# Analysis of Maize Protoplast 02/26/2013

Analysis: Aaron Collins

Raman imaging: T. Beechem and A. McDonald

# Notes

Samples from Wurtzel lab were received on morning of 02/26/2013

Samples were analyzed in the following order; Control (untransformed) , PSY1-P, PSY-T, PSYP-D/E. 4-6

4-6 images were acquired for each protoplast type.  Protoplasts were concentrated via centrifugation at 500 x g for 2 minutes, loaded into a in-situ frame adhered to a microscope slide and covered with a #1.5 coverslip.

Raman Parameters

 20X, 0.75 NA, Nikon Plan APO objective

 10 mW of 532 nm laser power

 300 nm pixel size

 10 or 20 ms integration time/pixel.

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We had some hiccups in the morning hours because the protoplasts where not giving the same intensity Raman signals as our first pass with these samples 3 weeks ago.  We double checked the instrument for performance that it was performing as expected so I think these protoplasts had less pigment in them. We were able to get good signal strength by increasing the laser power without noticeable photobleaching. Unfortunately this delayed us and we did not start acquiring data until about 1 pm.

Also Note that we were unable to use the 40X, 0.95NA objective as we had hoped as the working distance was not sufficient to get into the focal plan of the protoplasts.

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I was able to use a Lovins Field Finder to locate and alpha-numerical location of protoplast of interest using a epi-fluorescence (non-confocal) microscope using a 40X, 0.75 NA objective and a FITC filter cube. The resolution and lack of confocality make it challenging to determine if the PSY1-P protoplasts contained obvious fibrils. Transfer of slide from lab to lab did not change the location of the protoplasts in other words, they stayed in place.

# Example of field finder FOV
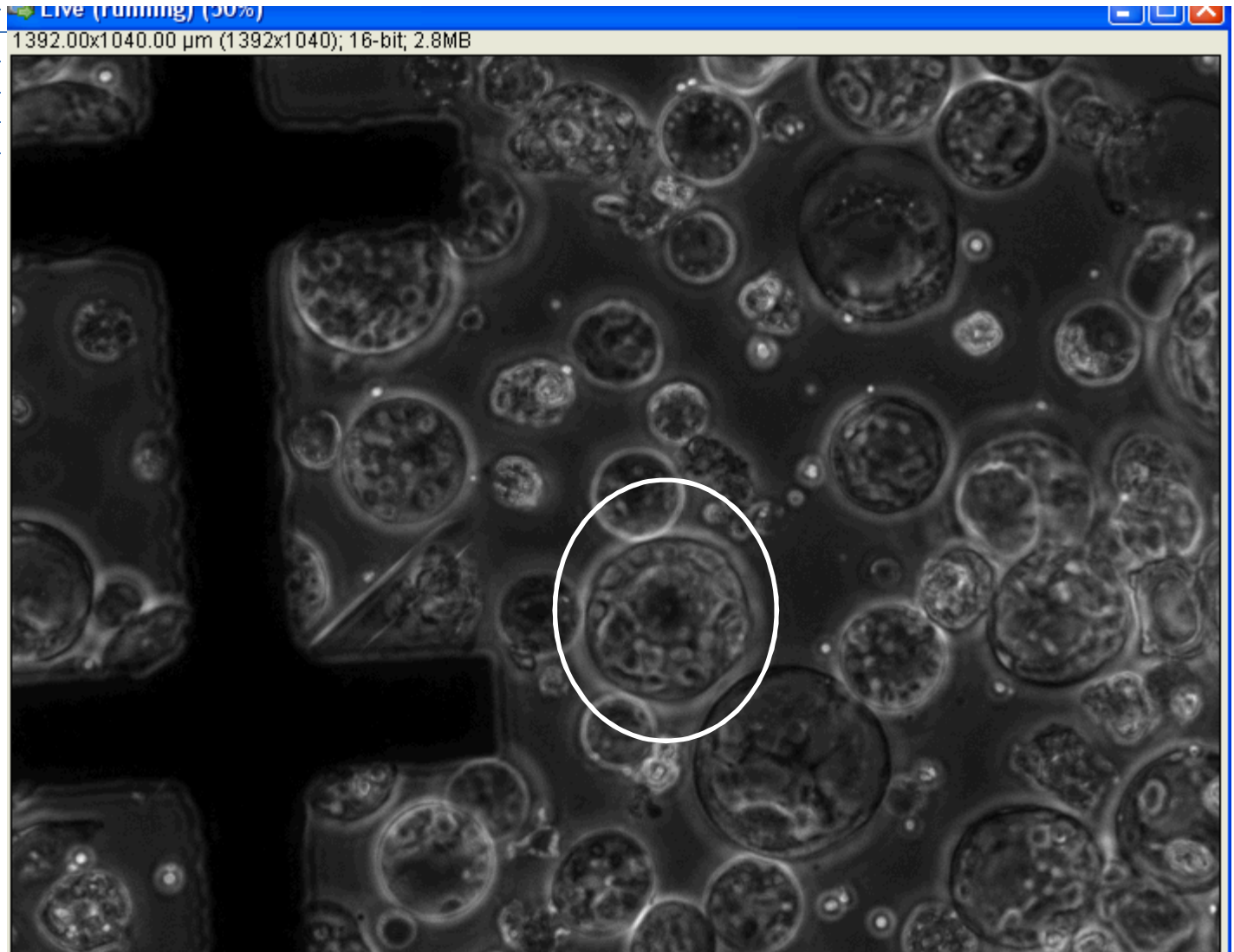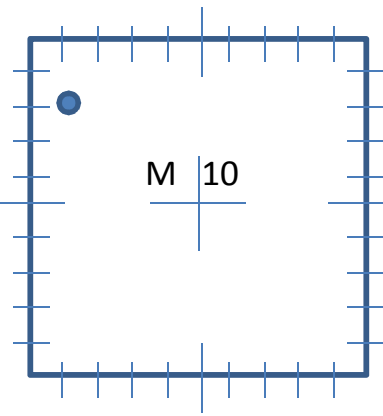


Brightfield image PSY1-P protoplast after identifying fluorescence with FITC cube.

# Data Analysis and MCR model

*.Dat files from the WiTec software were saved, converted into a format that could be analyzed with our multivariate algorithms. The converted files were combined into a composite dataset and multivariate curve resolution algorithms were used to mathematically model the spectral variance of the images. 19 images were combined from Control, PSY1-P, PSY1-T and PSYP-D/E.

## MCR Pure Component Spectra
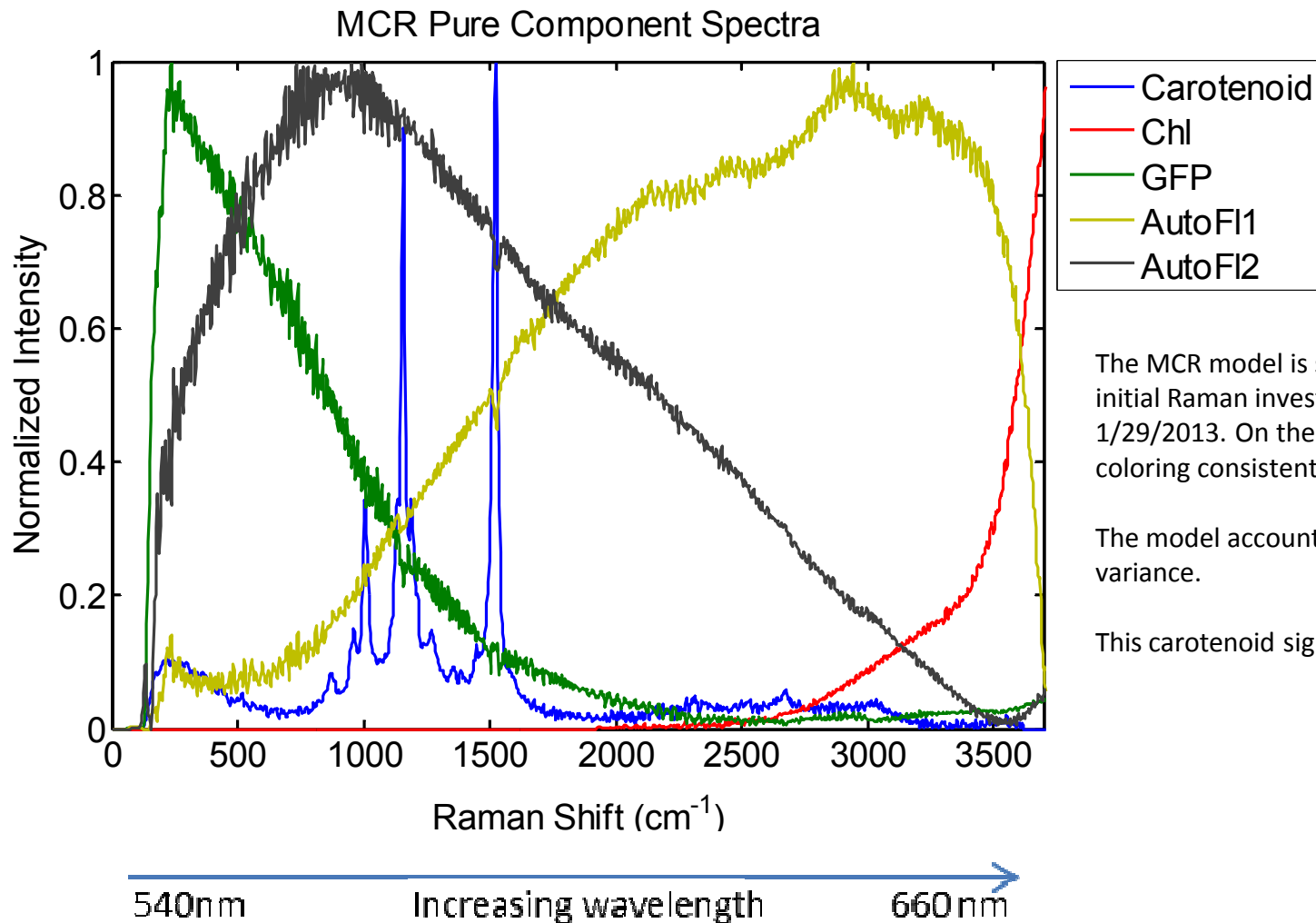


The MCR model is similar to the results of the initial Raman investigation of protoplasts from 1/29/2013. On the following slides I will use RGB coloring consistent with the model to the left ←.

The model accounts for > 99.5% of the spectral variance.

This carotenoid signature is spot on.

# Control – untransformed



Trace = 0, Carotenoid

48 µm
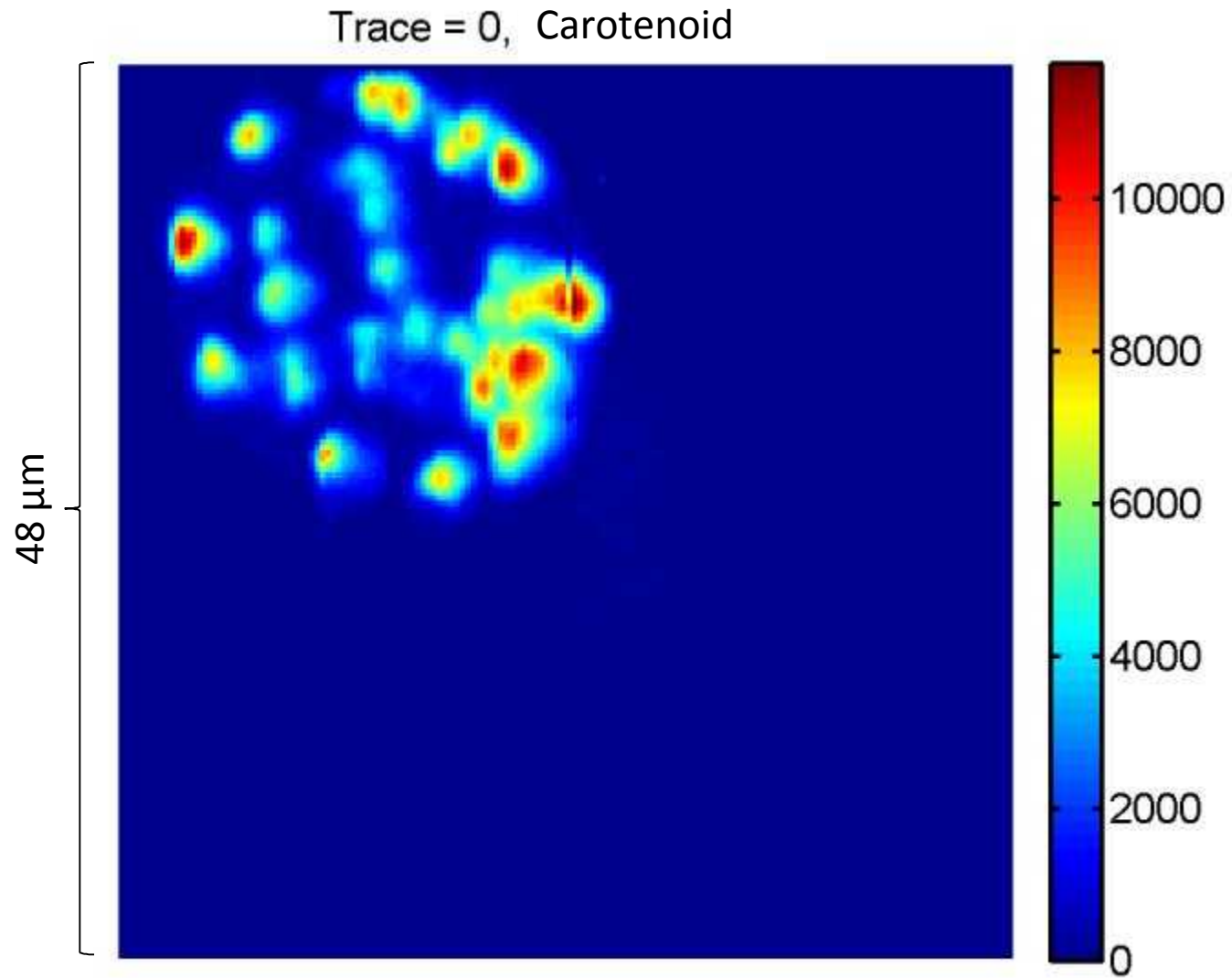
0.02 Sec integration time

# Control – untransformed



0.02 Sec integration time
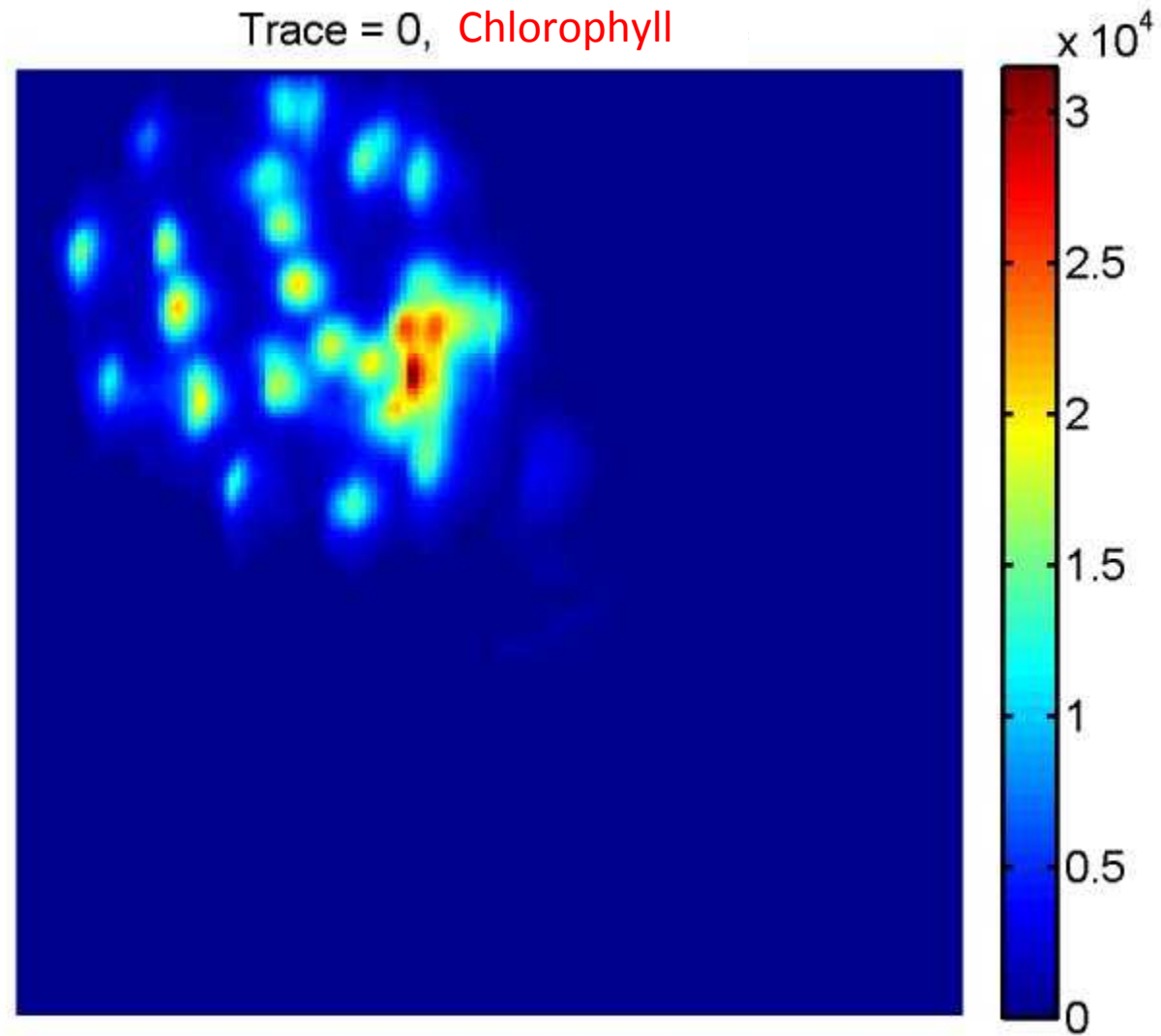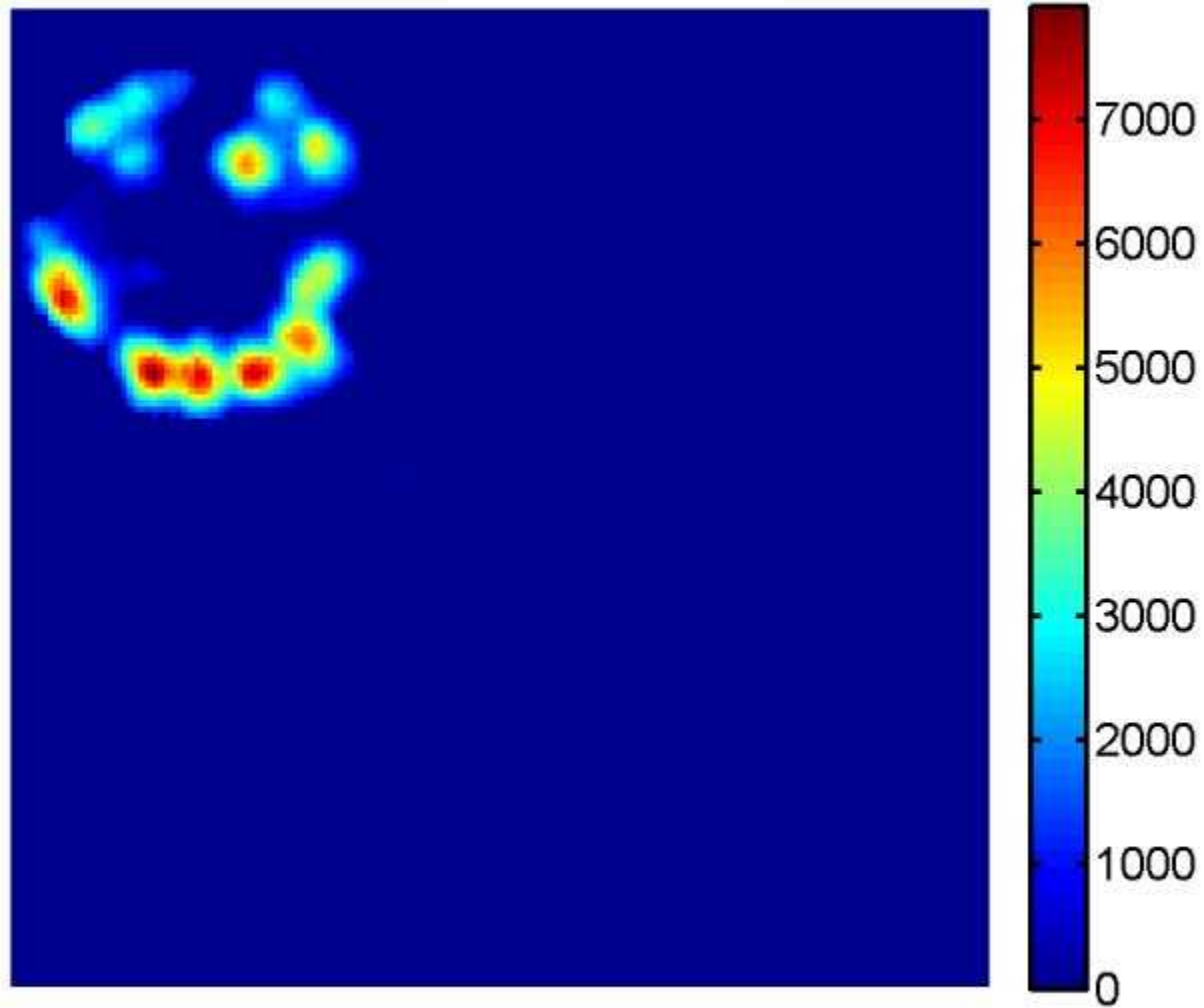
# Control – untransformed



Trace = 0,  GFP
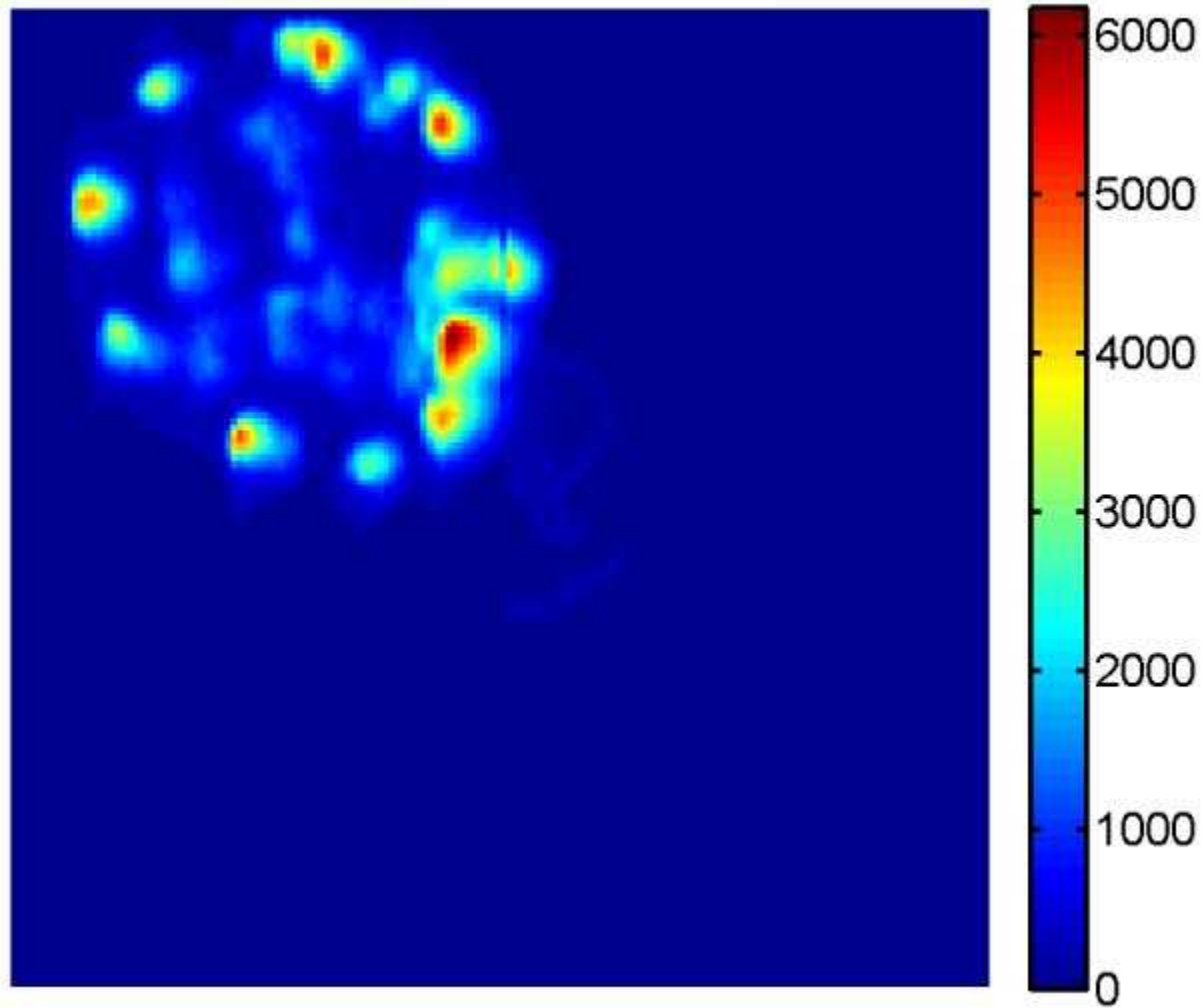
0.02 Sec integration time

