindles in any of the PSY1-P-GFP samples but at this resolution it might not be unexpected.

Summary

The initial Raman imaging work was profitable on many fronts;

1) We were able to obtain some evidence for GFP and definitive Chl fluorescence on the microscope
2) Carotenoid was identified but it's identity is unknown at this point – Does the Wurtzel lab know what is the most abundant carotenoid is in these protoplast?
3) It should be possible to quantify relative carotenoid abundances between protoplasts.
4) We experienced no issue with the protoplasts moving during imaging. It might be possible to use a gridded slide/coverslip to locate cells on a fluorescence microscope before Raman imaging or before sending to us. http://www.rpicorp.com/products/prod_info.html?products_front_id=1901&catid=5&cat_id=71

Issues to be resolved

1) Raman imaging is time intensive – each scan took 3-15 minutes depending on scan area.
2) Identifying transformed cells is not straightforward on the Raman scope.
3) Need to work out timing of sampling. We can't investigate more than 2-3 mutants per day. As a note, when I checked the protoplast this morning (1/30) most of the protoplasts were ruptured and little to no GFP could be found with a traditional fluorescence scope. Because of this, our line-scanner Raman system was not tested to see if a speed increase was possible.
4) Moving to a high magnification objective will make finding transformants even more difficult due to the limited field of view.

Wurtzel lab - Let's get into the nitty gritty of what you would like us to investigate on the next pass.