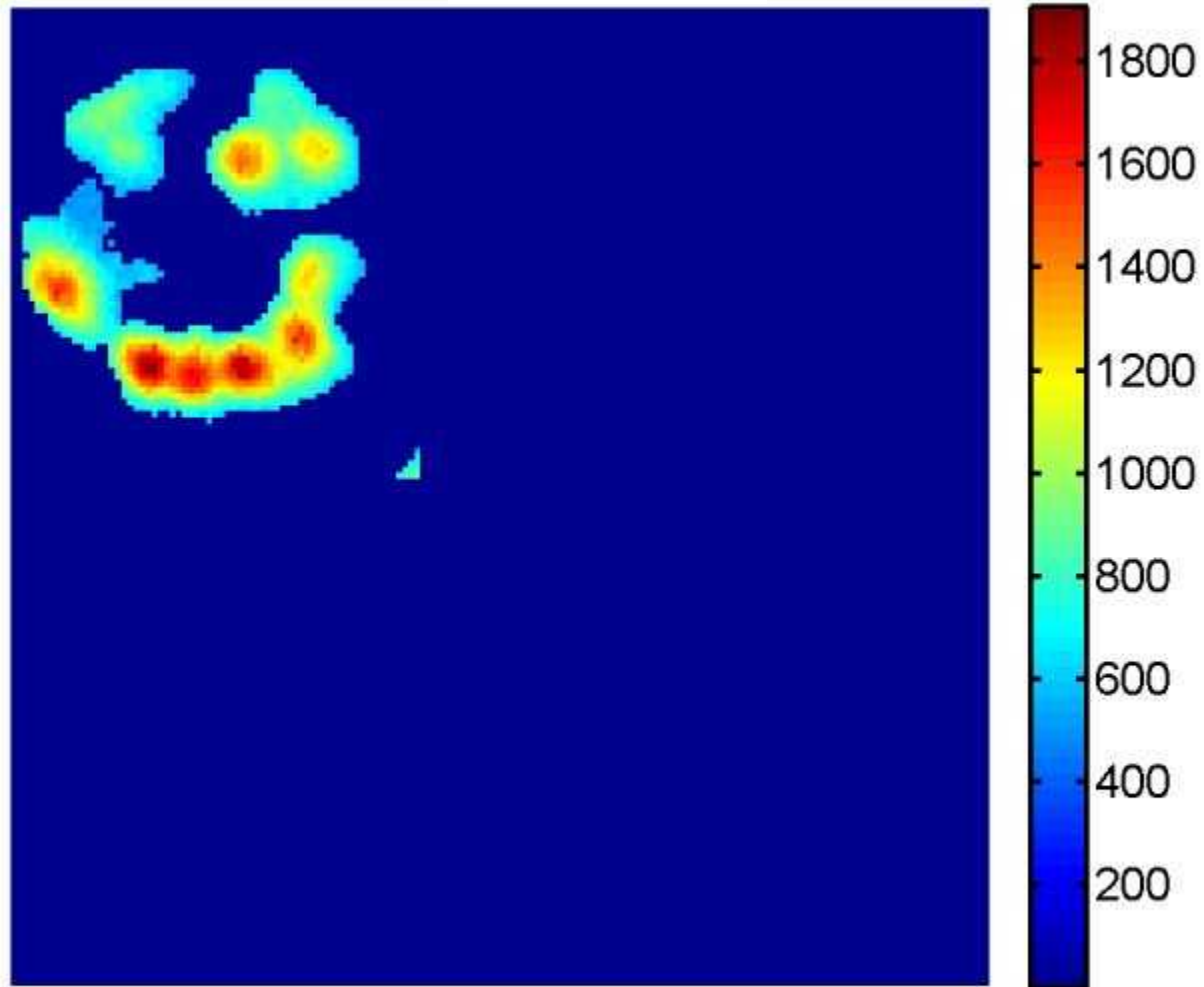# Control – untransformed



Trace = 1, Carotenoid

0.02 Sec integration time
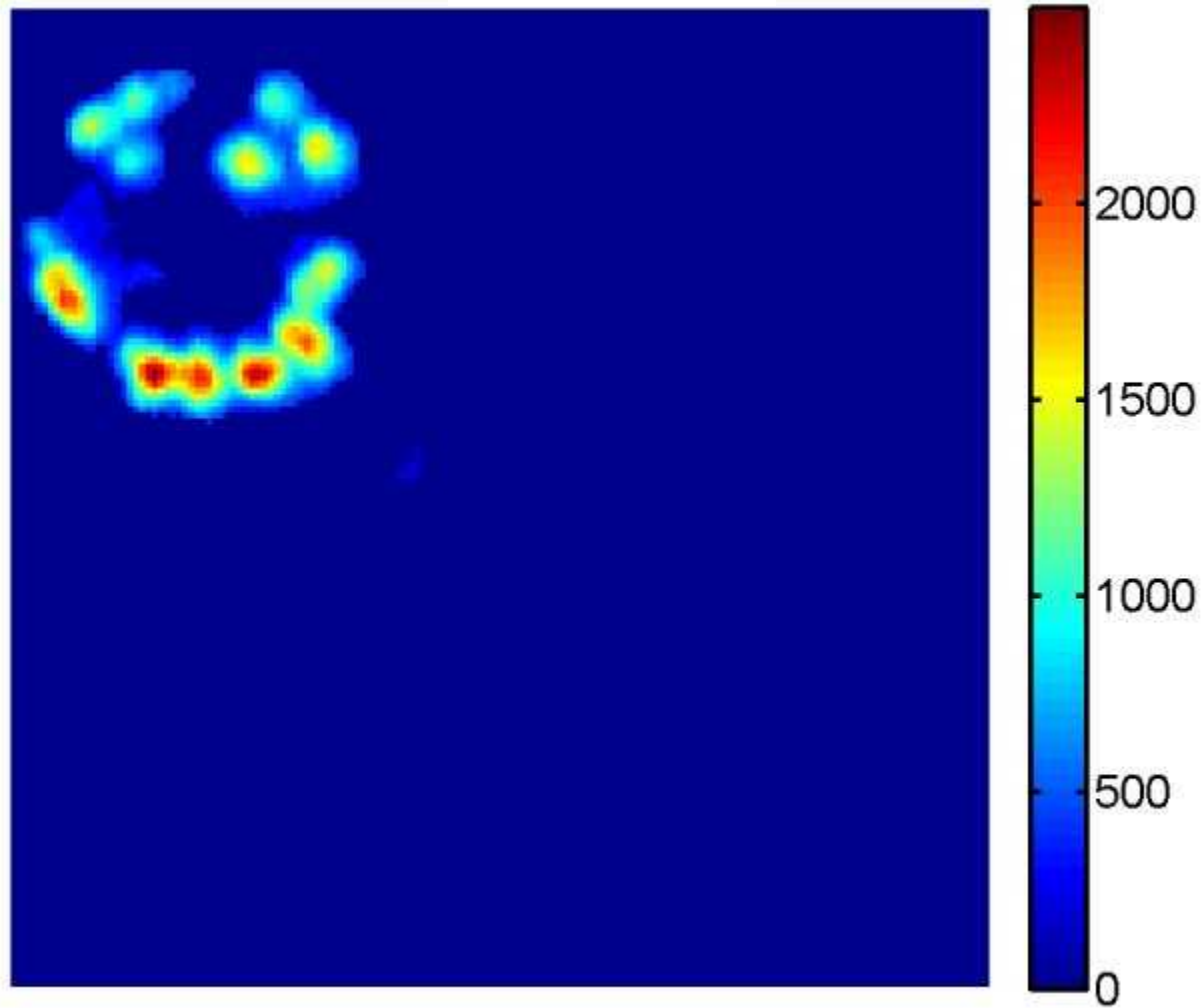
# Control – untransformed



Trace = 1, Chlorophyll
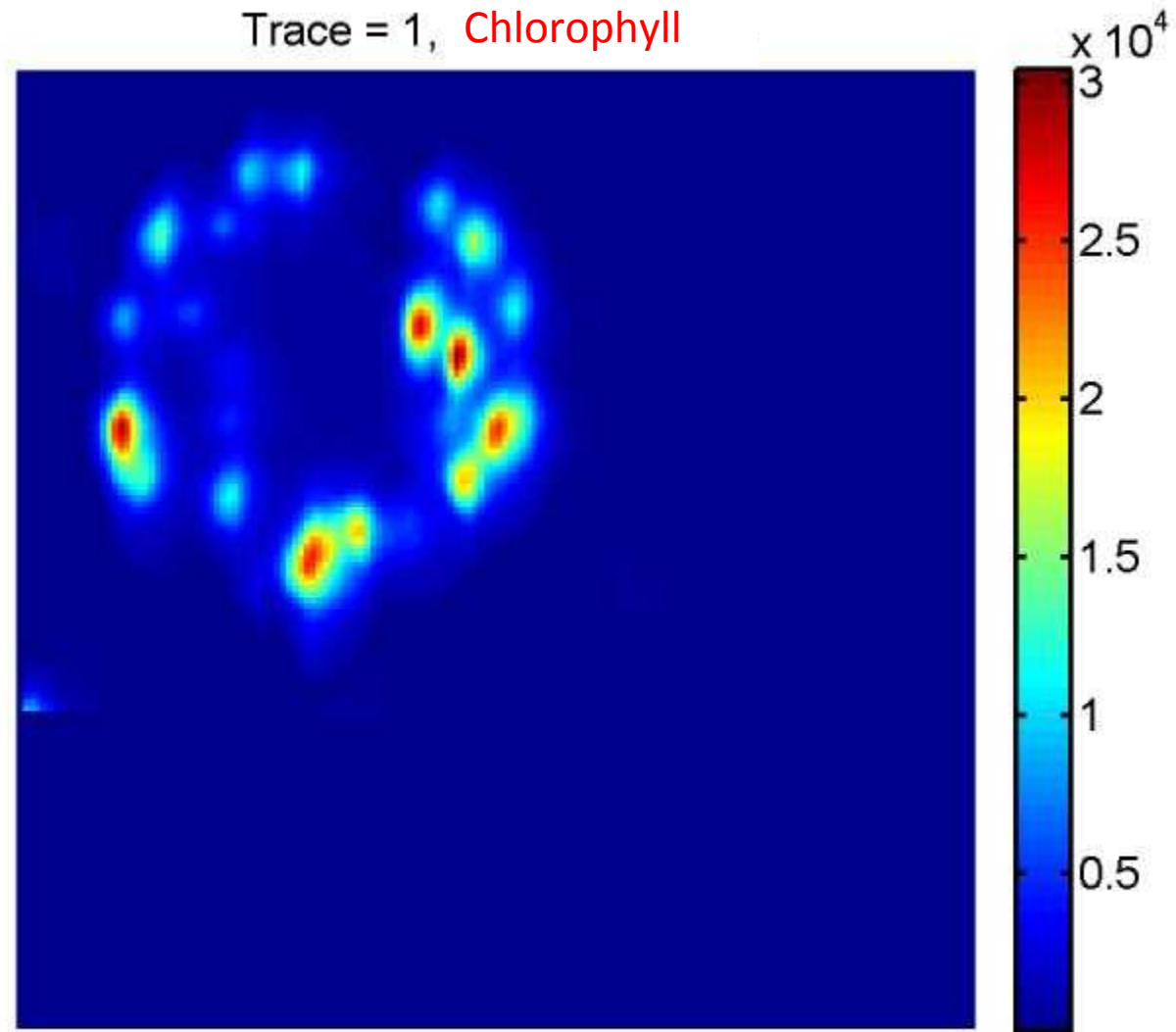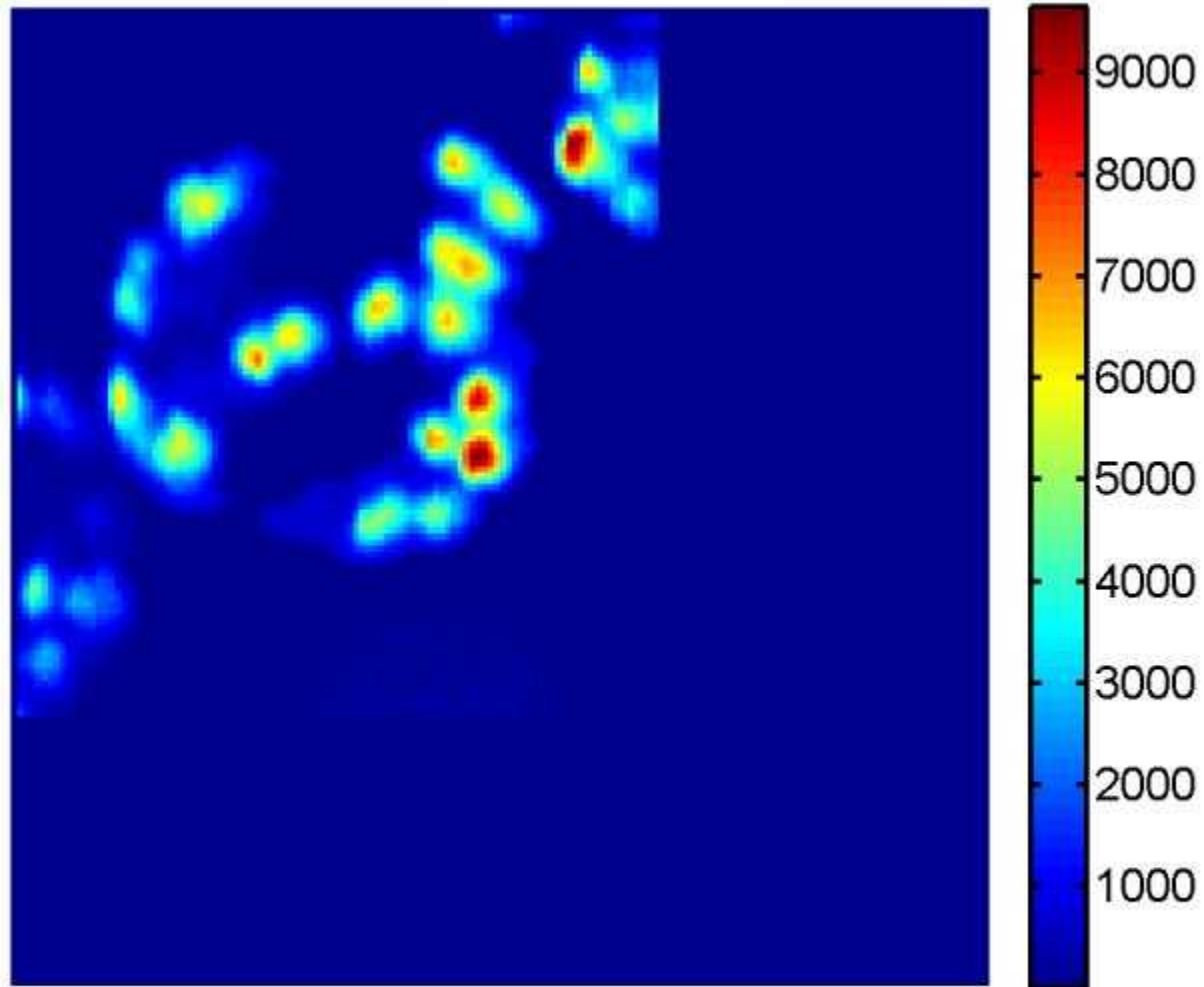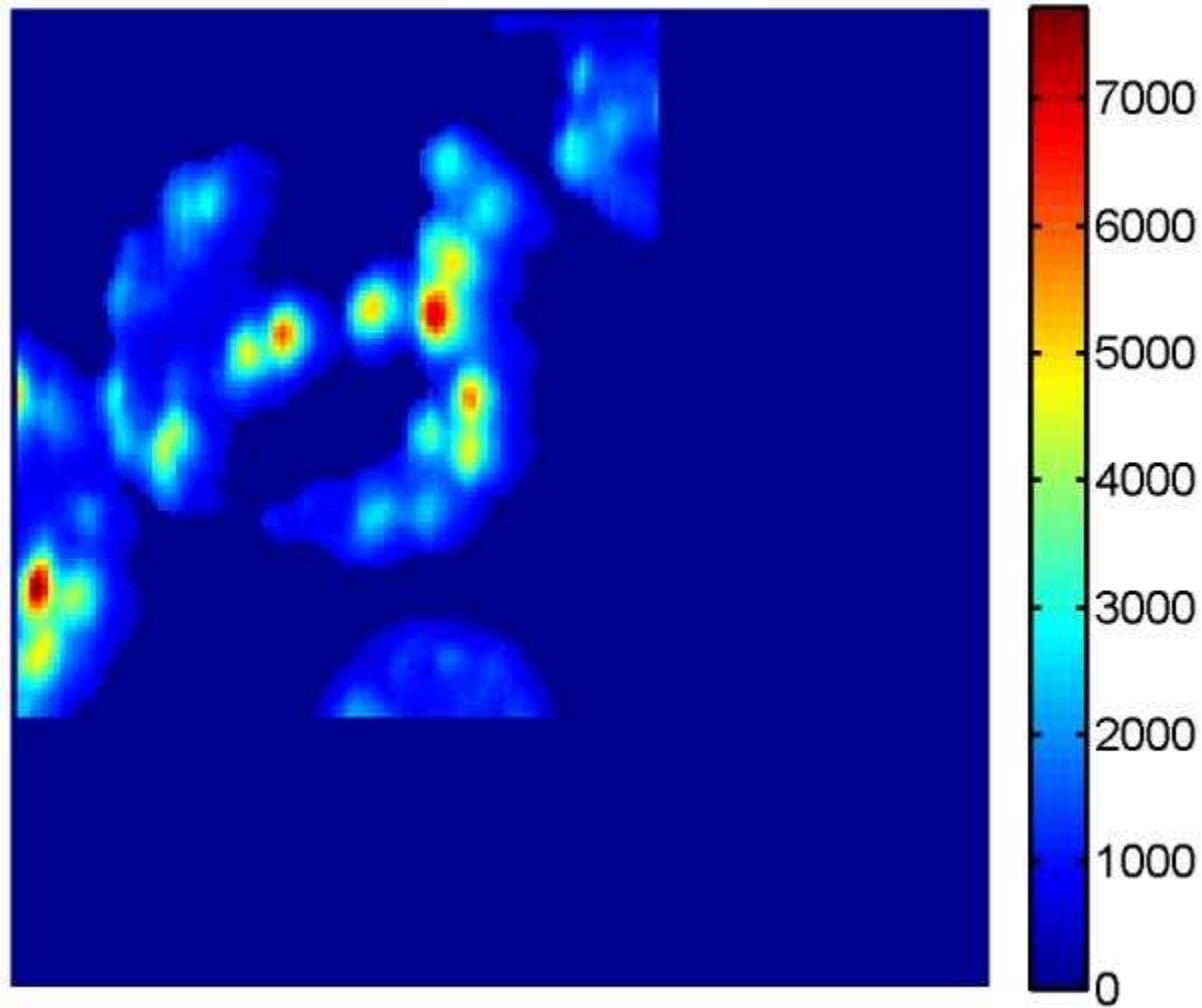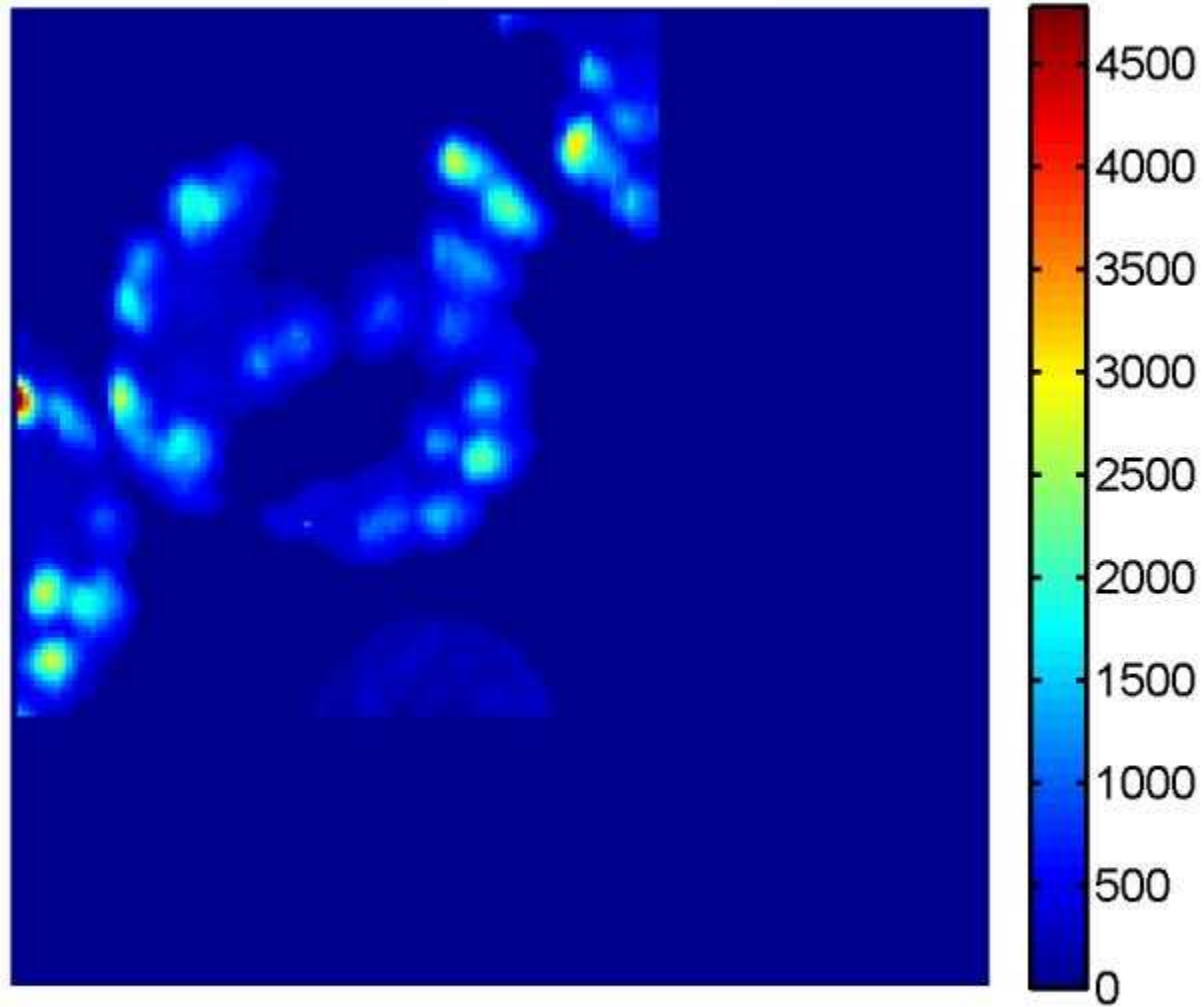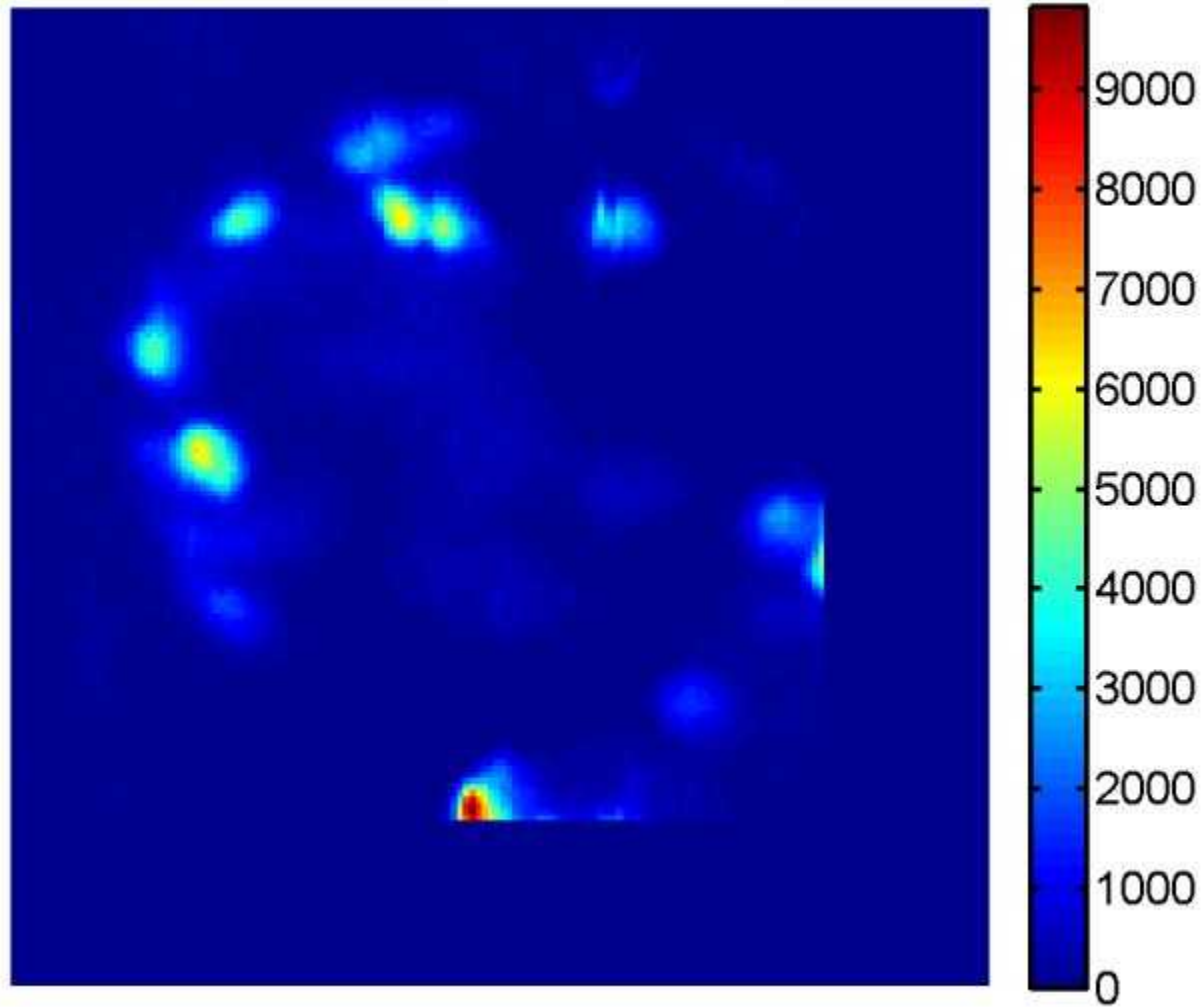
0.02 Sec integration time

# Control – untransformed



Trace = 1,     GFP
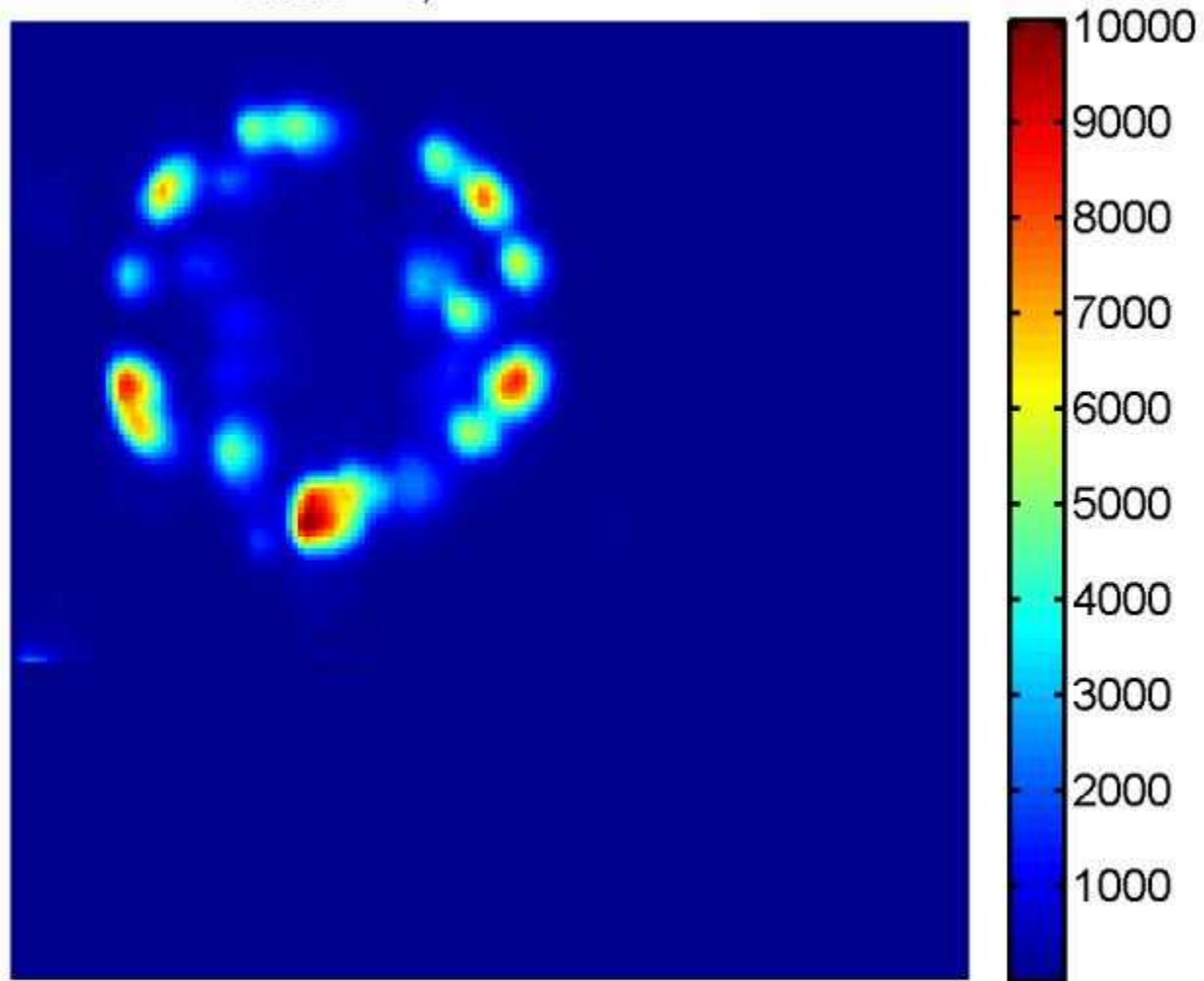
0.02 Sec integration time
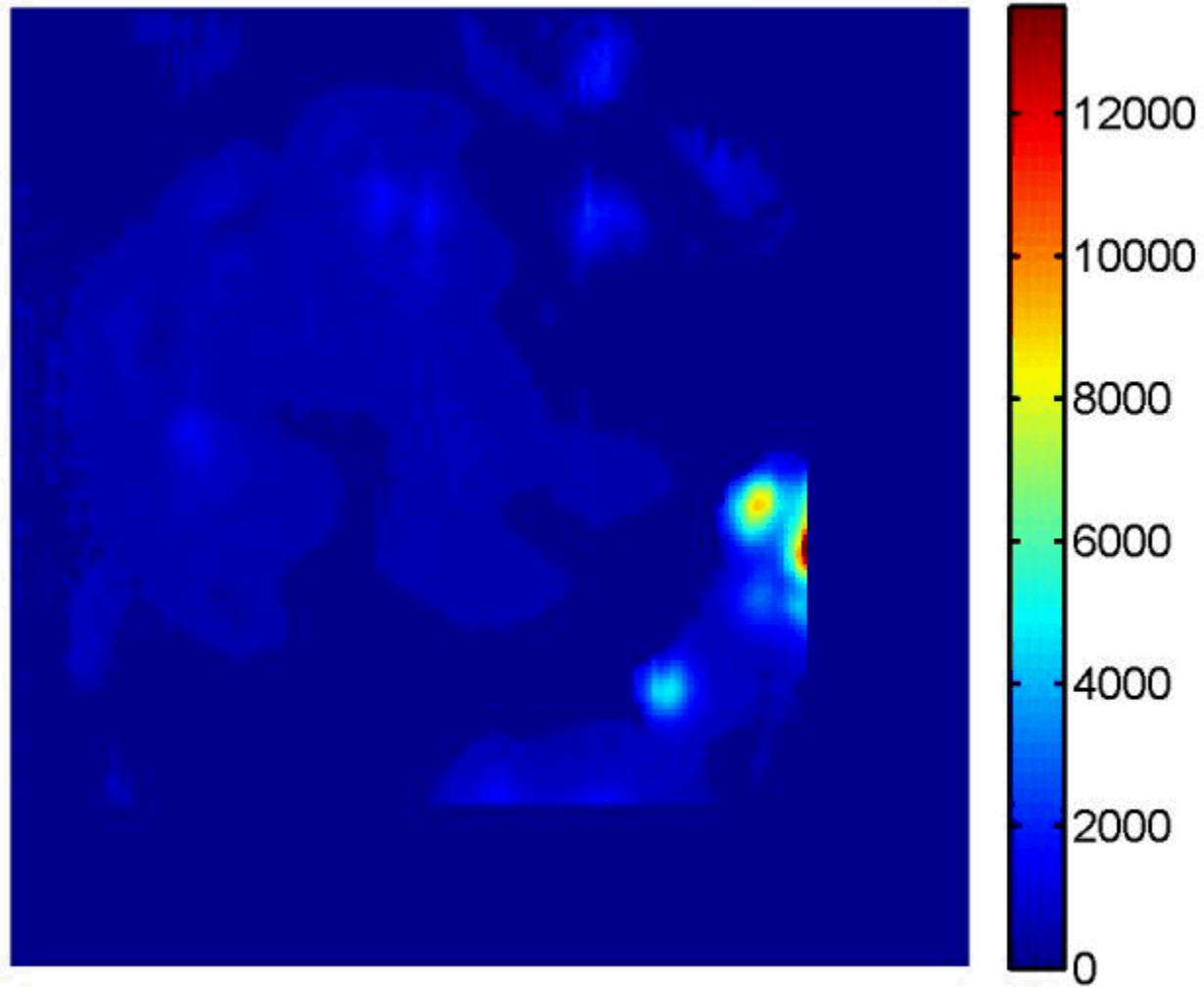
# Control – untransformed



Trace = 2, Carotenoid

0.02 Sec integration time

# Control – untransformed



Trace = 2, Chlorophyll

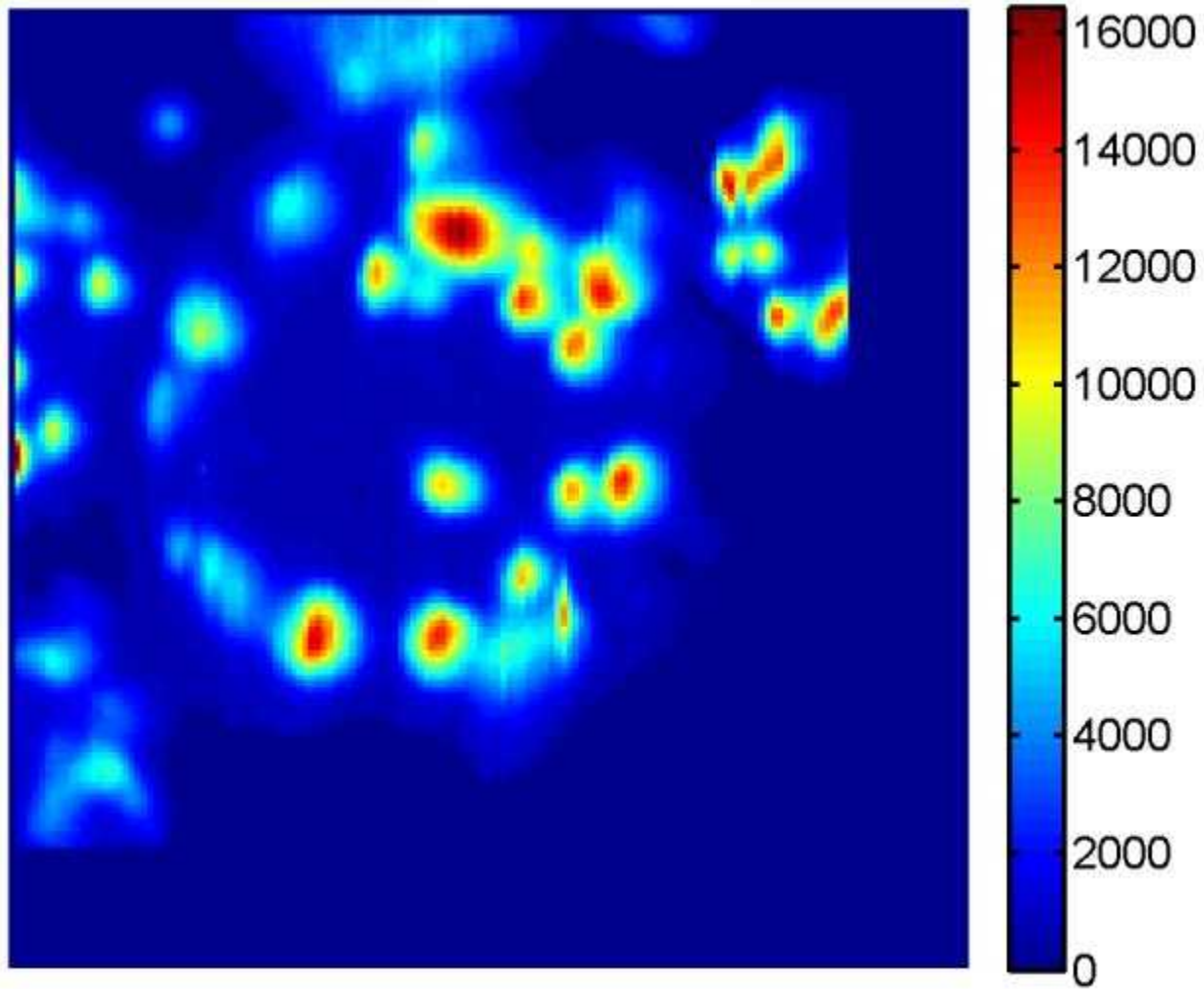0.02 Sec integration time

# Control – untransformed



Trace = 2,    GFP

0.02 Sec integration time

# Control – untransformed



Trace = 3, Carotenoid

0.02 Sec integration time

# Control – untransformed



Trace = 3, Chlorophyll

0.02 Sec integration time

# Control – untransformed



Trace = 3,  GFP

0.02 Sec integration time

# Control – untransformed



Trace = 4, Carotenoid

0.02 Sec integration time

# Control – untransformed



Trace = 4, Chlorophyll

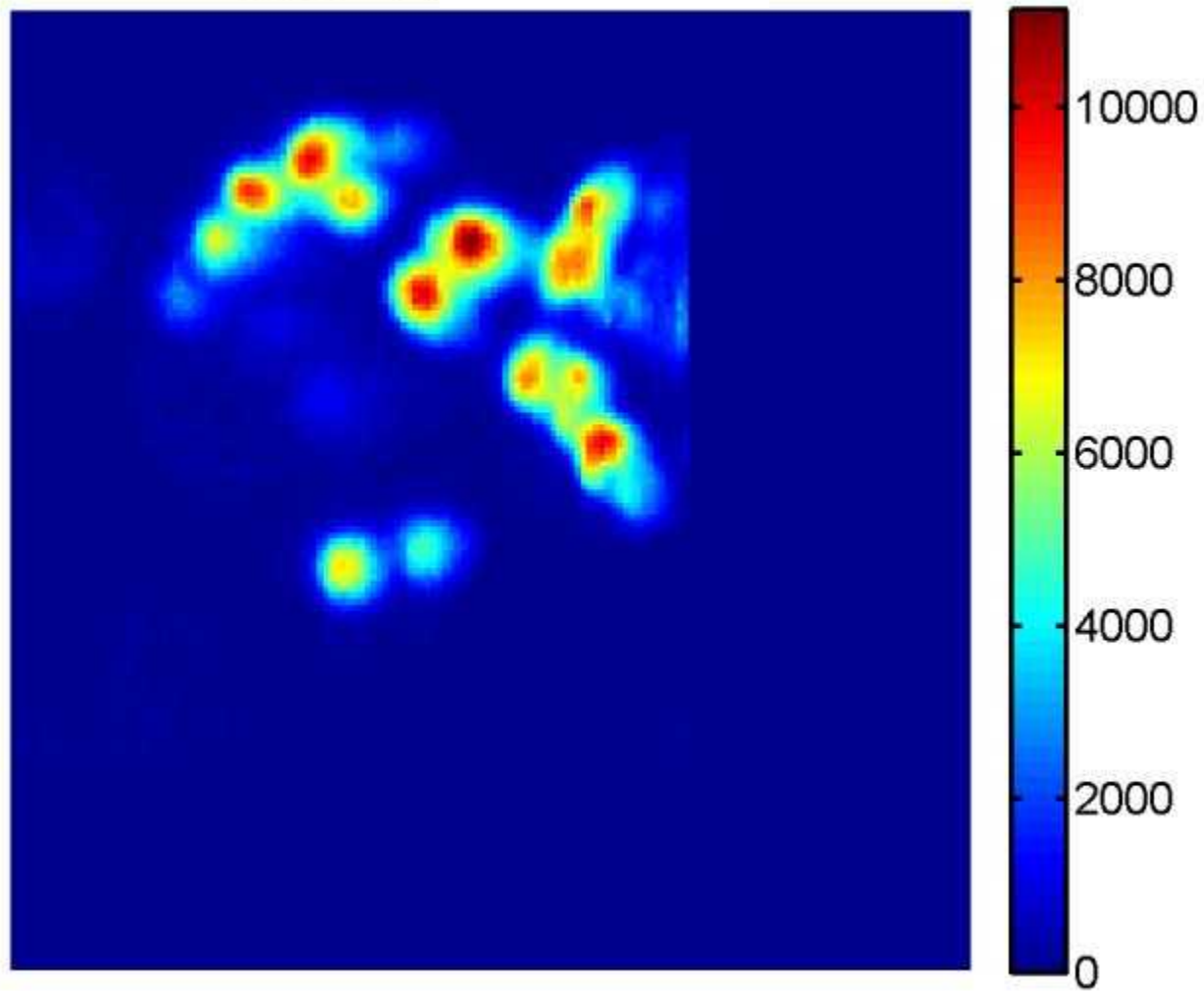0.02 Sec integration time

# Control – untransformed



Trace = 4,    GFP
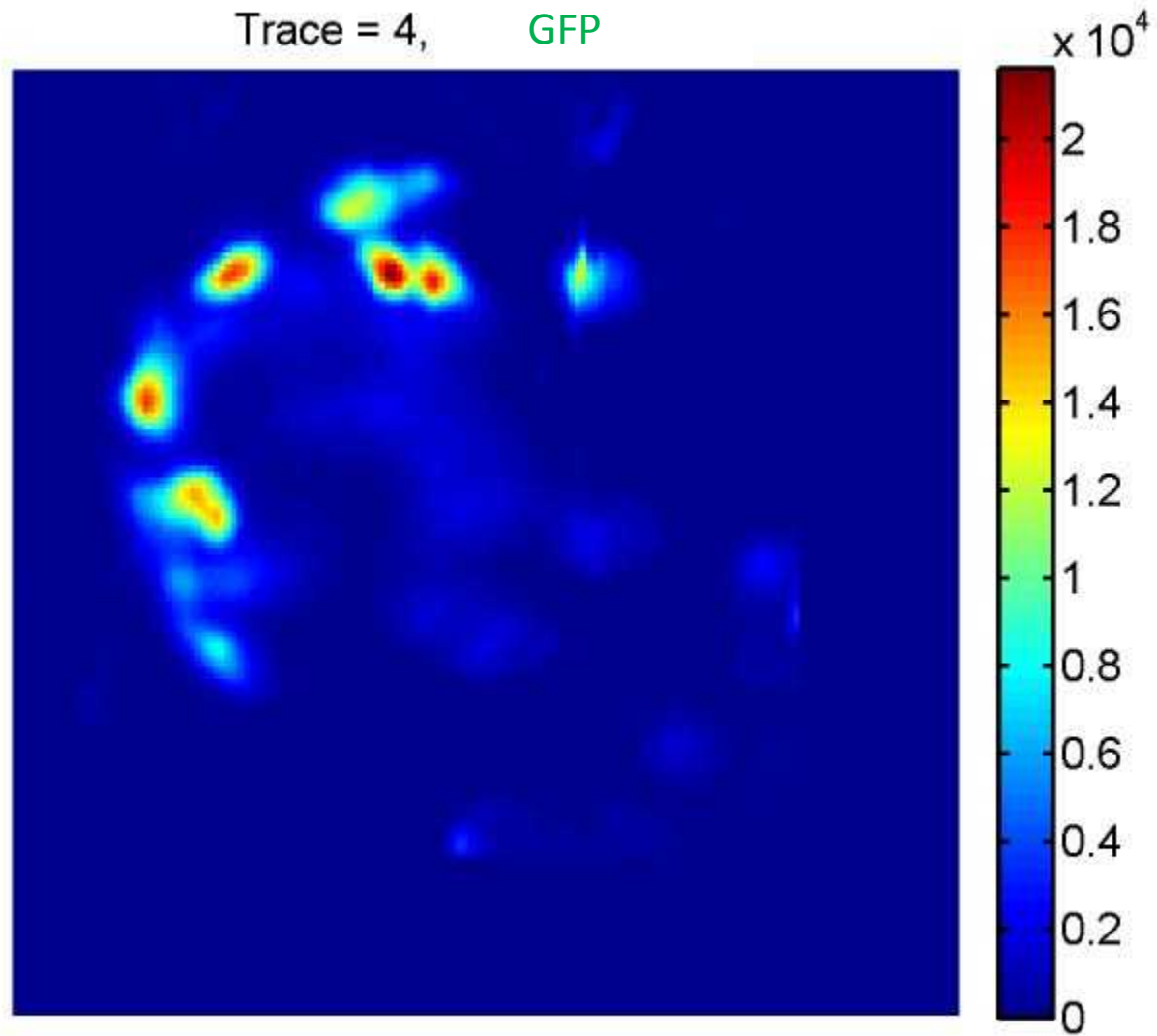
0.02 Sec integration time

# PSY1-P



Trace = 5, Carotenoid
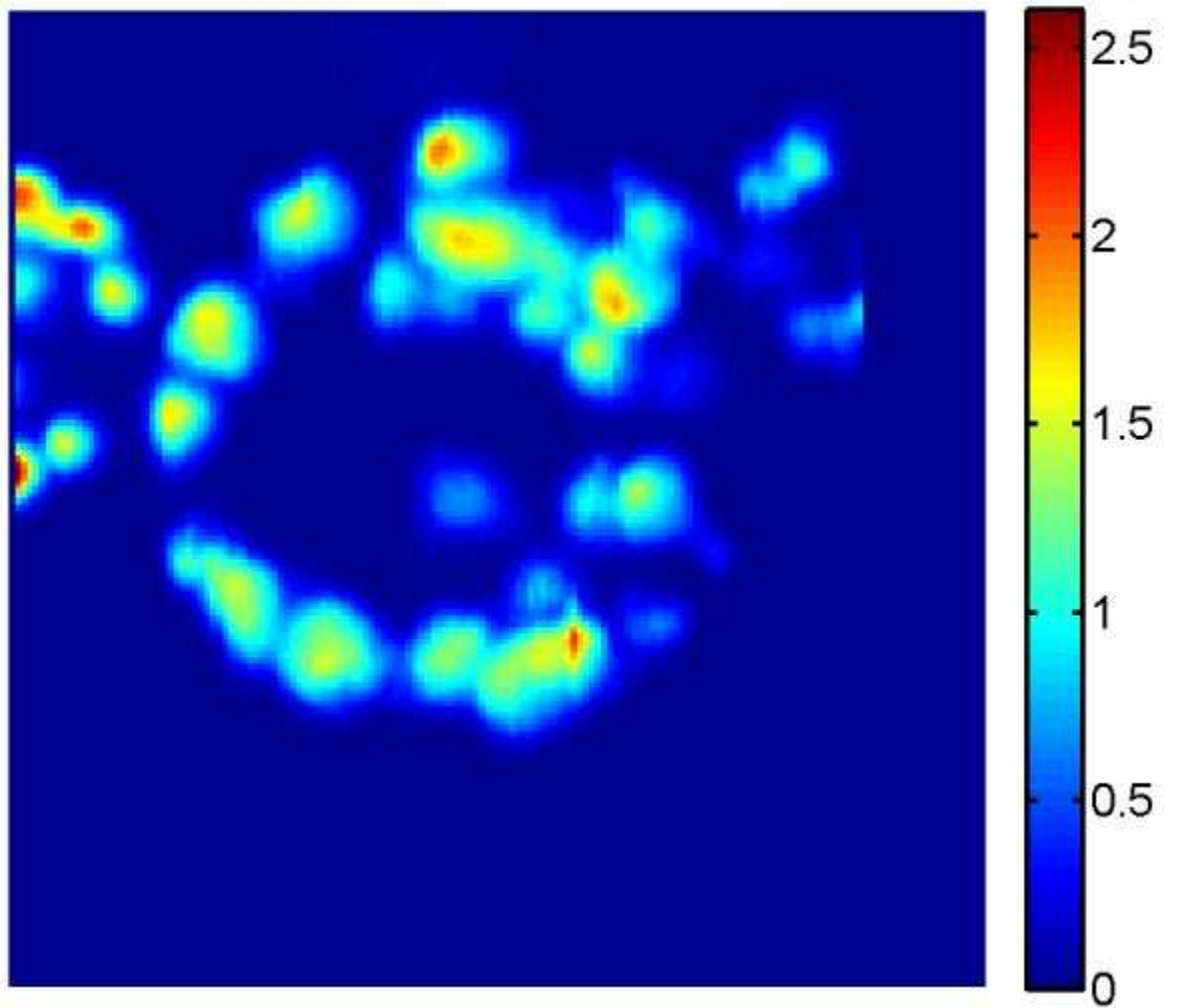
0.02 Sec integration time

Trace = 5, Chlorophyll

0.02 Sec integration time
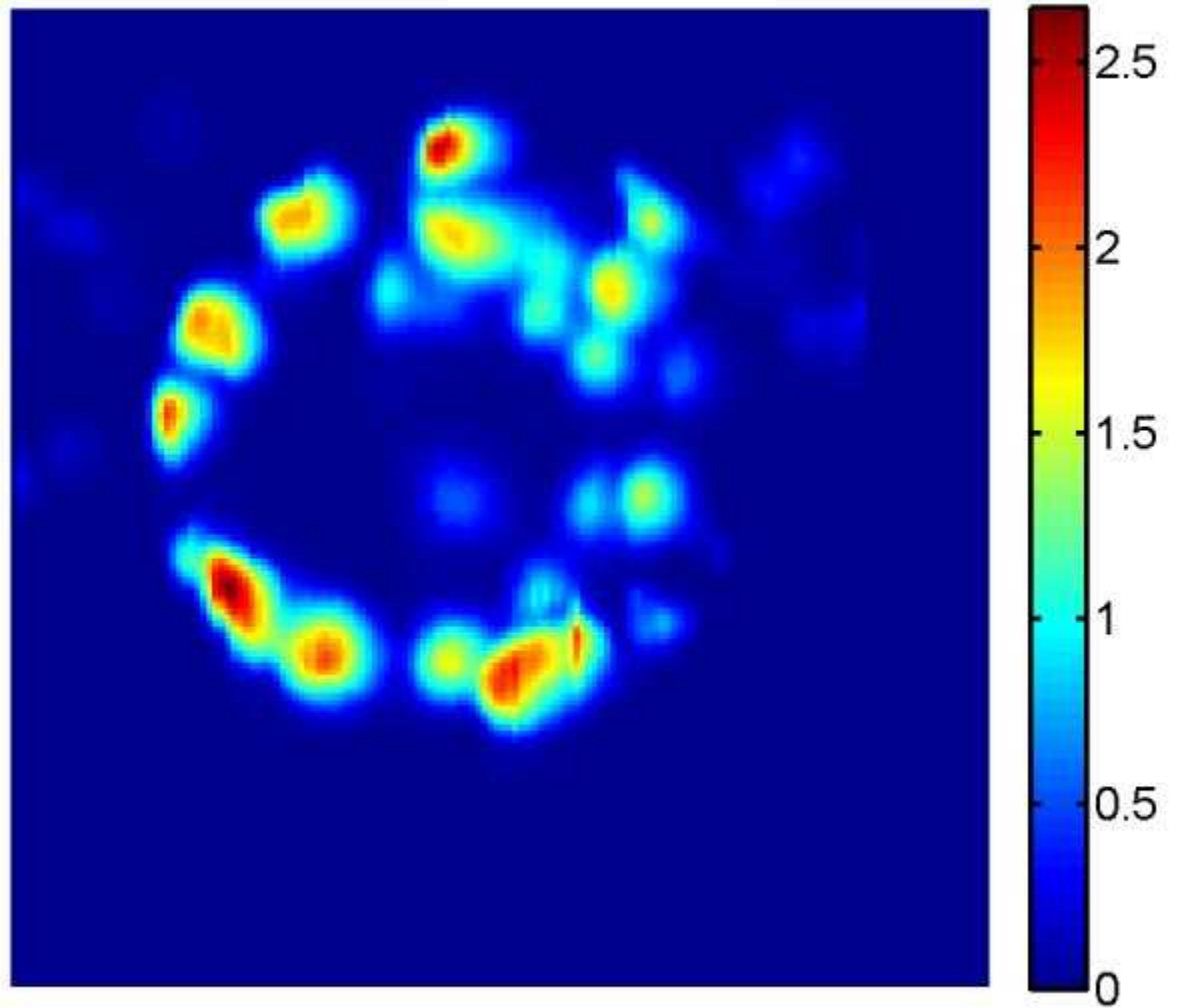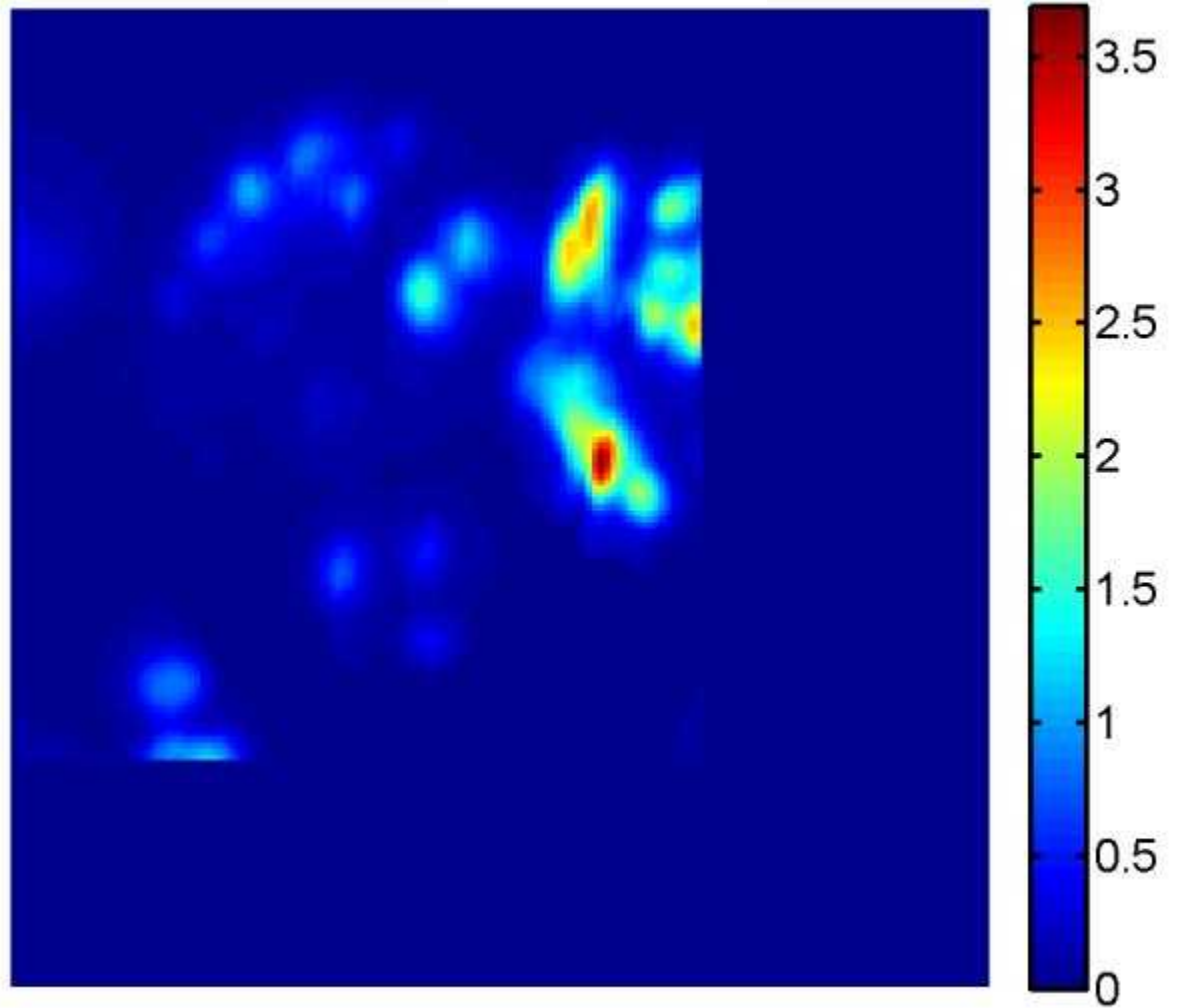
# PSY1-P



0.02 Sec integration time

# PSY1-P



Trace = 6, Carotenoid

0.02 Sec integration time
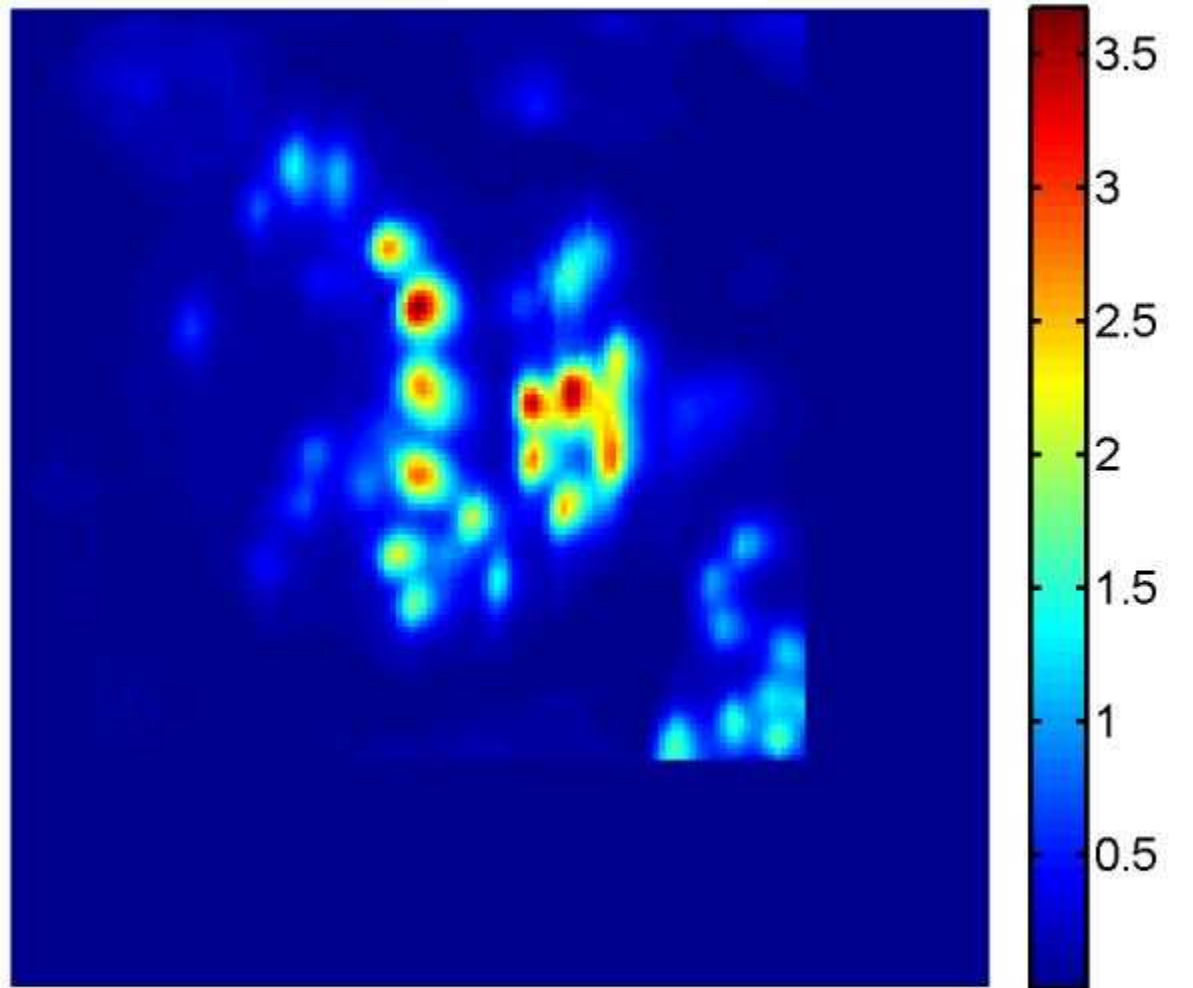
# PSY1-P



0.02 Sec integration time

# PSY1-P



Trace = 6, GFP
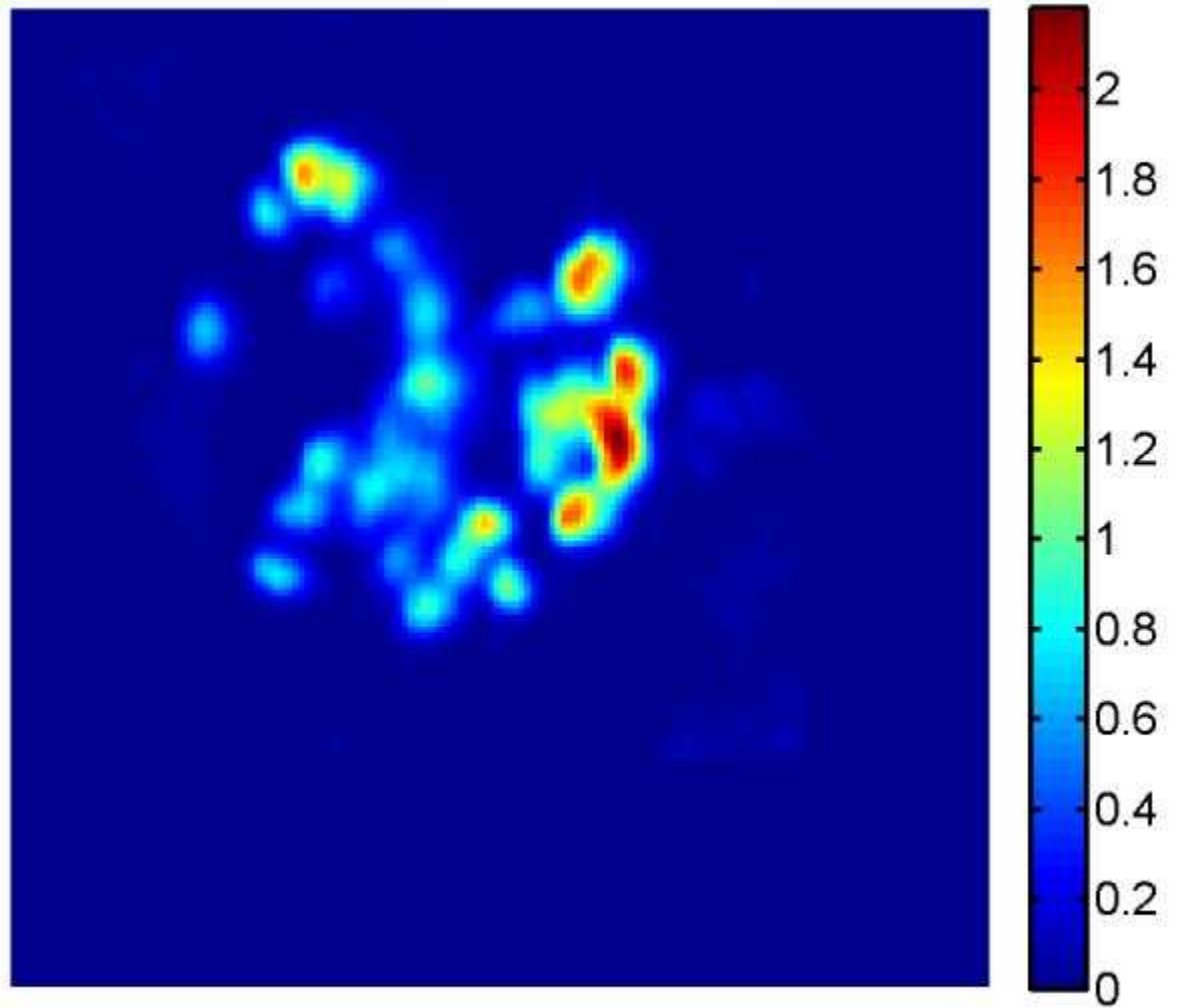
0.02 Sec integration time

# PSY1-P


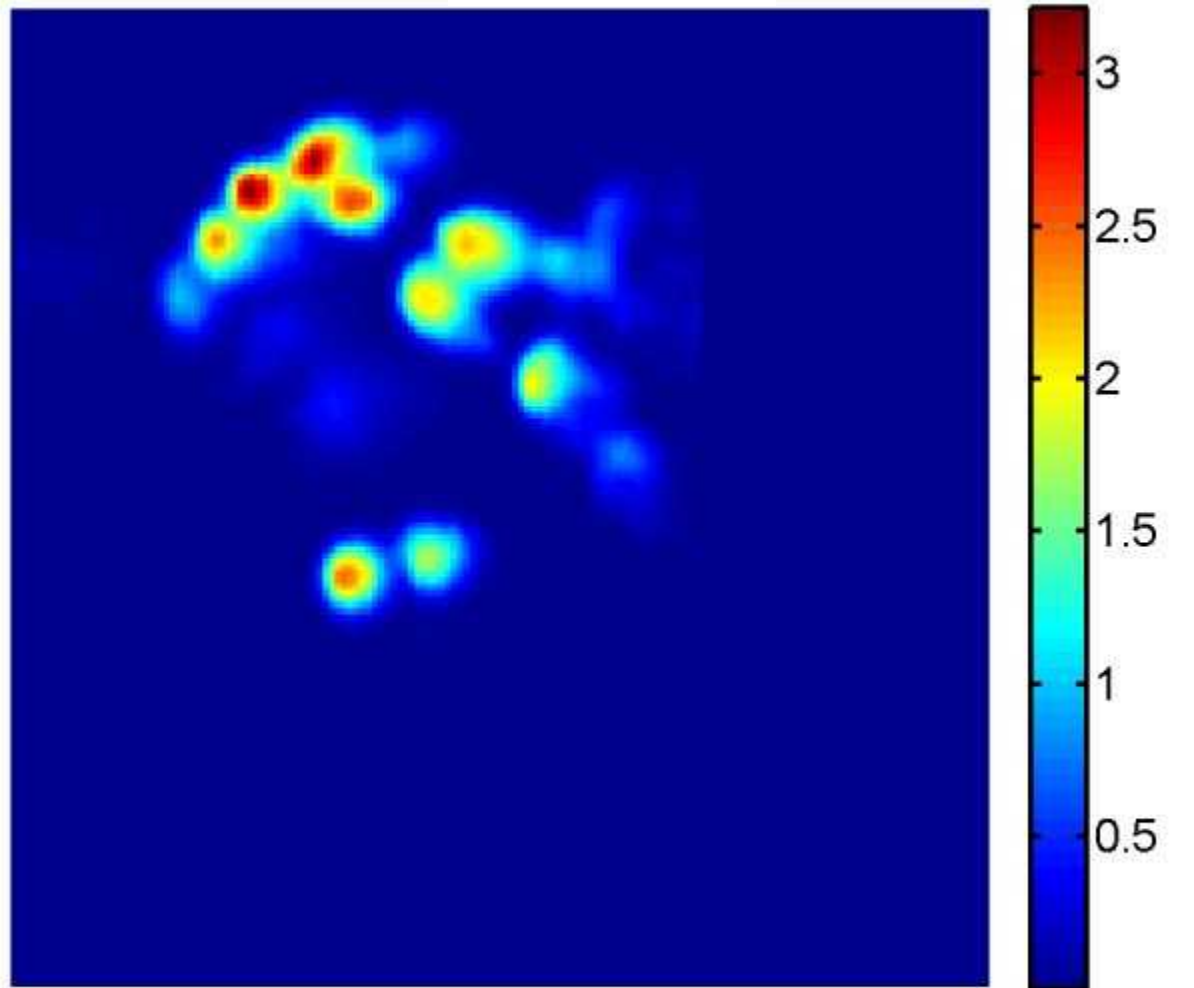
Trace = 7, Carotenoid

0.02 Sec integration time

# PSY1-P



Trace = 7, Chlorophyll
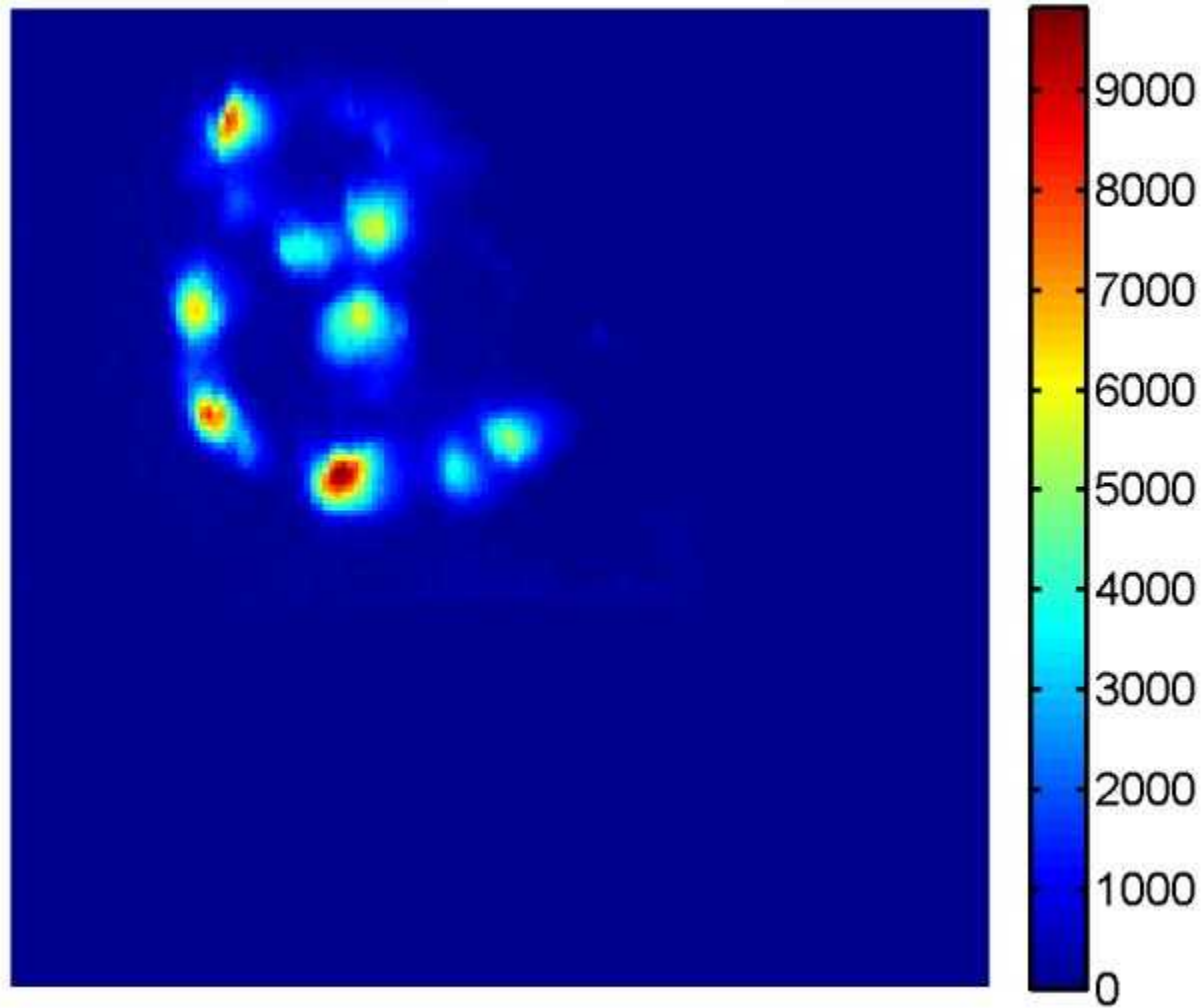
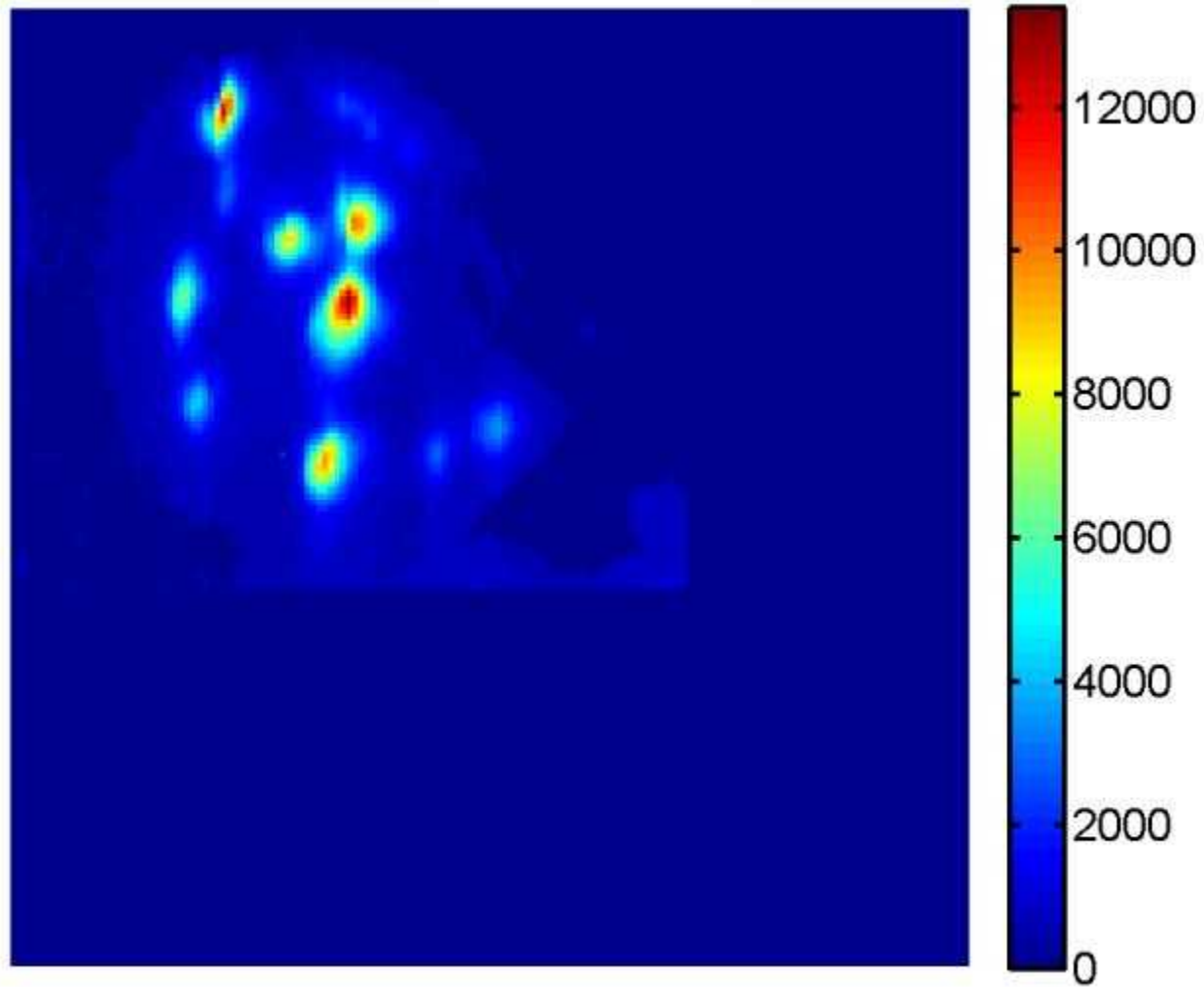0.02 Sec integration time

# PSY1-P



Trace = 7, GFP

# PSY1-P



Trace = 8,  Carotenoid

0.02 Sec integration time

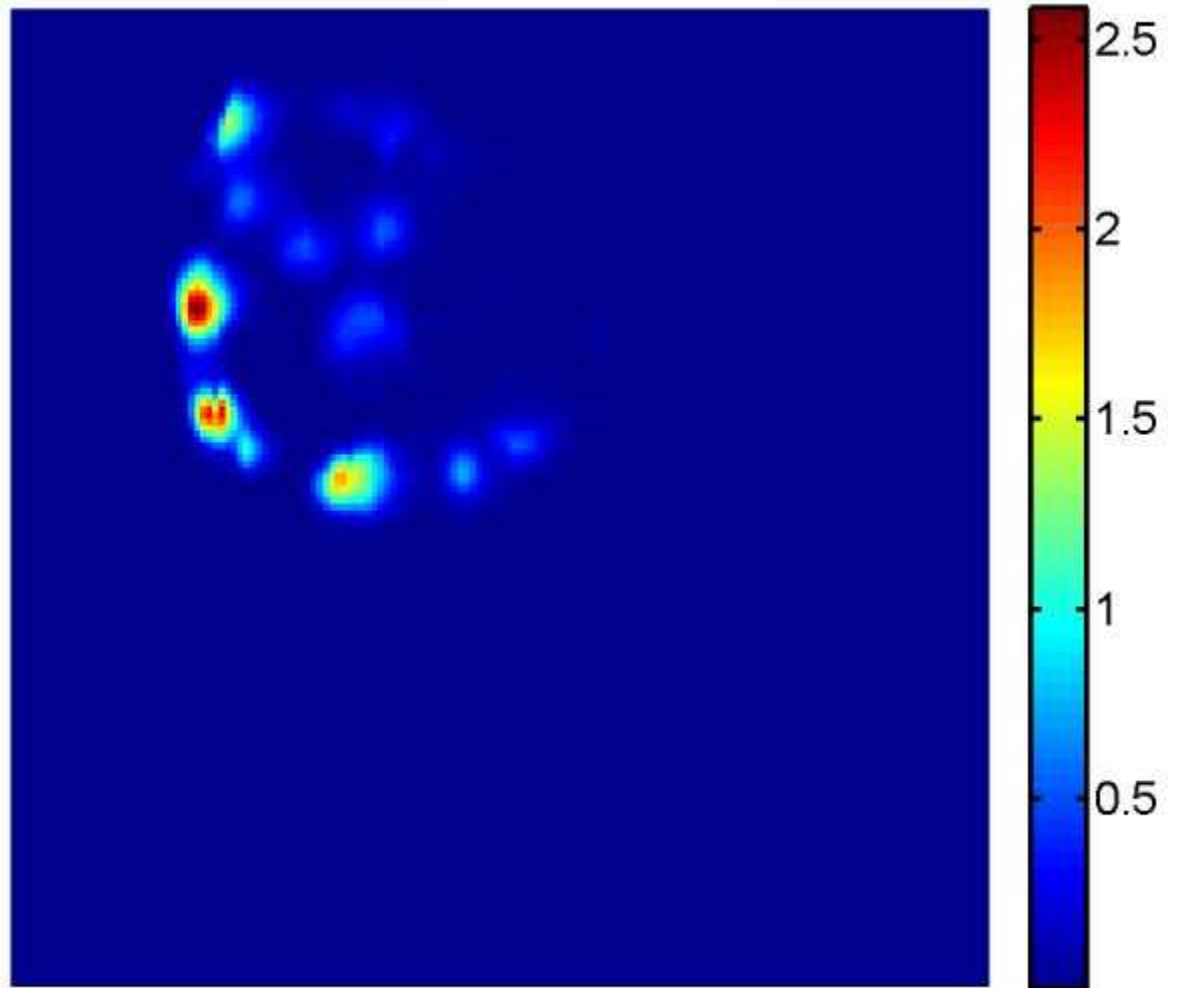# PSY1-P



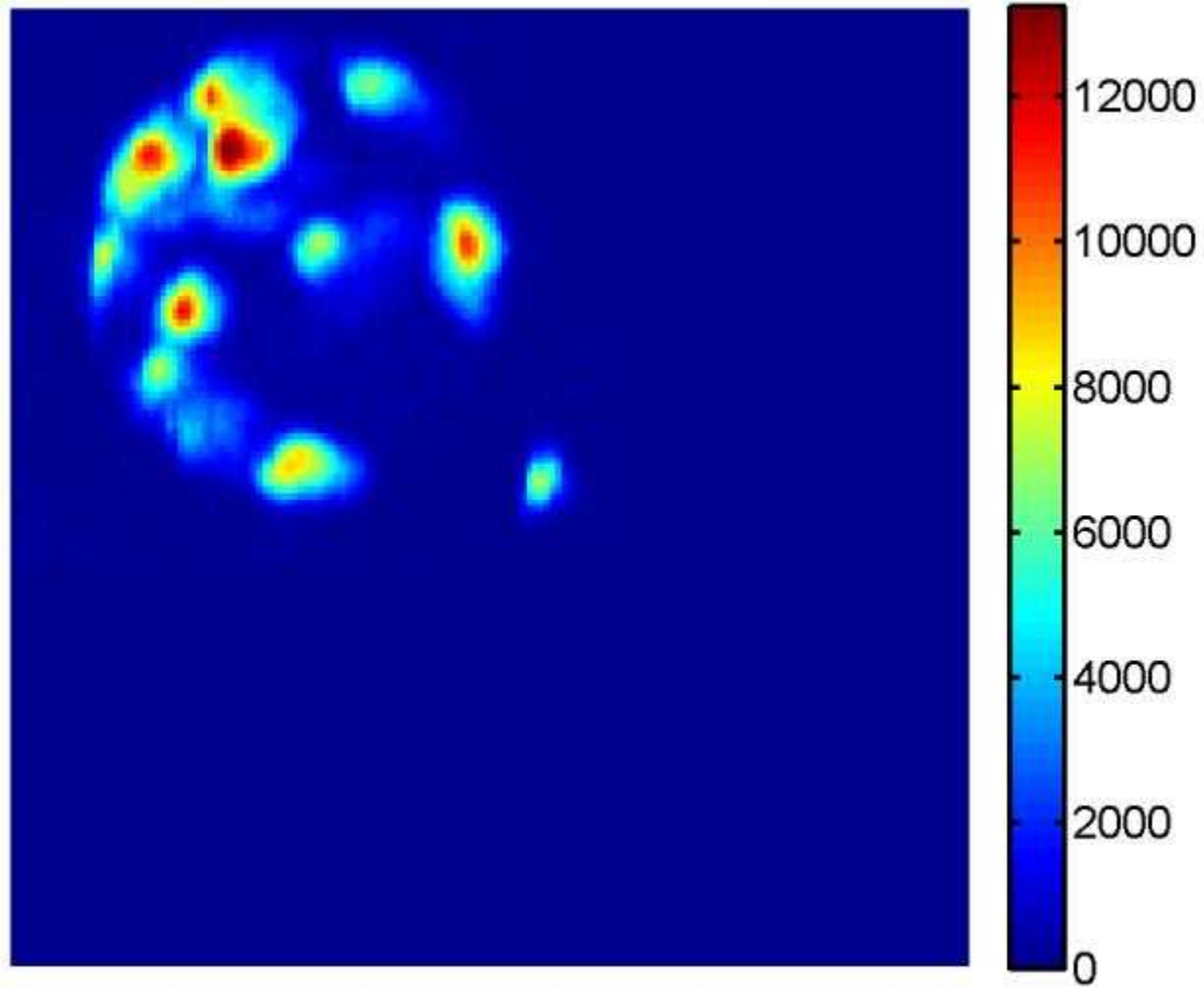Trace = 8, Chlorophyll

0.02 Sec integration time
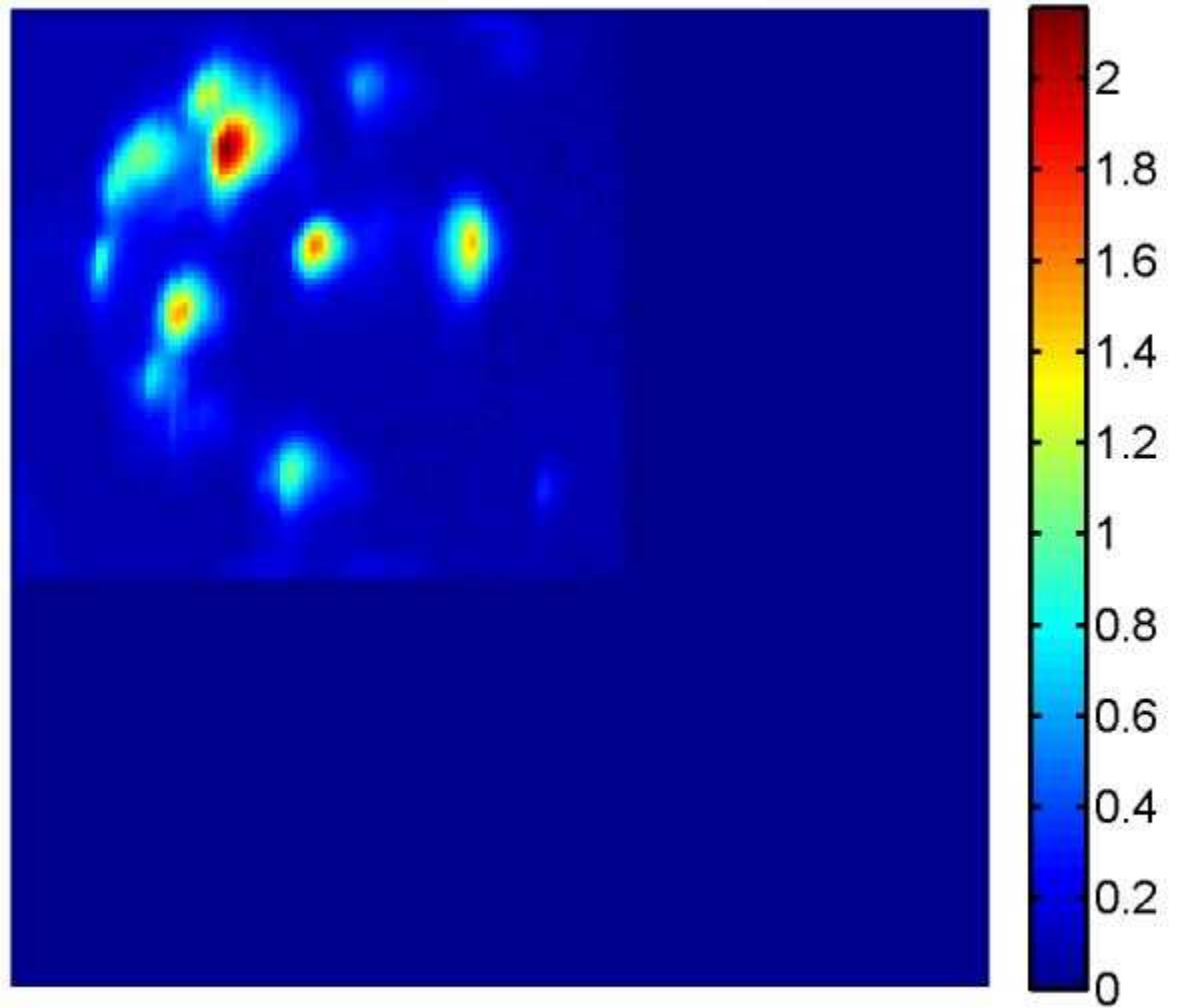
# PSY1-P



Trace = 8, GFP

0.02 Sec integration time

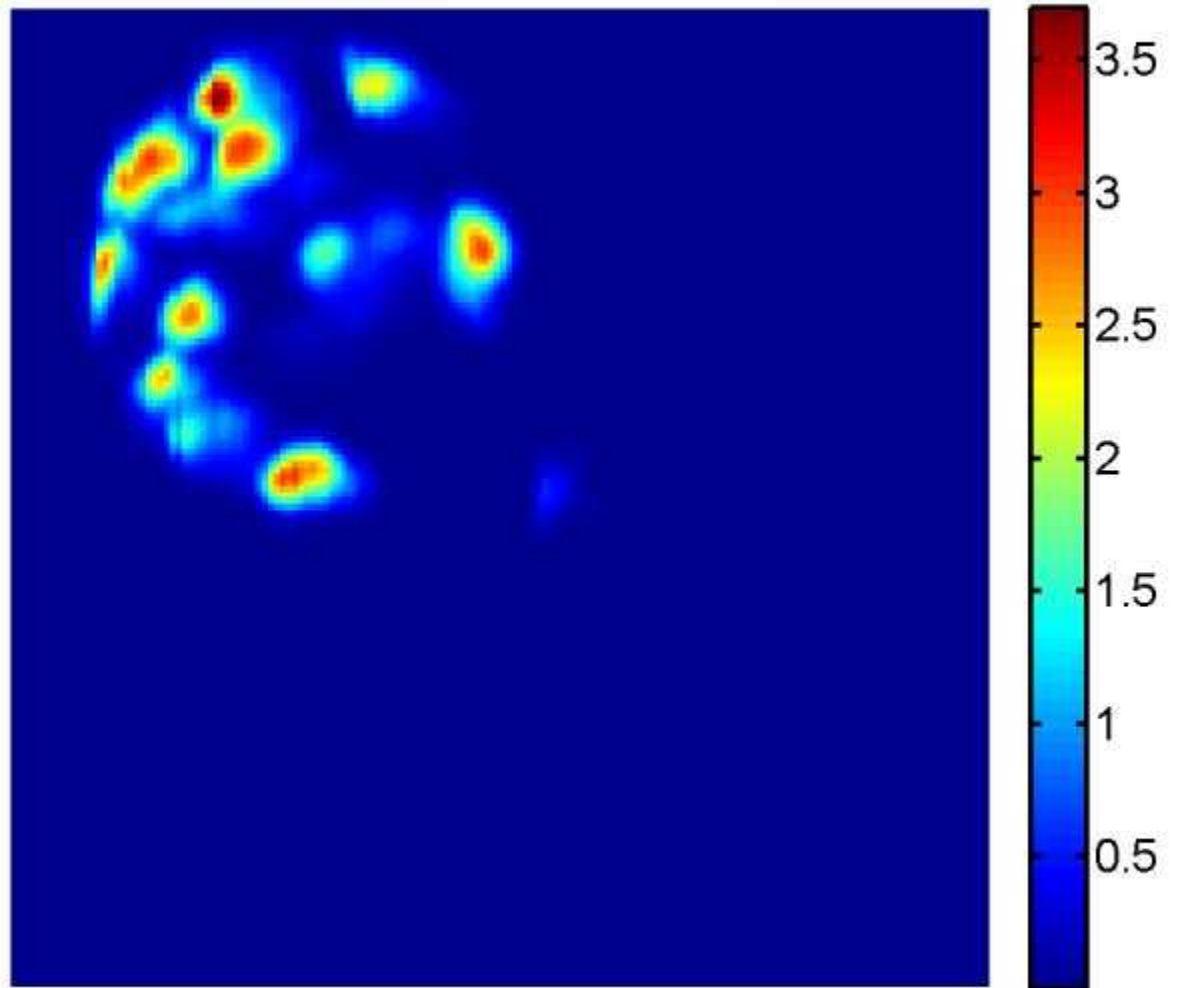# PSY1-T



Trace = 9, Carotenoid

0.02 Sec integration time

# PSY1-T



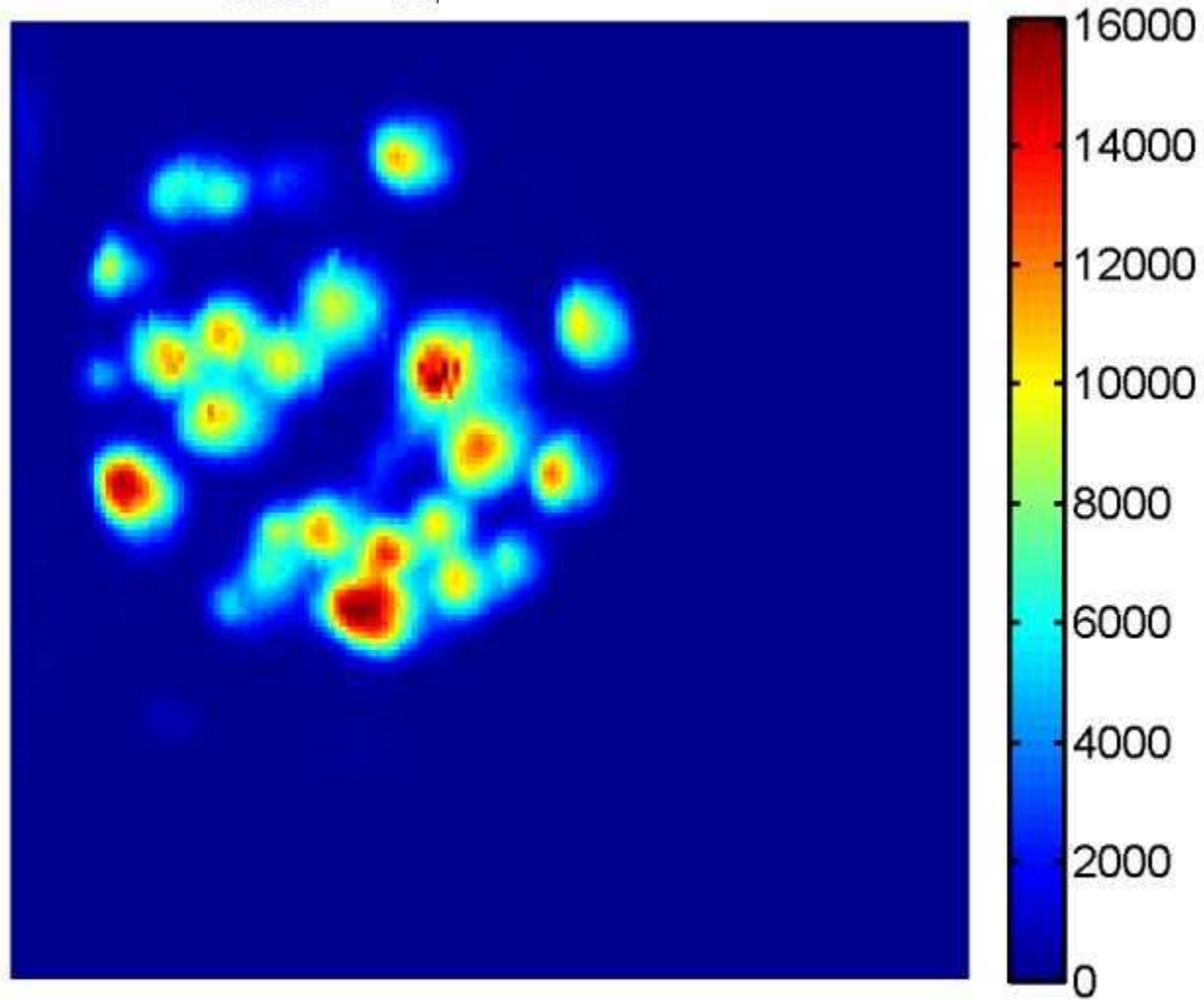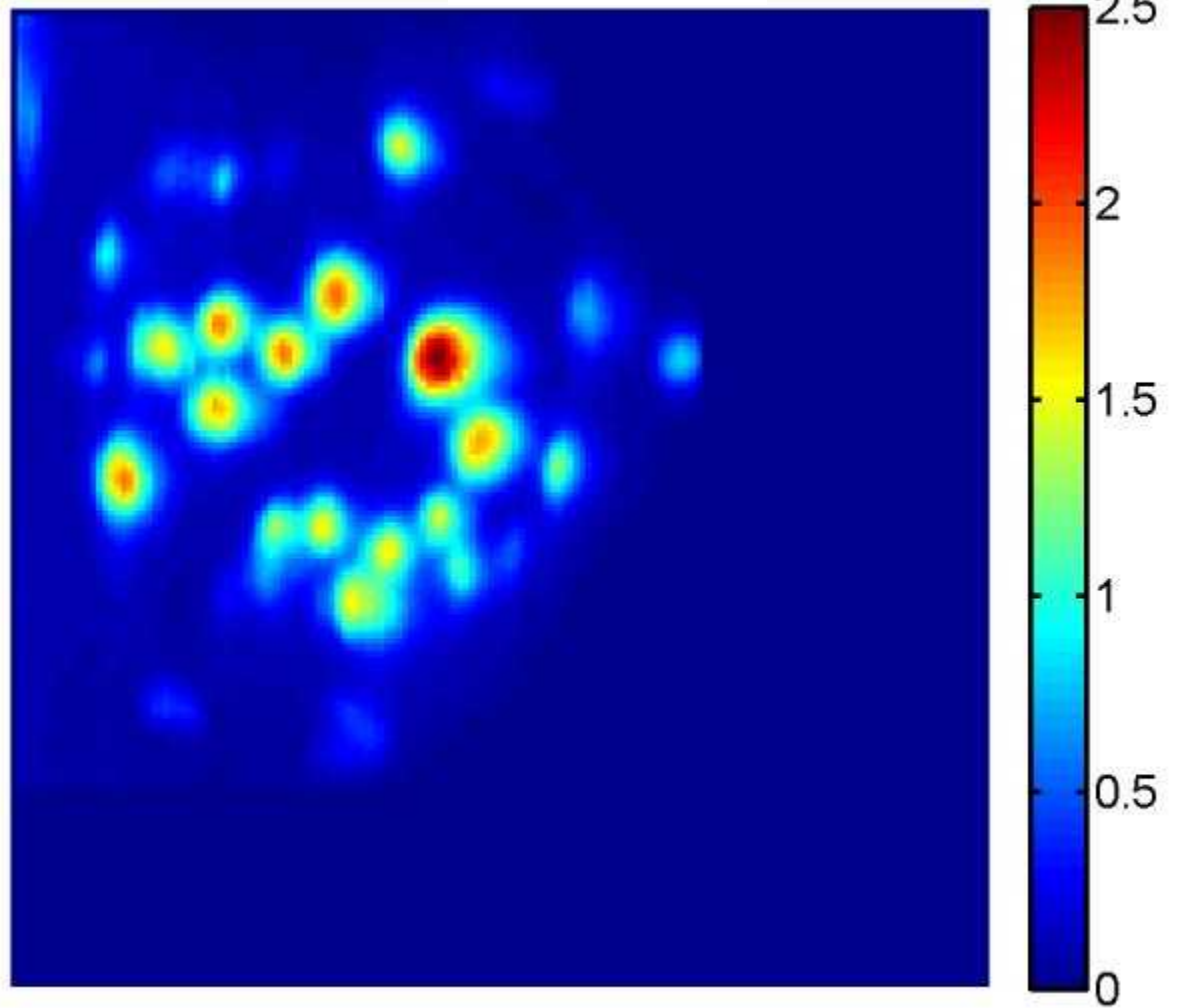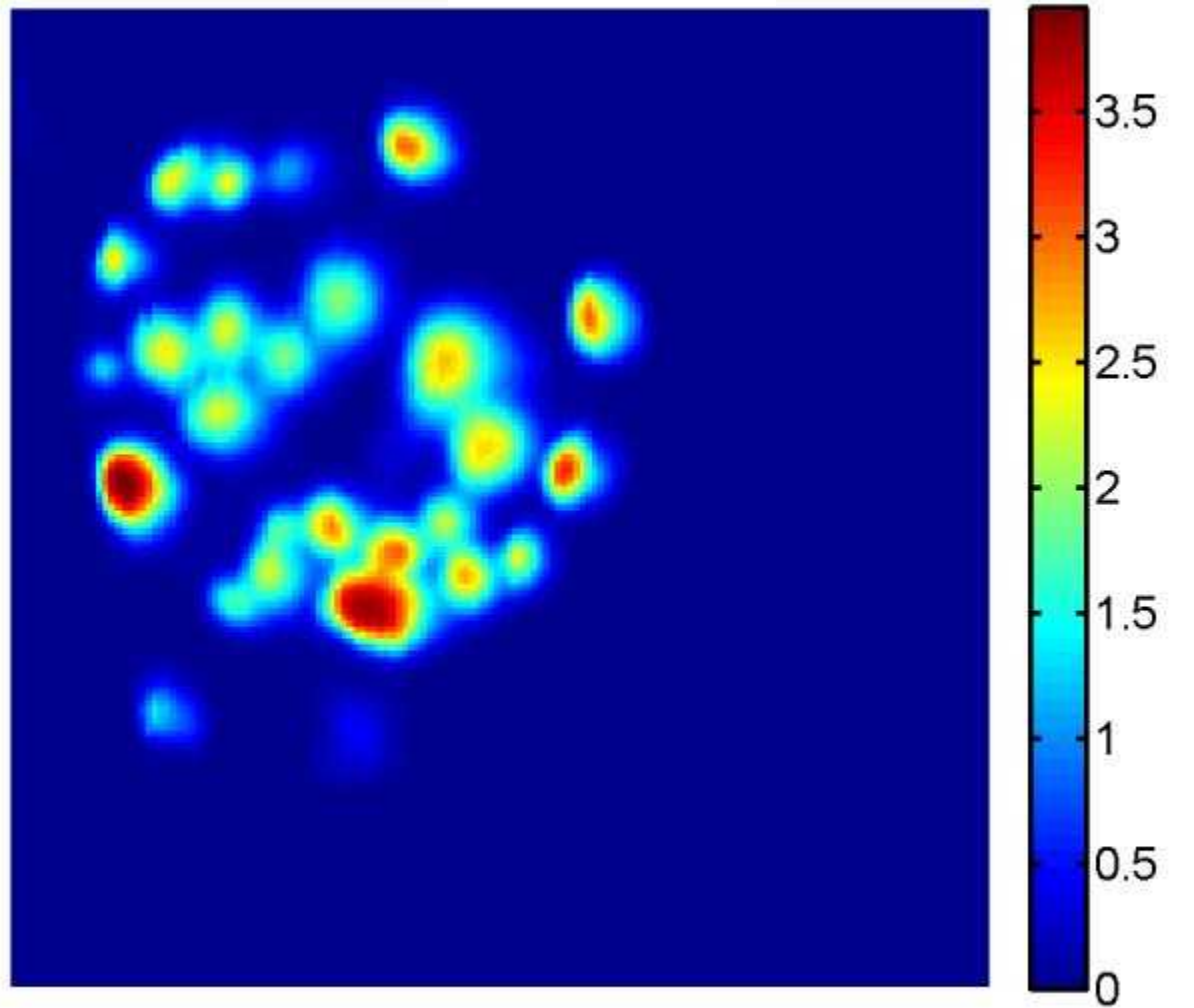0.02 Sec integration time

# PSY1-T



Trace = 9,  GFP

0.02 Sec integration time

# PSY1-T



Trace = 10, Carotenoid

0.02 Sec integration time

# PSY1-T
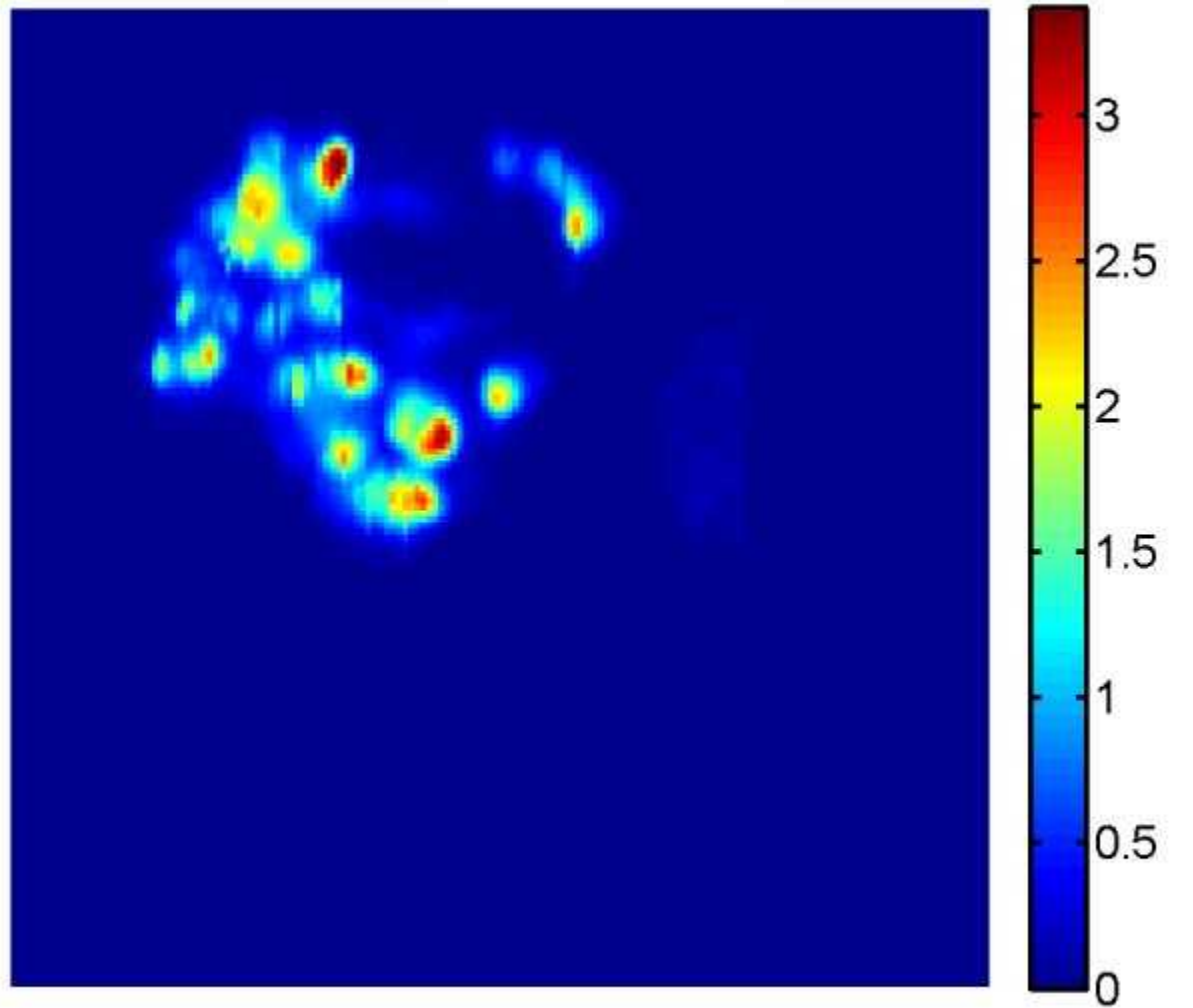


Trace = 10, Chlorophyll

0.02 Sec integration time

# PSY1-T



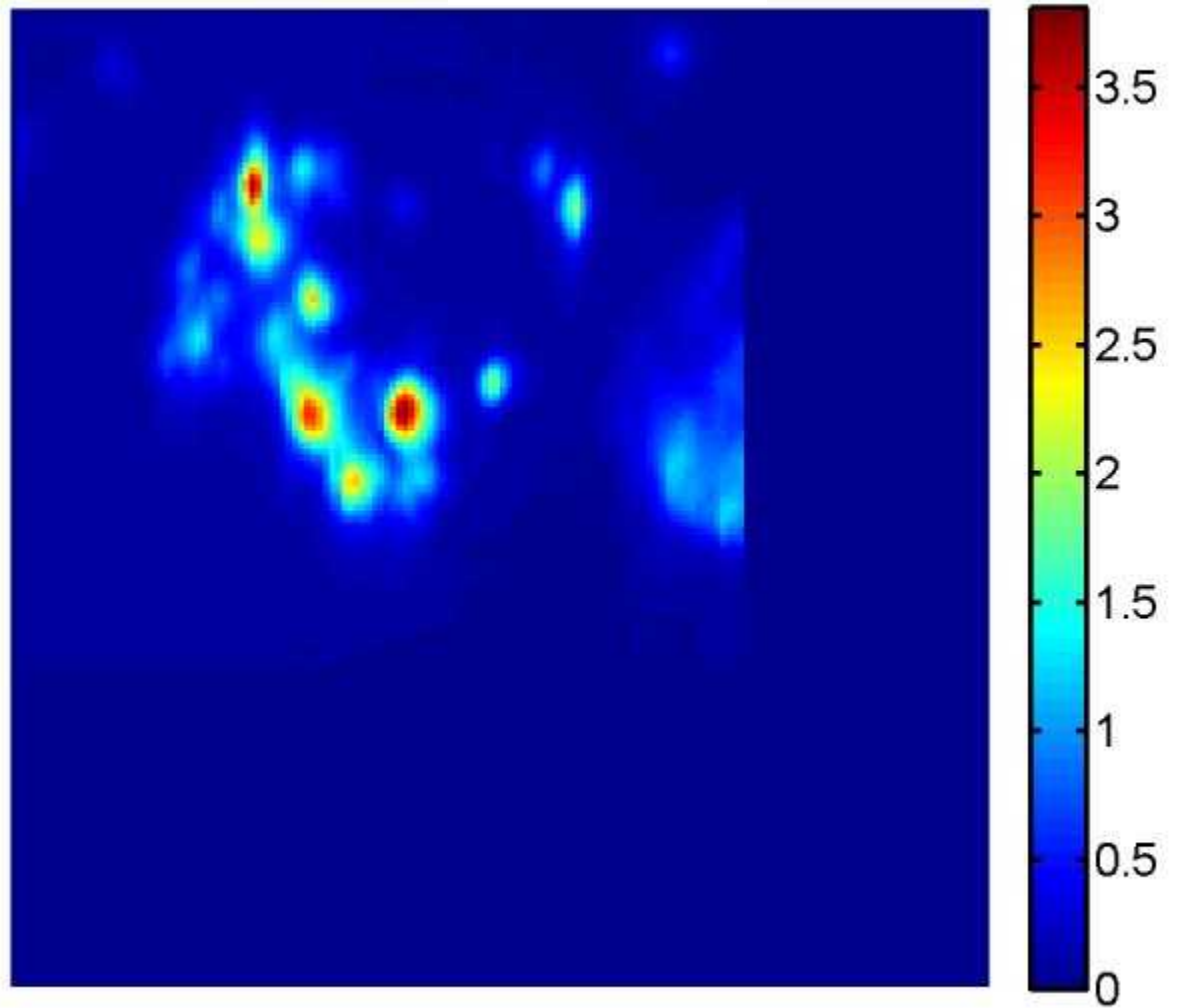0.02 Sec integration time

# PSY1-T



Trace = 11, Carotenoid

0.02 Sec integration time
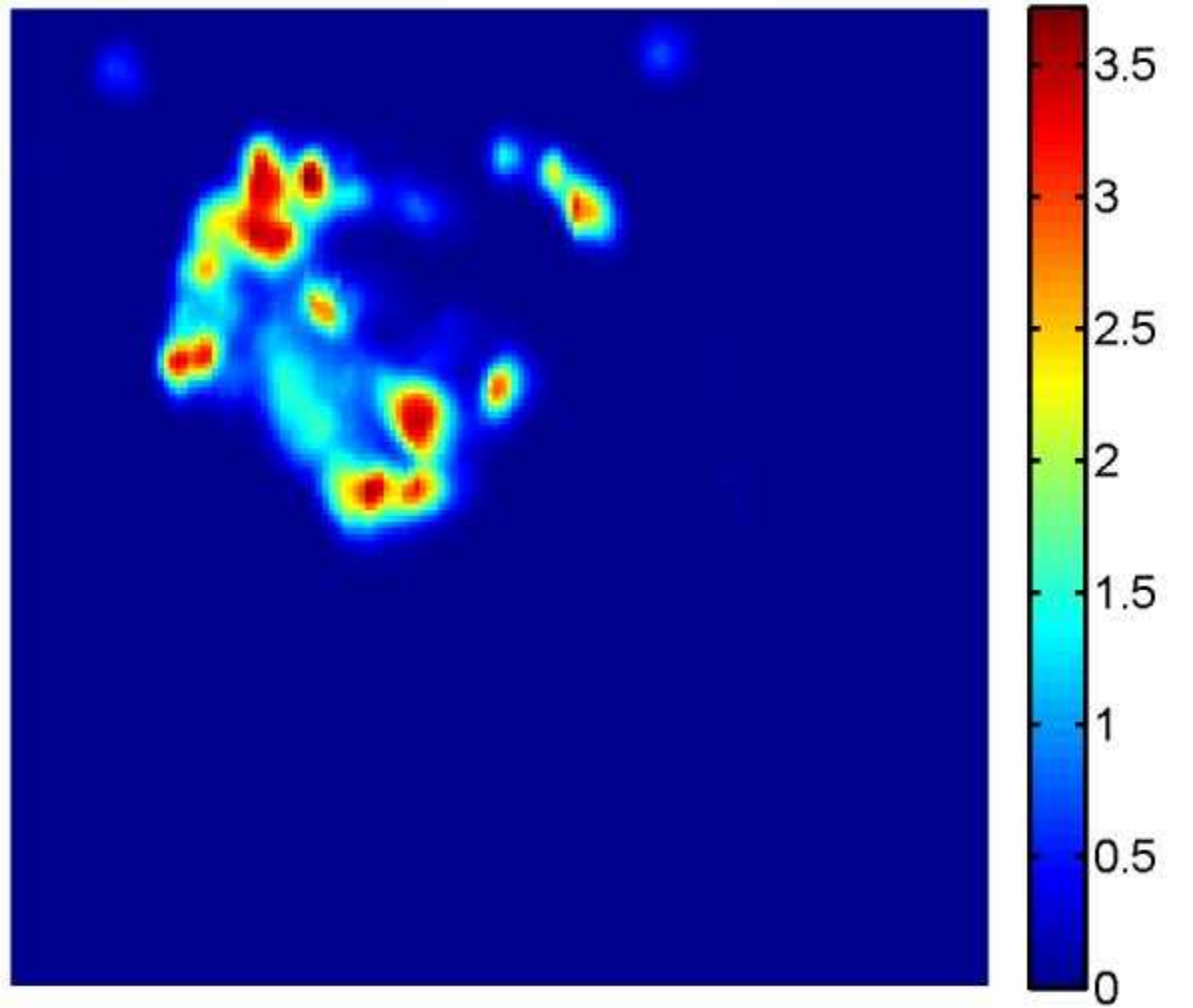
# PSY1-T



Trace = 11, Chlorophyll

0.02 Sec integration time
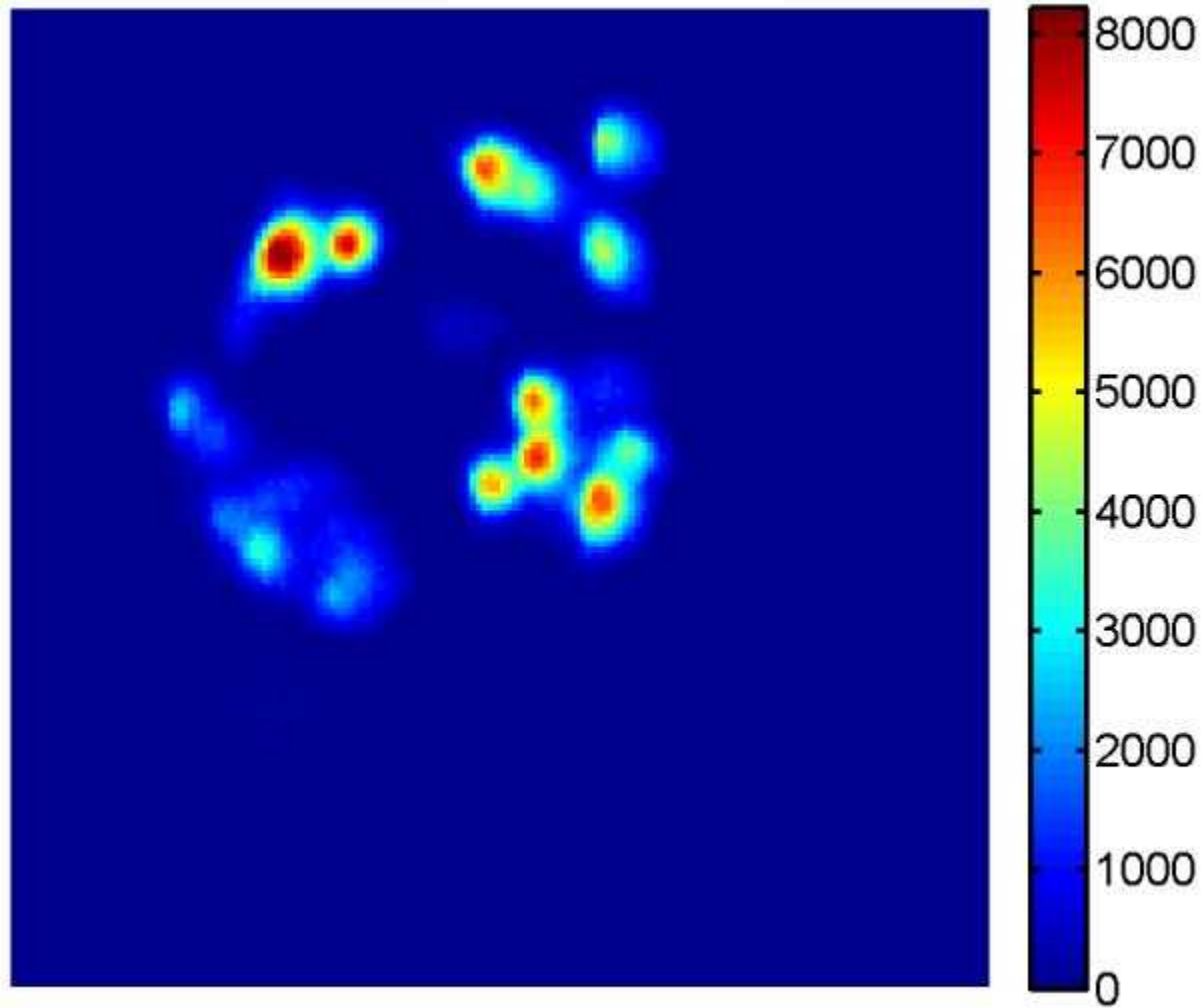
# PSY1-T



Trace = 11,    GFP

0.02 Sec integration time
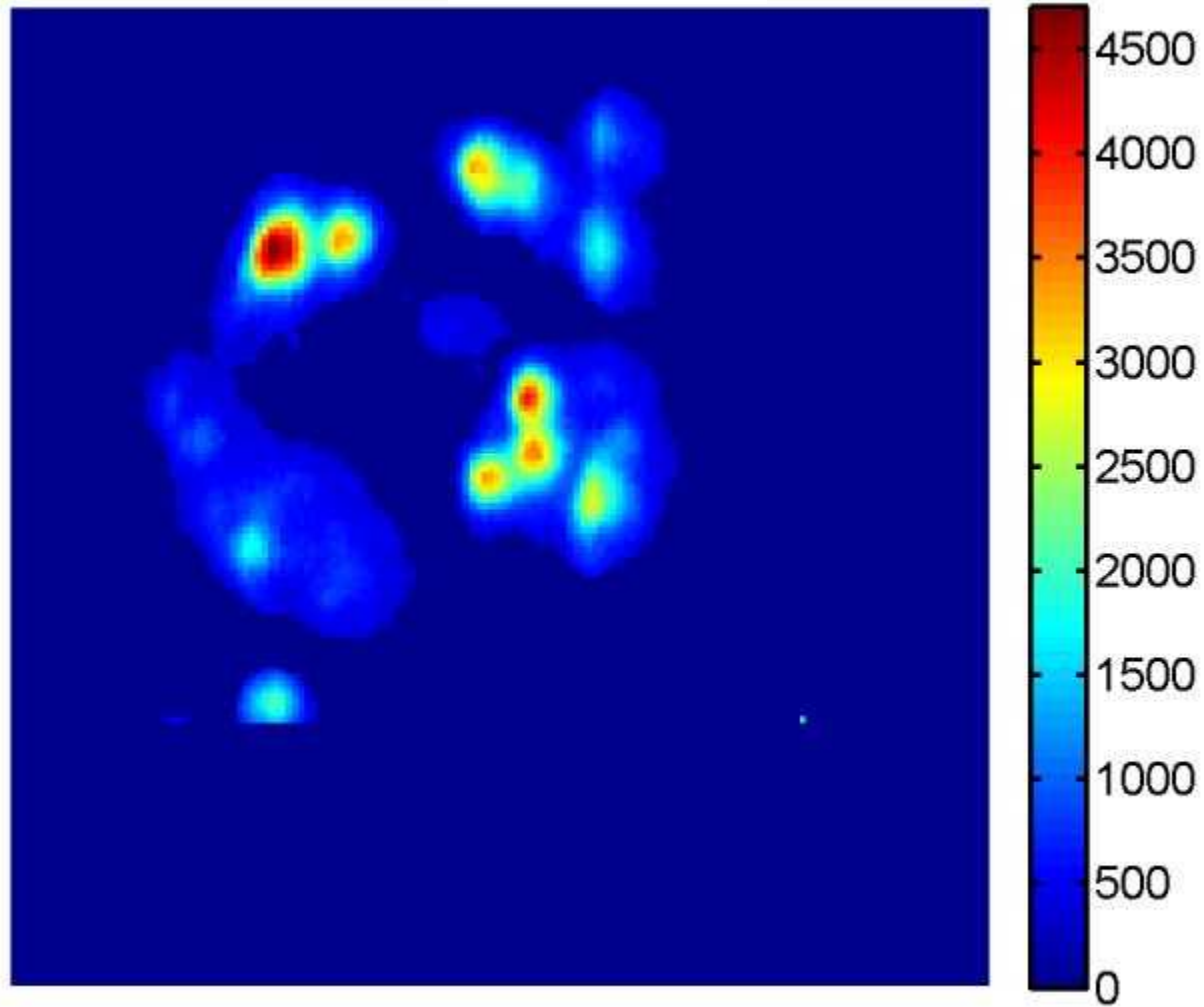
# PSY1-T



Trace = 12, Carotenoid
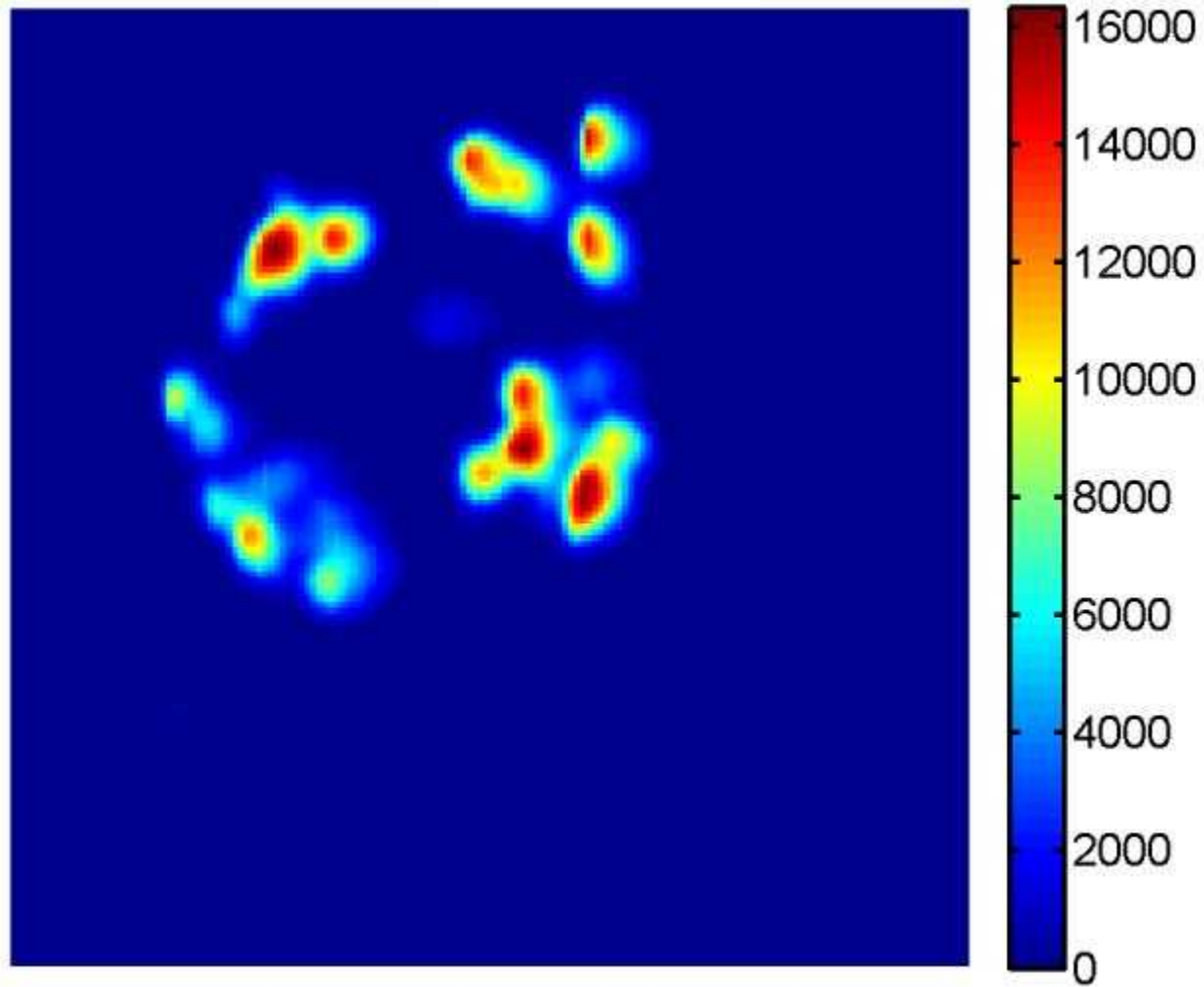
0.01 Sec integration time

# PSY1-T



Trace = 12, Chlorophyll

0.01 Sec integration time
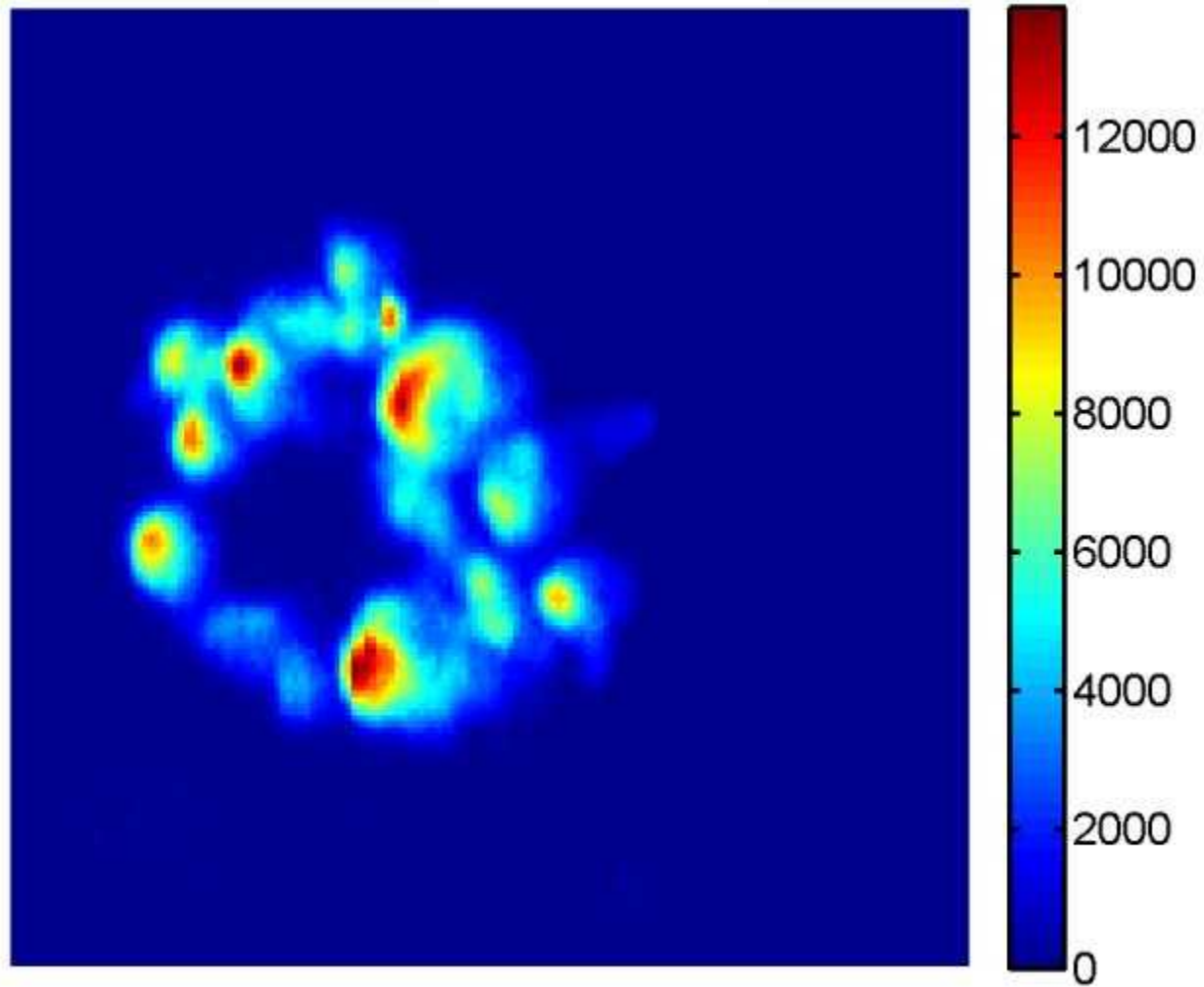
# PSY1-T

Trace = 12, GFP
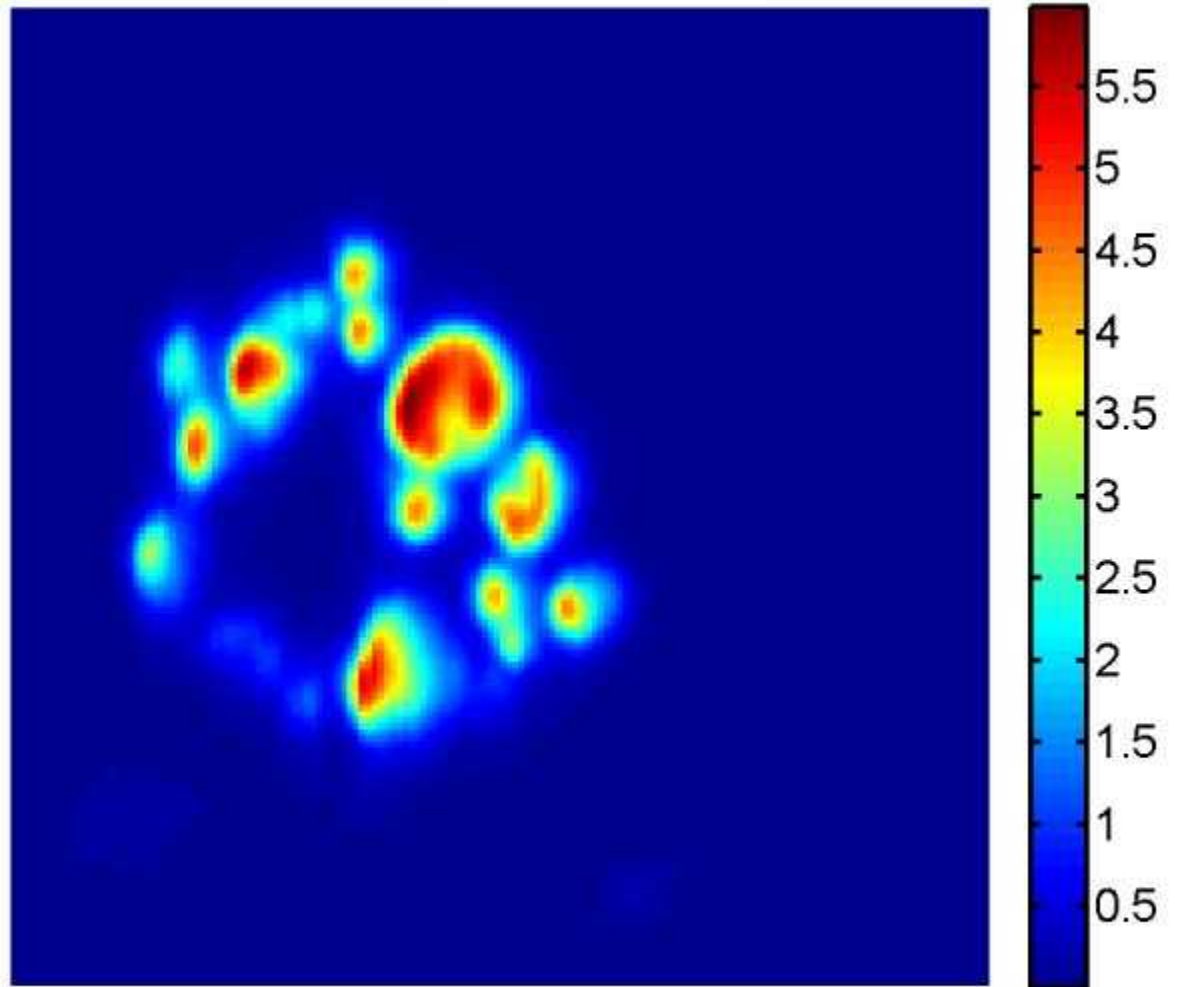


0.01 Sec integration time

# PSY1-T



Trace = 13, Carotenoid

0.01 Sec integration time
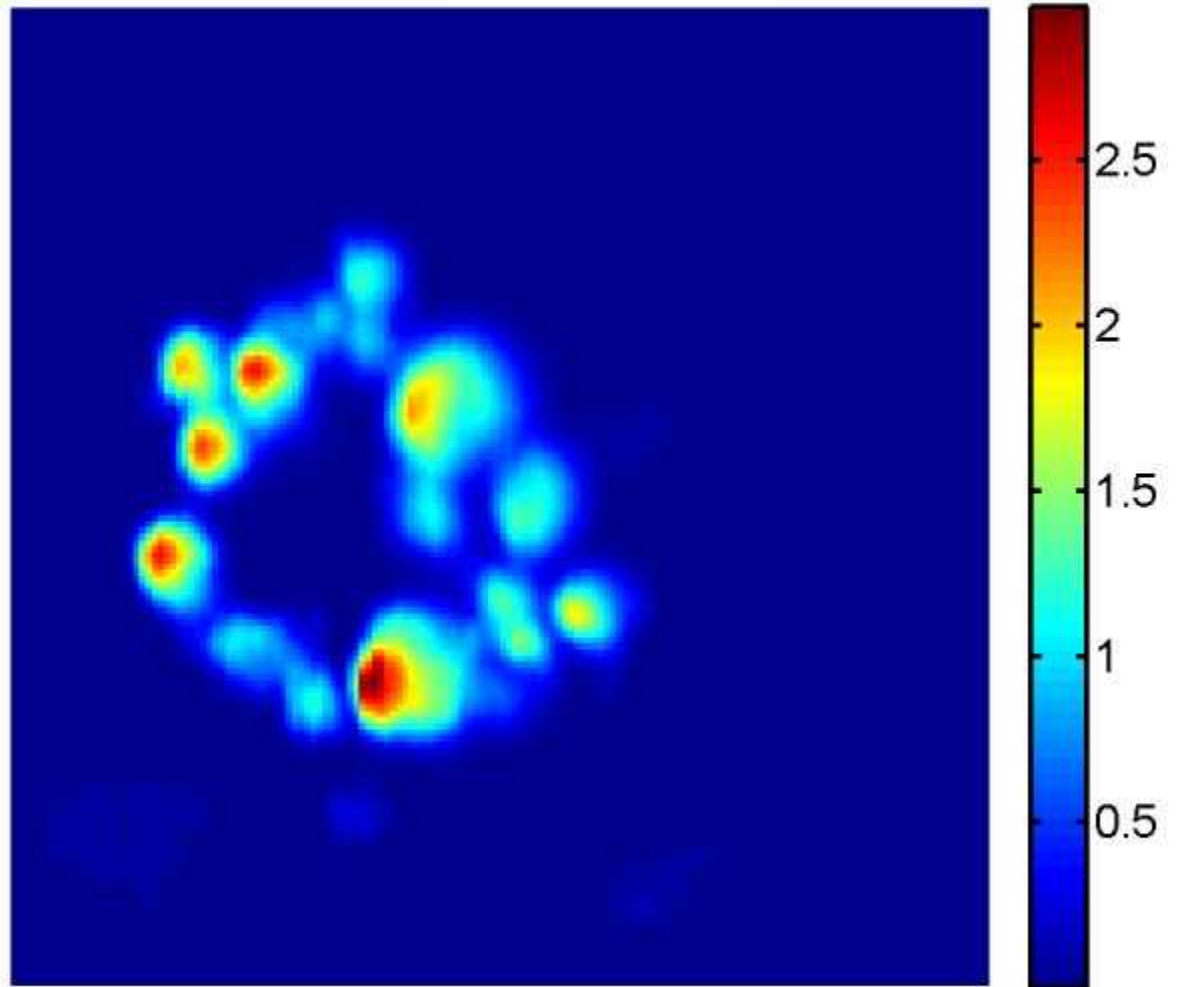
# PSY1-T
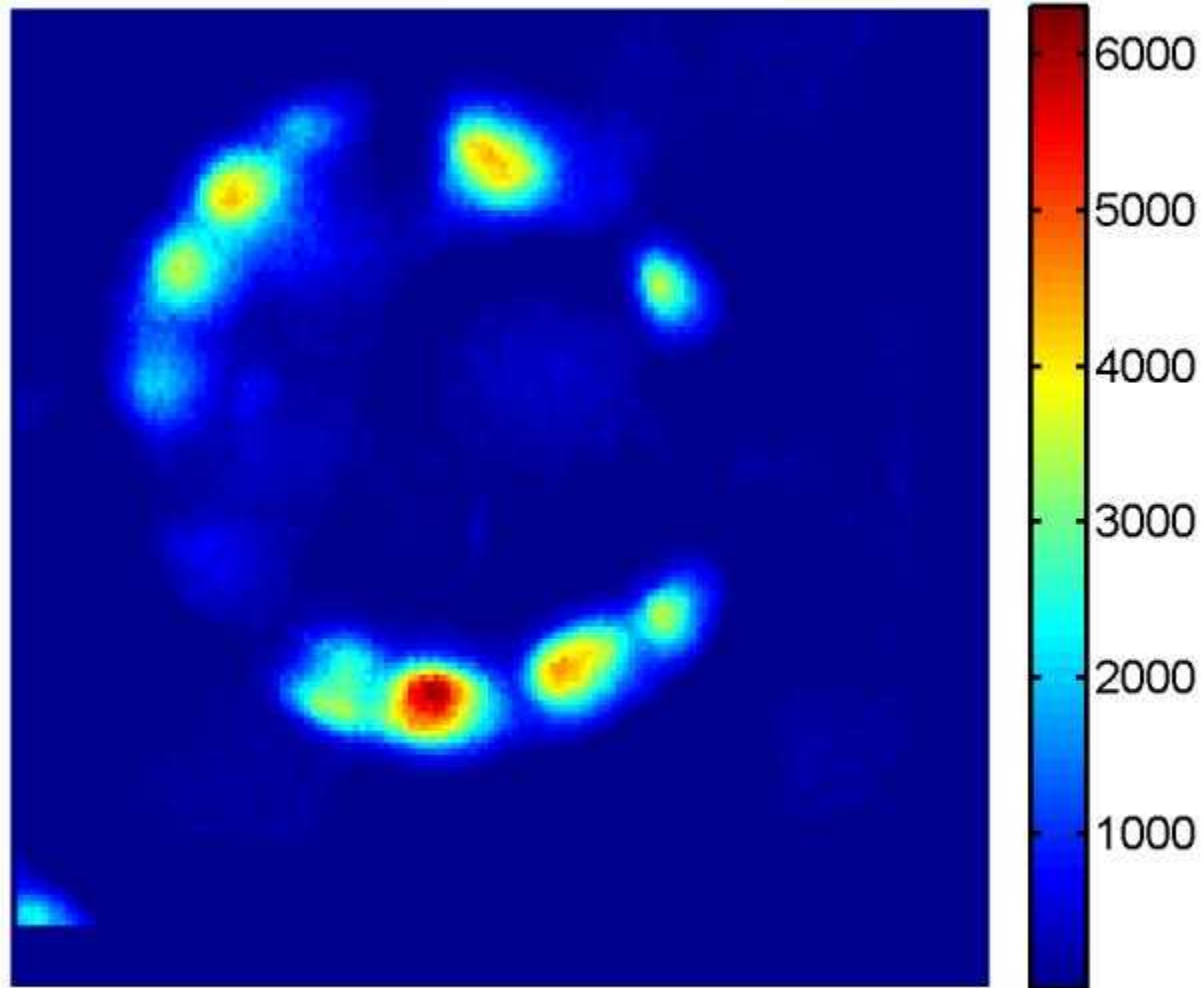


Trace = 13, Chlorophyll

0.01 Sec integration time

# PSY1-T



Trace = 13,  GFP

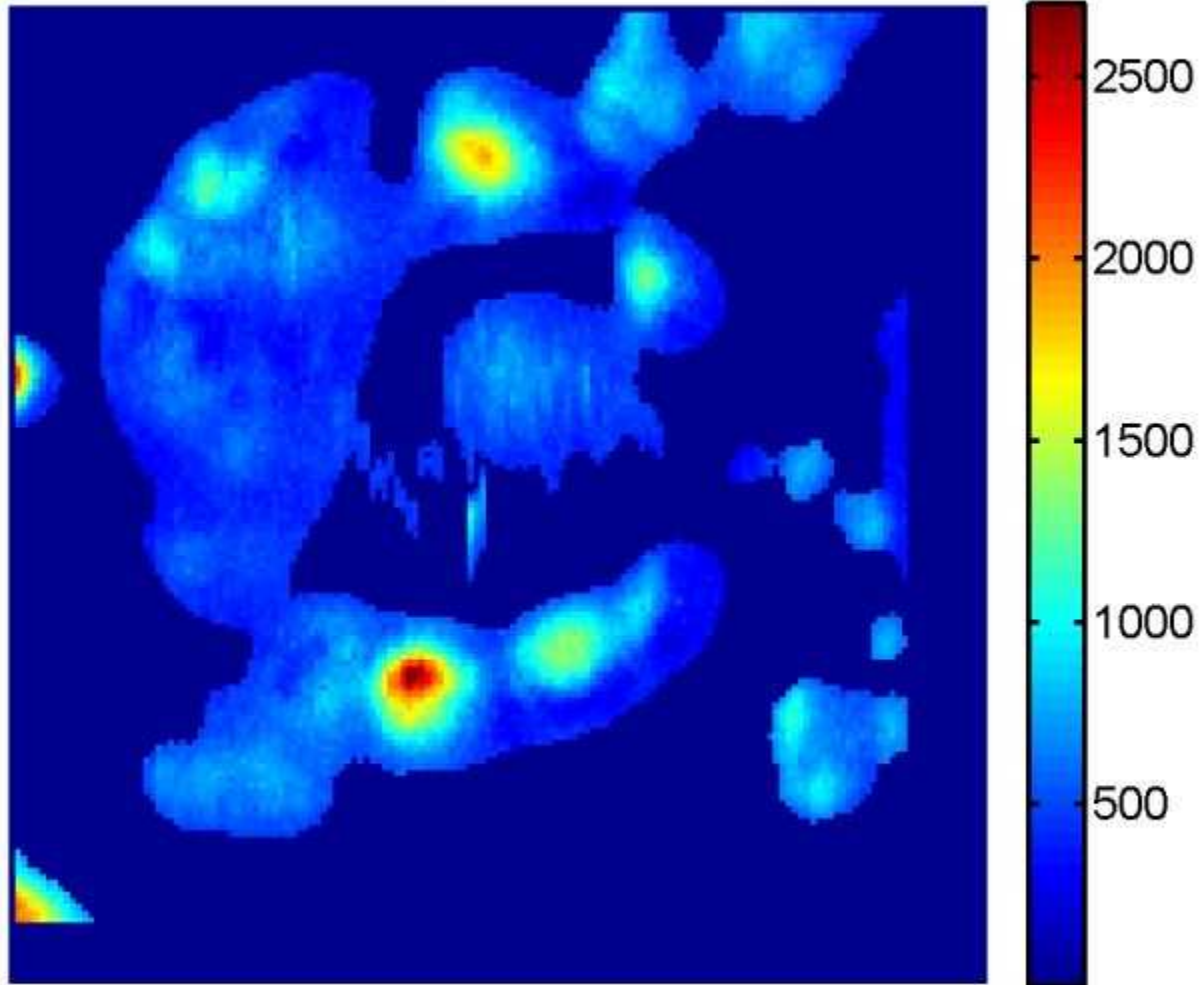0.01 Sec integration time

# PSYP-D/E



Trace = 14, Carotenoid

0.01 Sec integration time

# PSYP-D/E



Trace = 14, Chlorophyll

0.01 Sec integration time

# PSYP-D/E



Trace = 14,    GFP

0.01 Sec integration time

# PSYP-D/E



Trace = 15, Carotenoid

0.01 Sec integration time
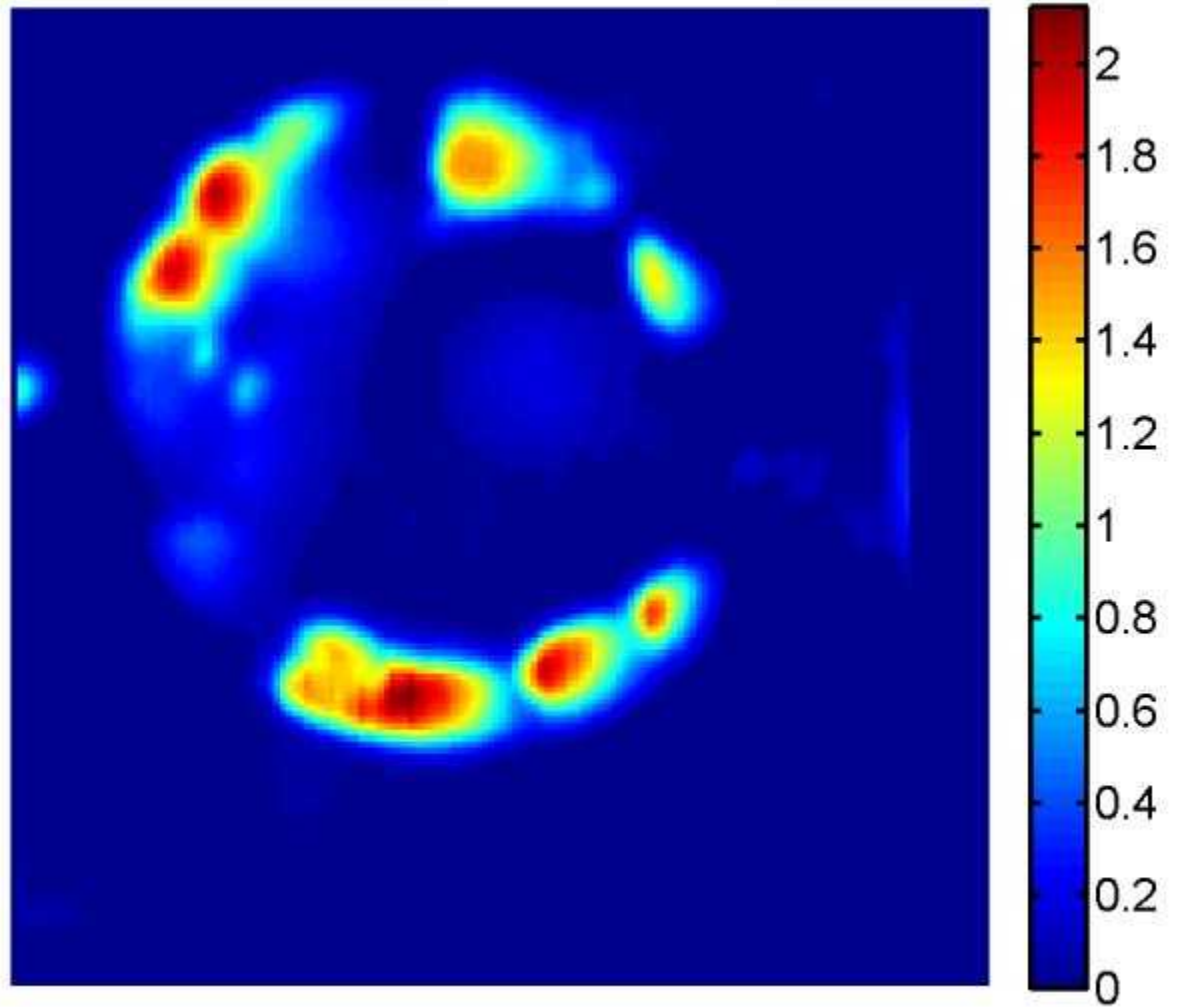
# PSYP-D/E



Trace = 15, Chlorophyll

0.01 Sec integration time

# PSYP-D/E



Trace = 15,      GFP

0.01 Sec integration time

# PSYP-D/E



Trace = 16, Carotenoid

0.01 Sec integration time

# PSYP-D/E



Trace = 16, Chlorophyll

0.01 Sec integration time

# PSYP-D/E
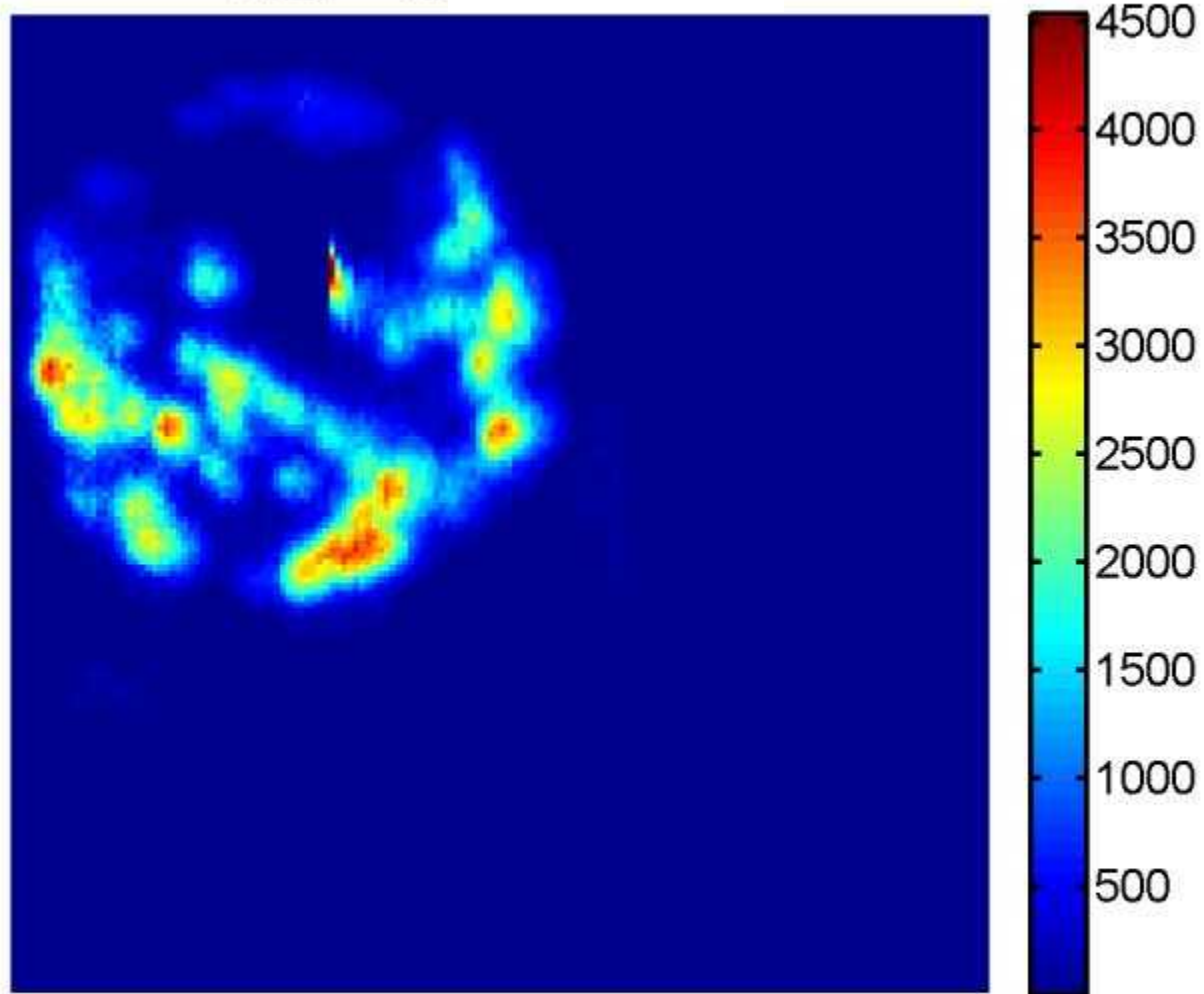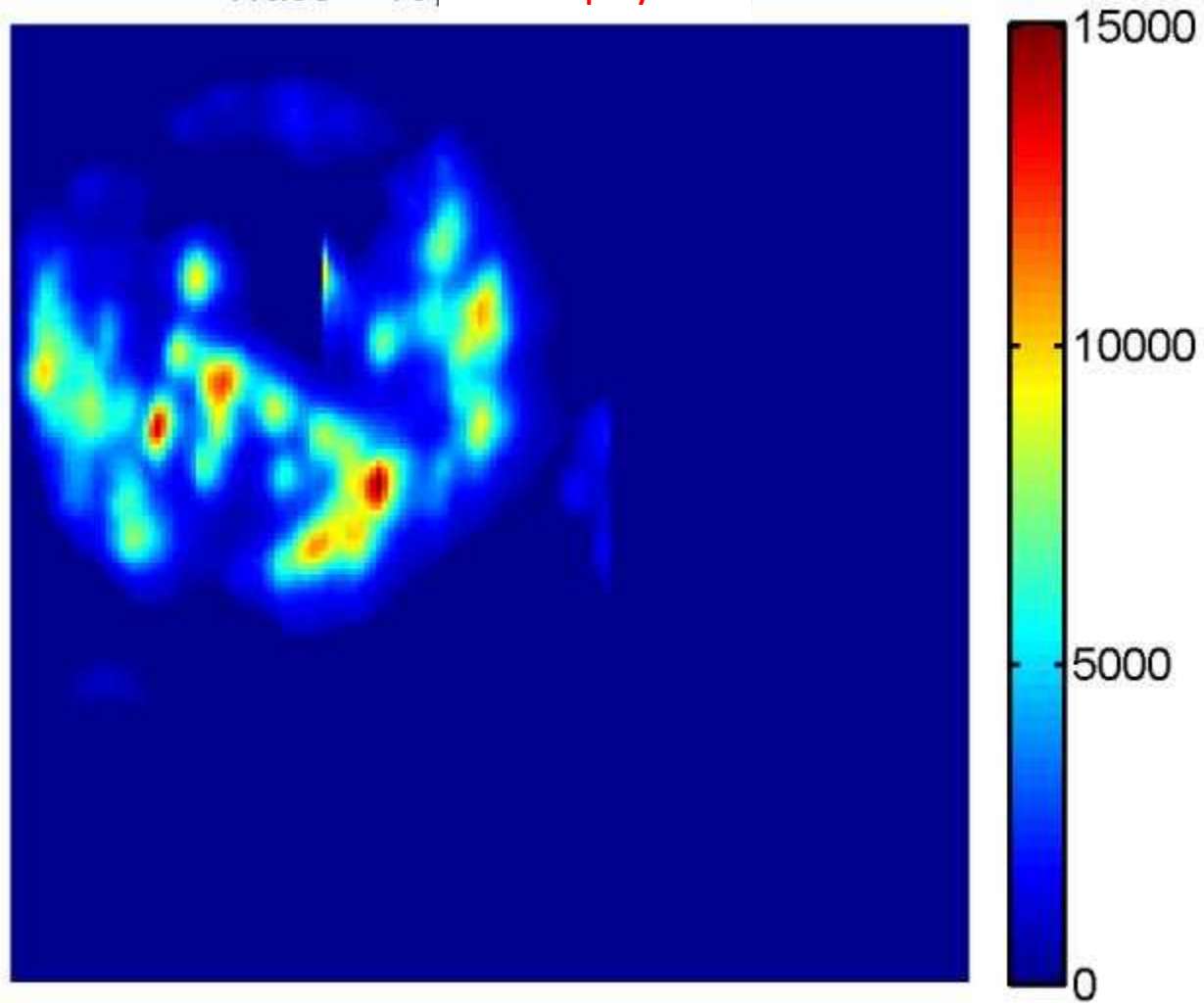


Trace = 16,    GFP

0.01 Sec integration time

# PSYP-D/E



Trace = 17, Carotenoid

0.01 Sec integration time

# PSYP-D/E



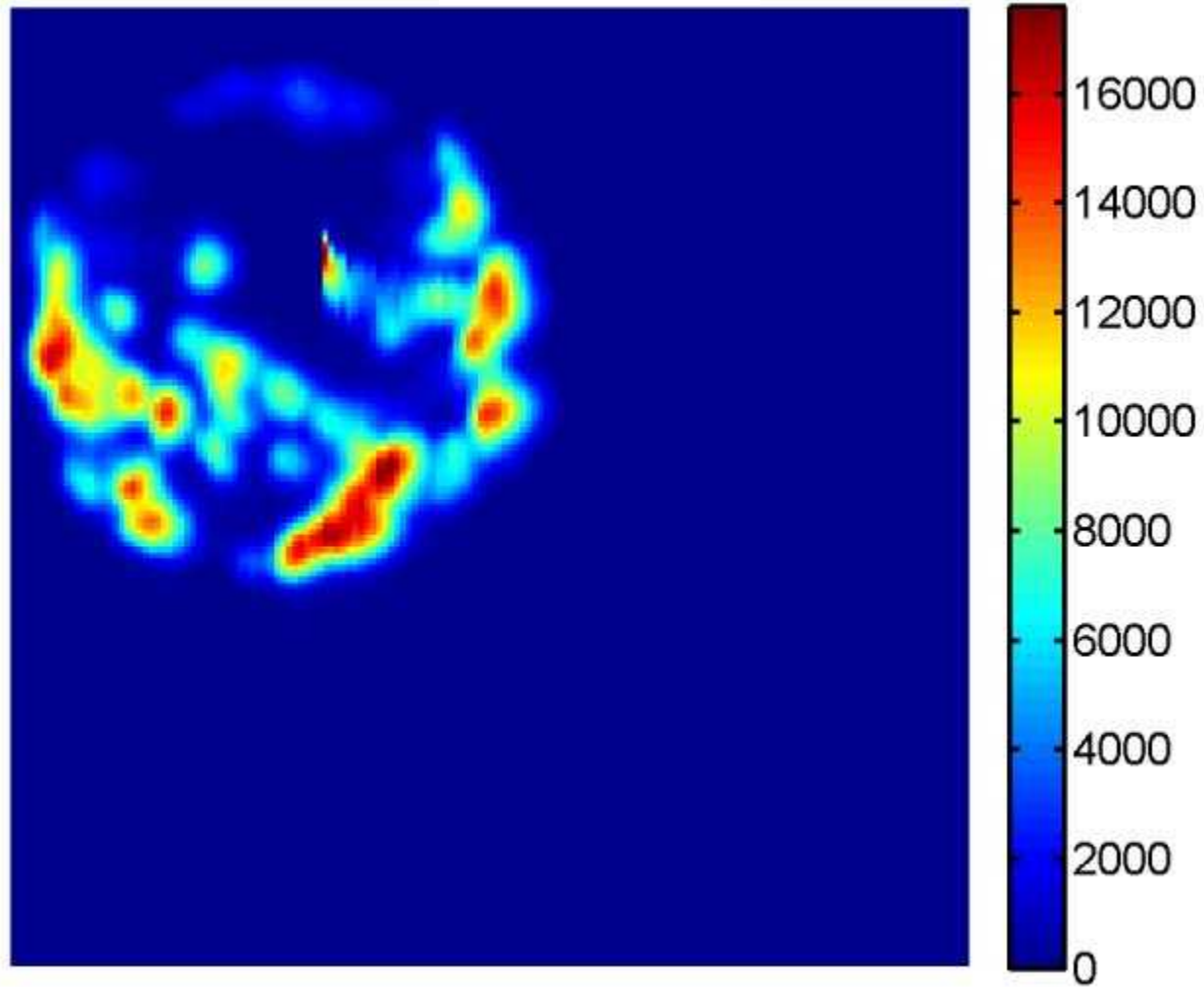Trace = 17, Chlorophyll

0.01 Sec integration time
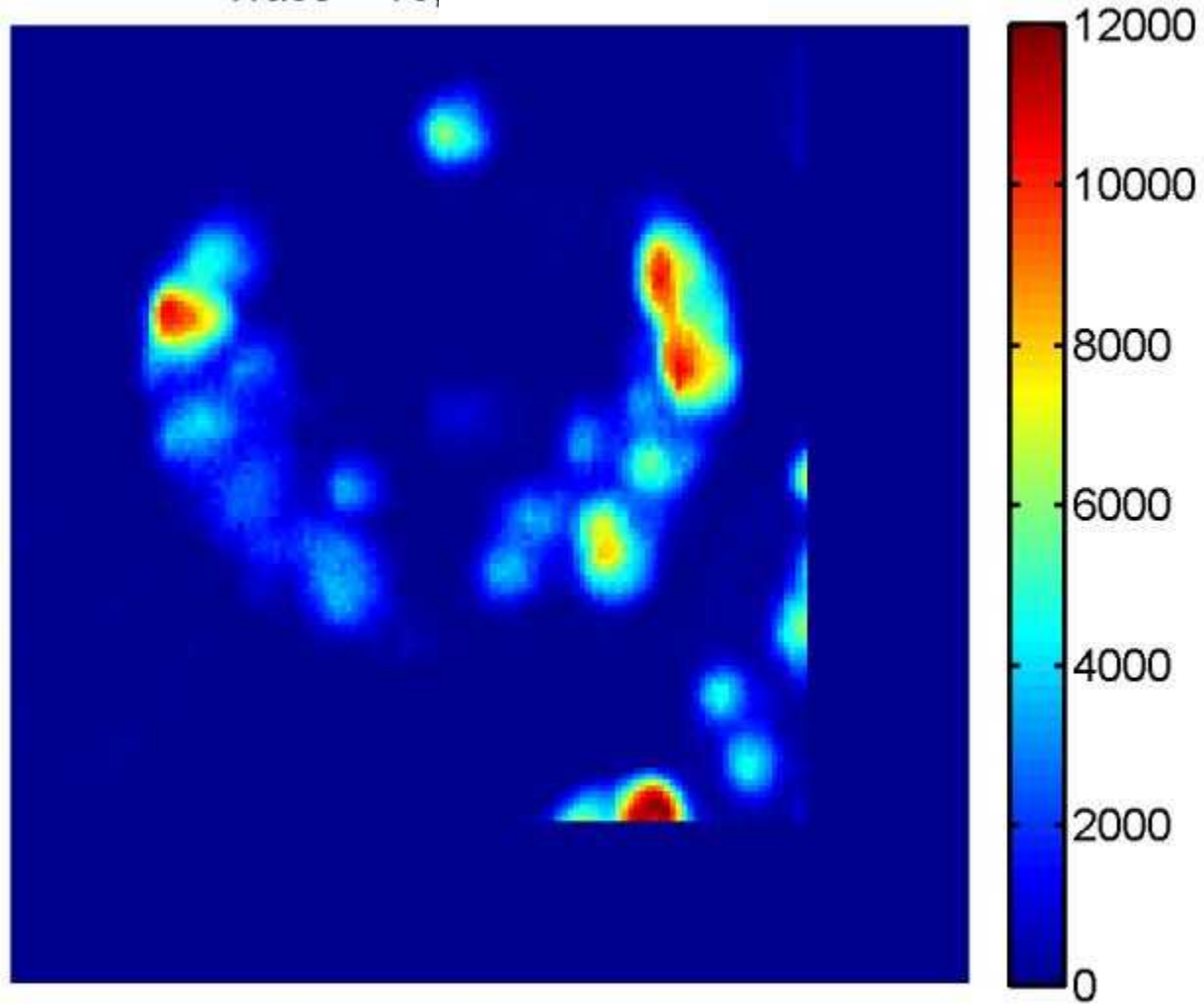
# PSYP-D/E



Trace = 17, GFP

0.01 Sec integration time
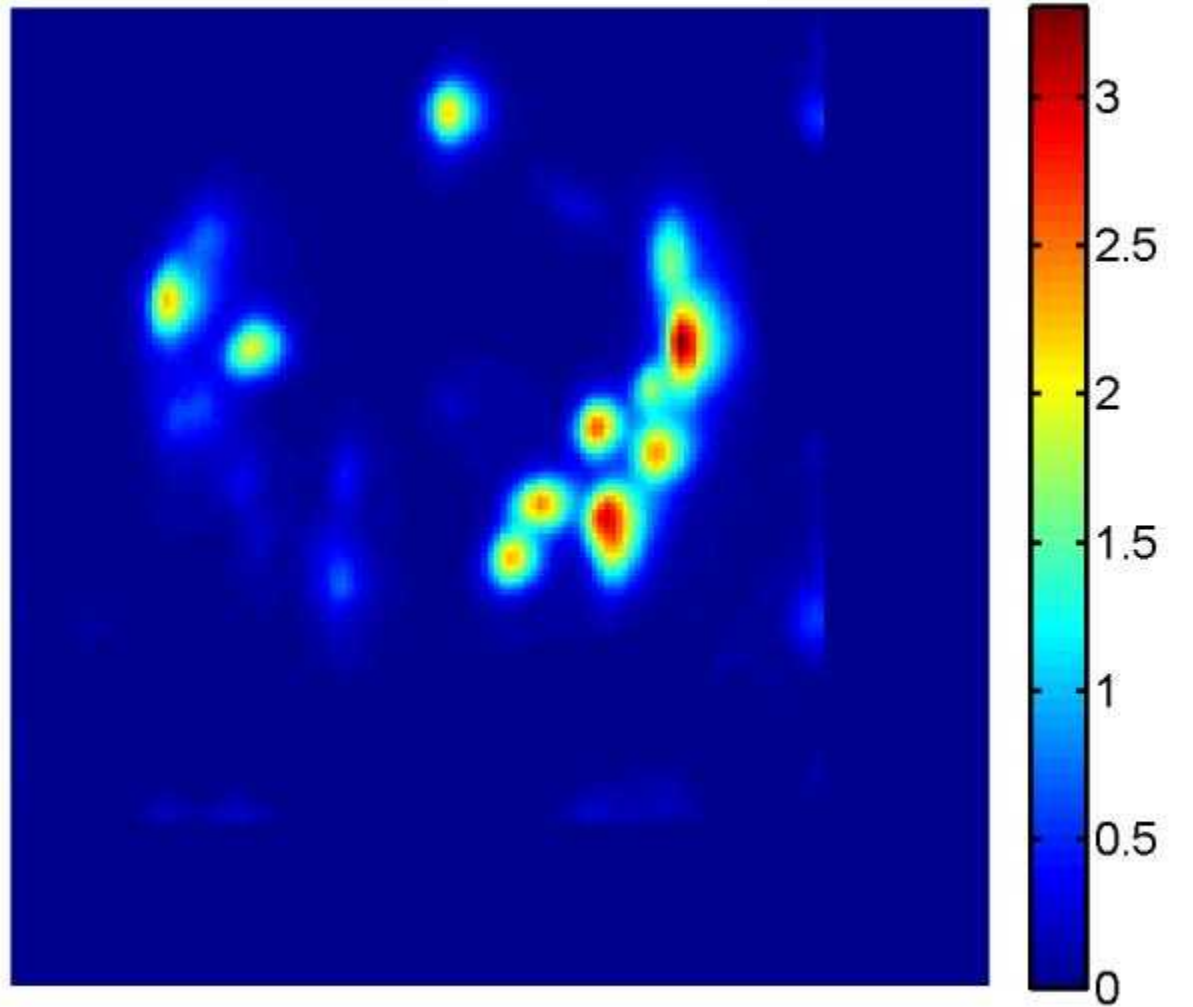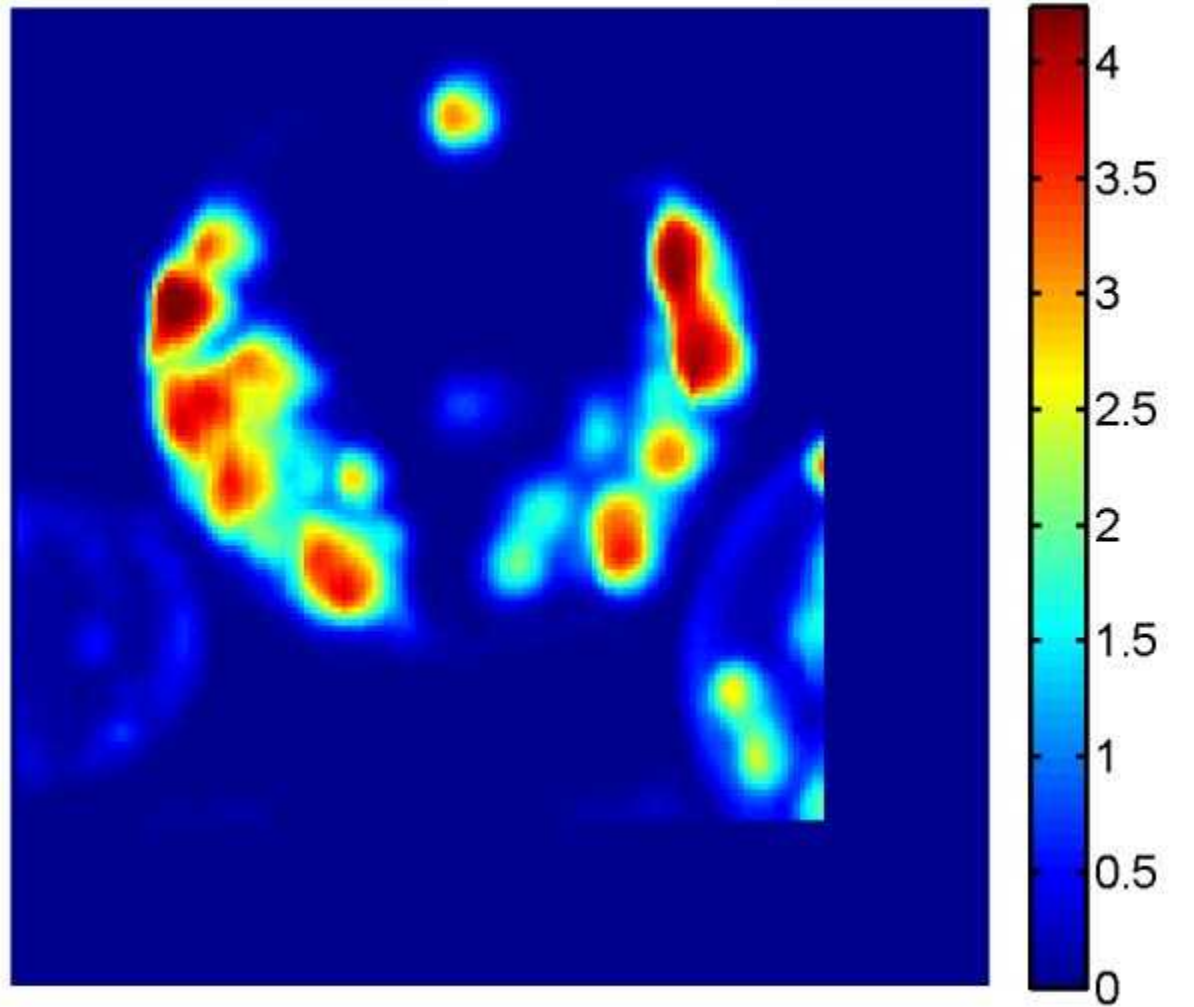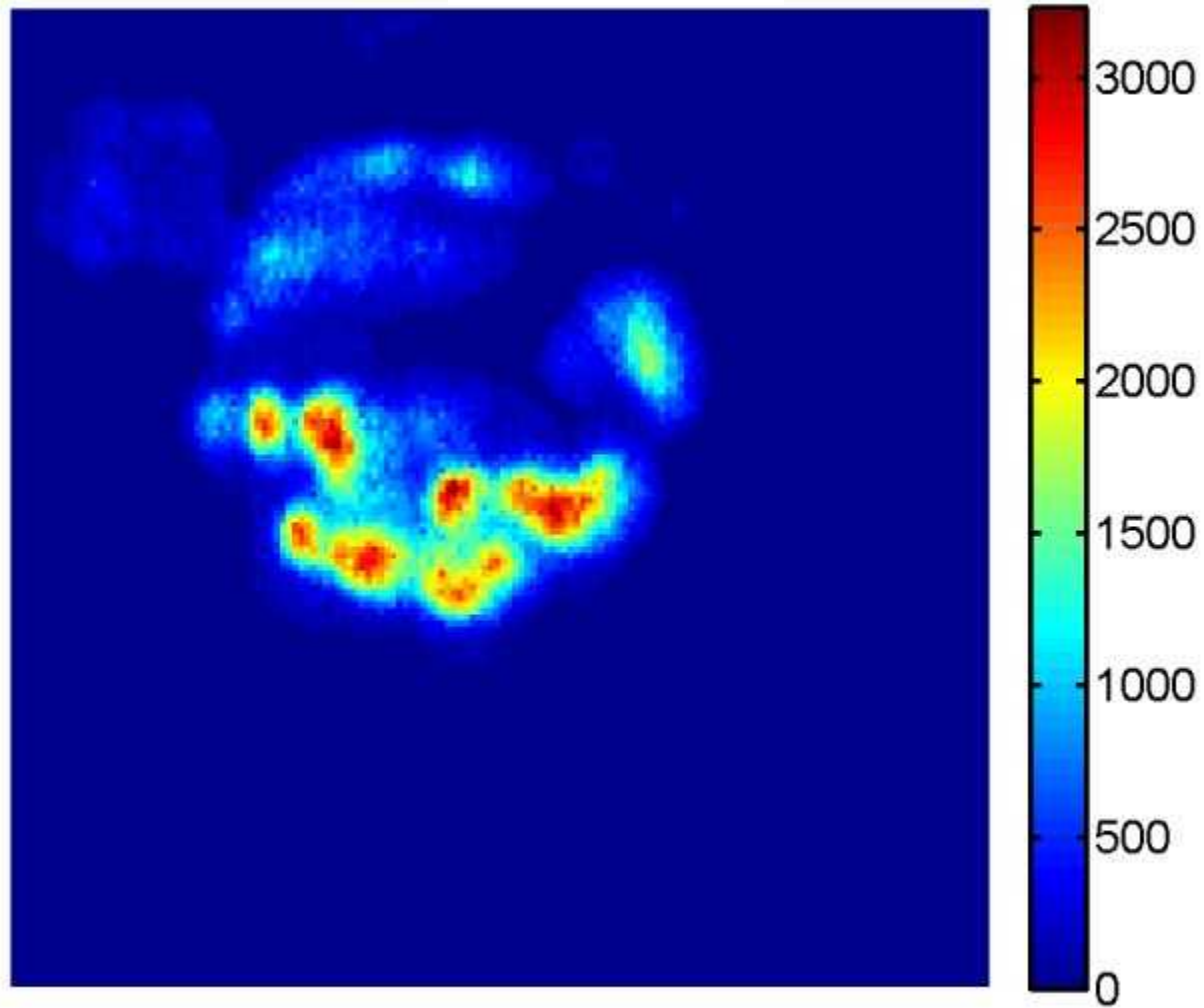
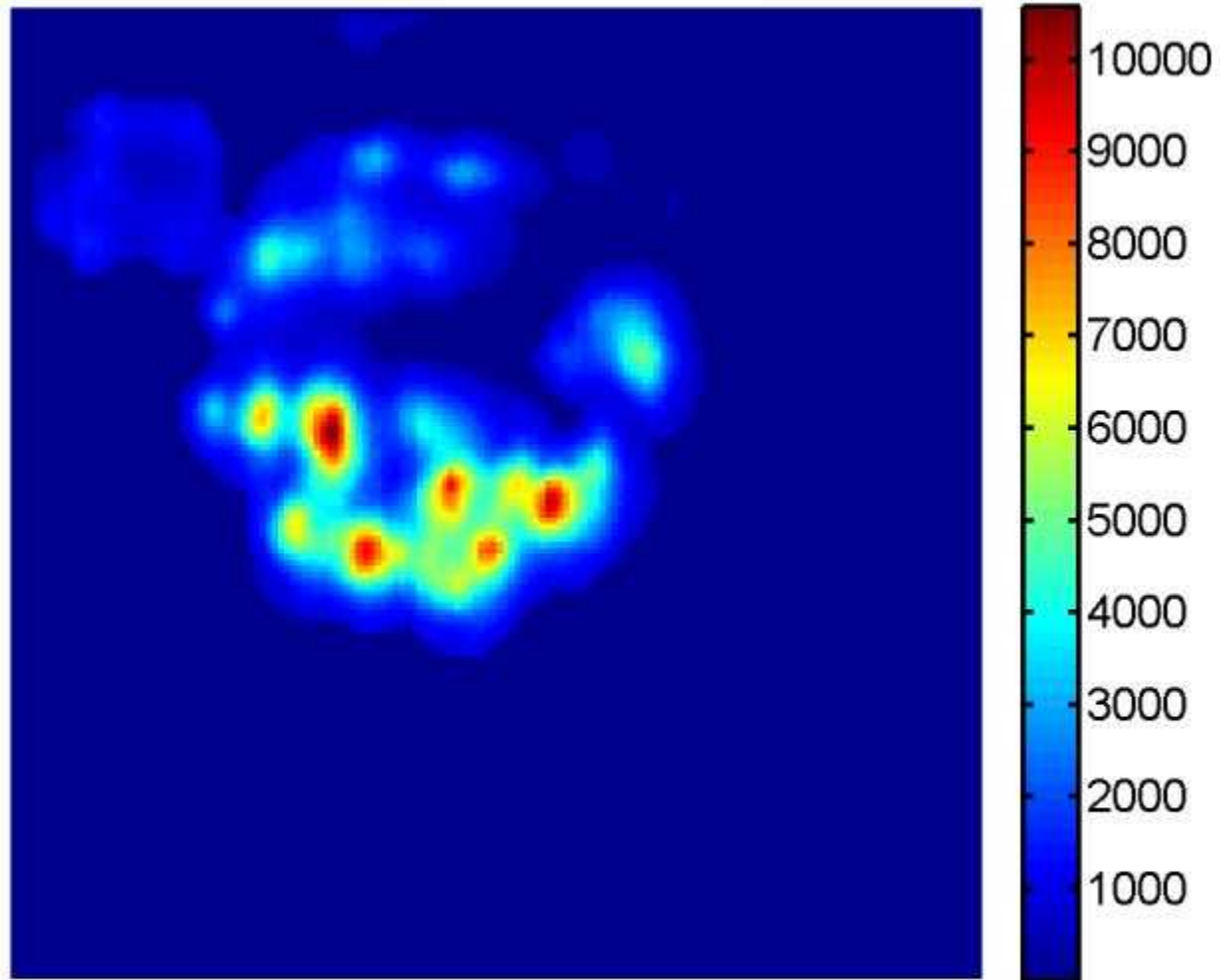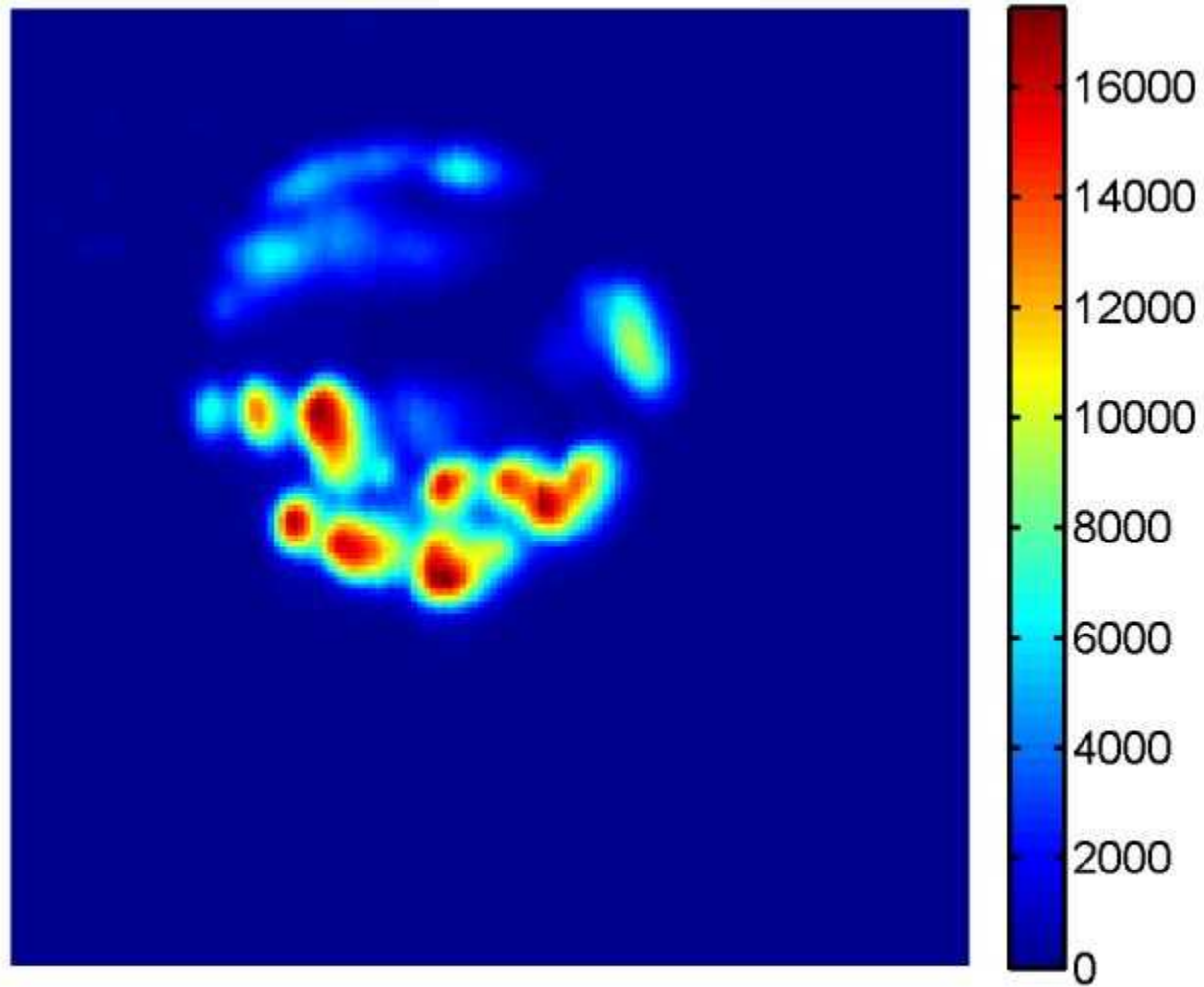# PSYP-D/E

Trace = 18, Carotenoid
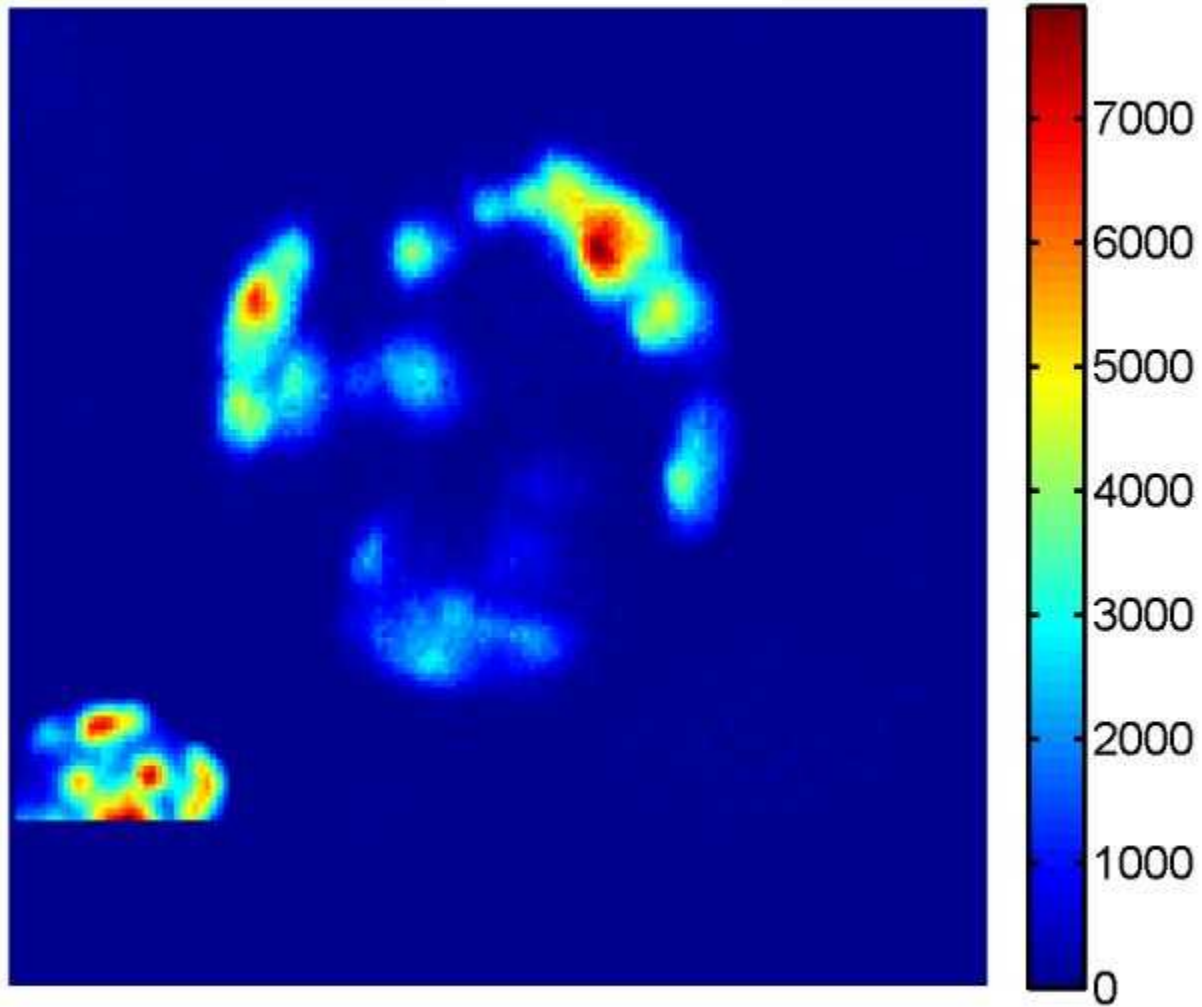


0.01 Sec integration time

# PSYP-D/E



Trace = 18, Chlorophyll
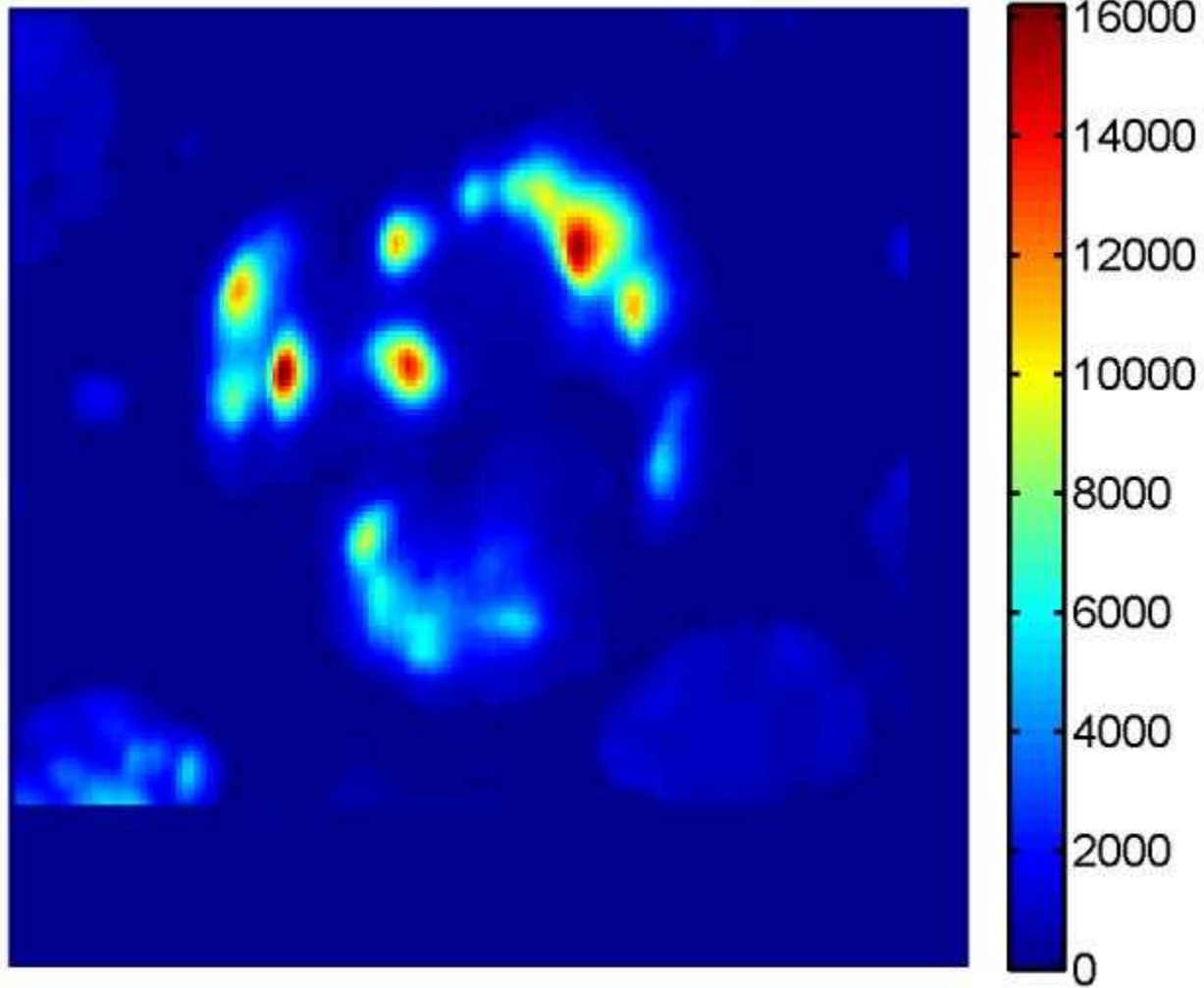
0.01 Sec integration time

# PSYP-D/E



Trace = 18, GFP

0.01 Sec integration time

RGB Composite maps of all types – R = Chl, G = GFP, B = Carotenoid

Control: Trace 1, Trace 2, Trace 3, Trace 4, Trace 5

PSY1-P: Trace 6, Trace 7, Trace 8, Trace 9

PSY1-T: Trace 10, Trace 11, Trace 12, Trace 13, Trace 14

PSYP-D/E: Trace 15, Trace 16, Trace 17, Trace 18, Trace 19

Auto-scaled on a per-image basis

# Single Protoplast Analysis

Create a tight mask around the protoplast plastid and then calculate the mean-per component concentration.
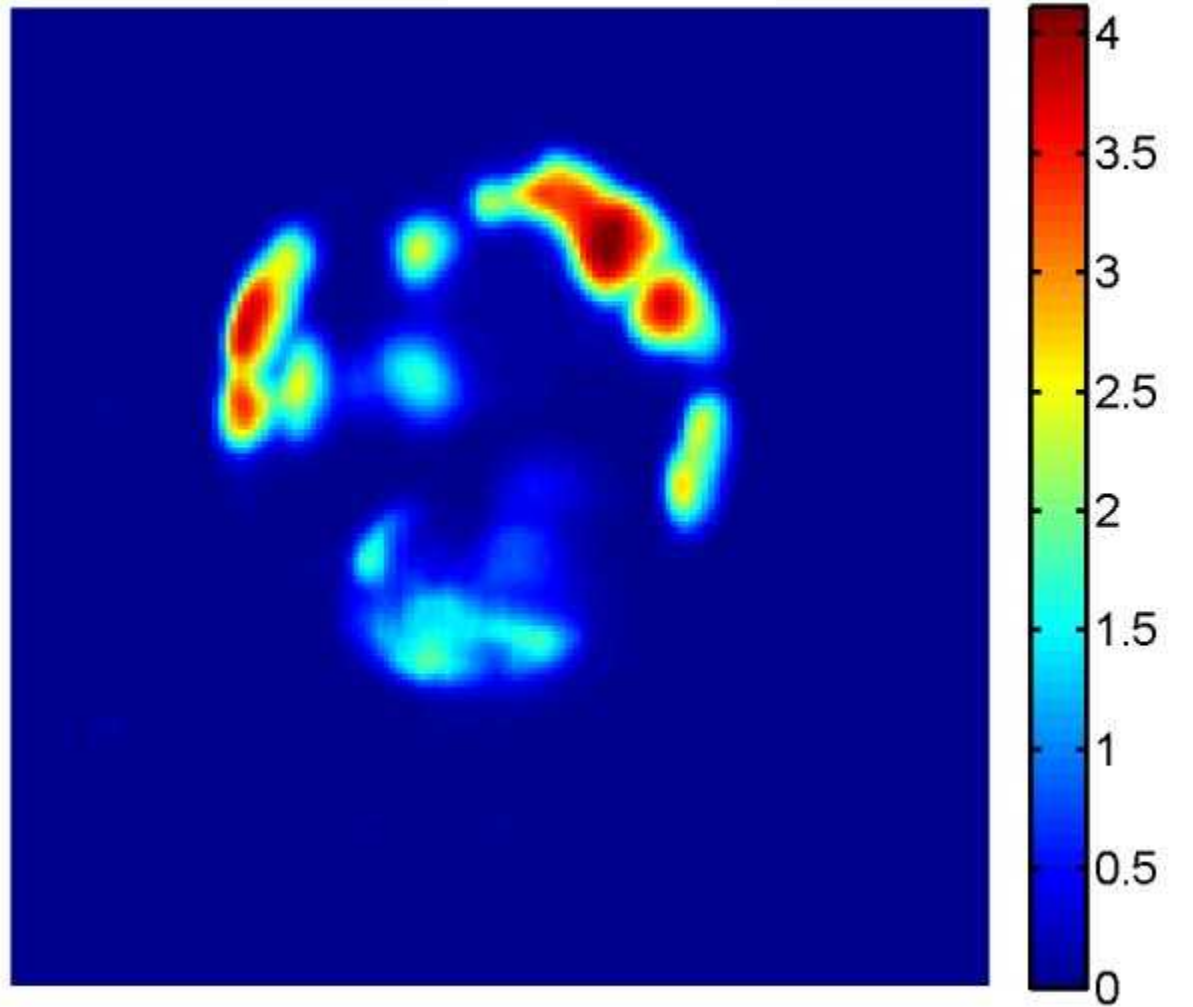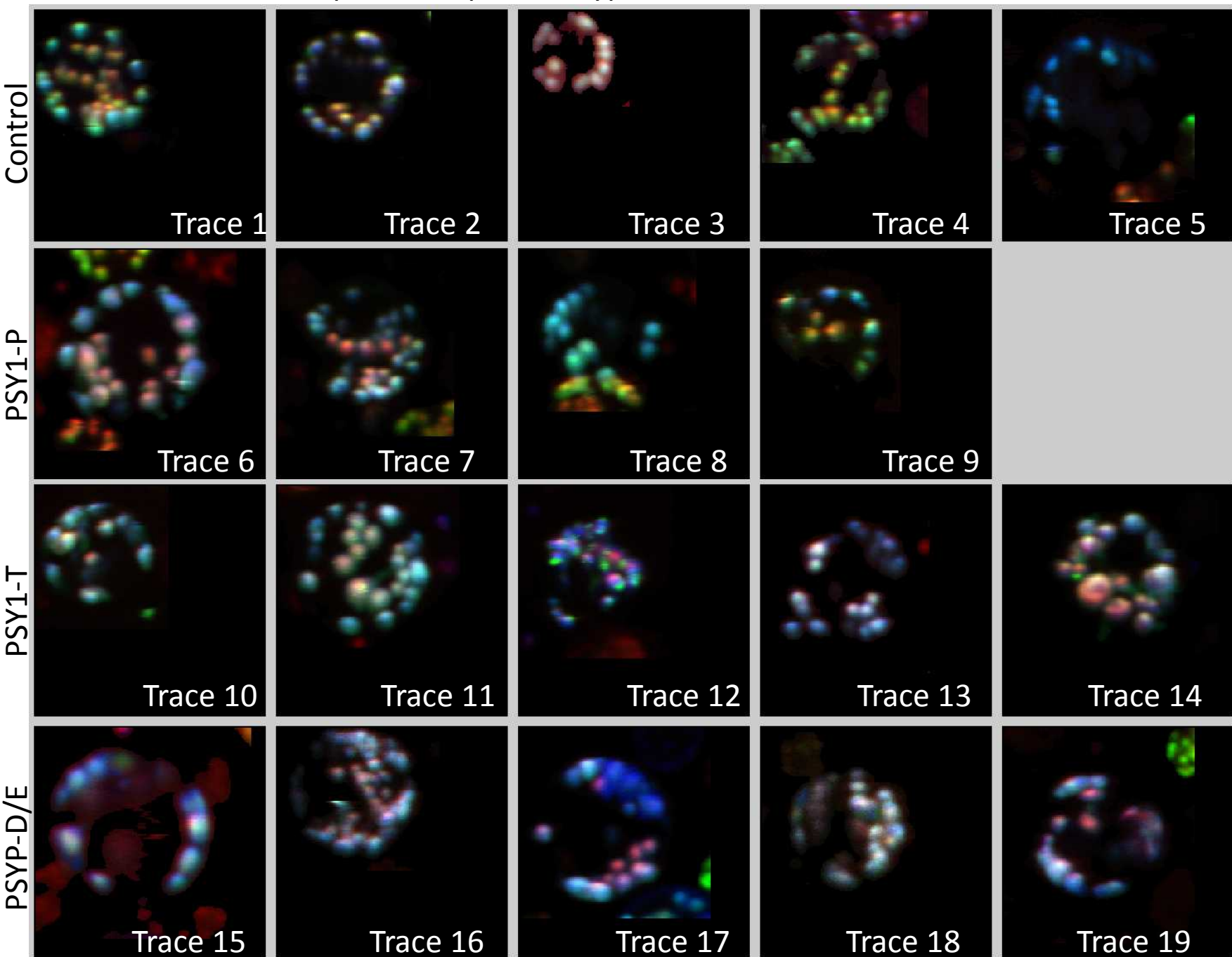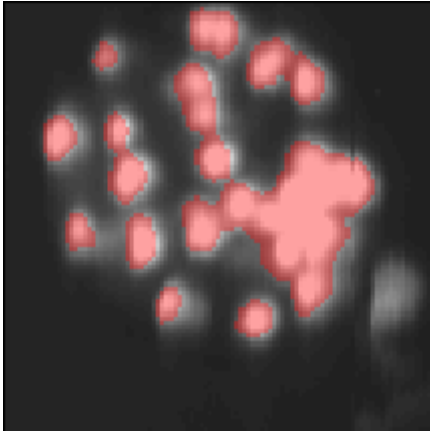


Here, pink denotes pixels included in the mean calculation. Note we don't want to decrease the mean value by including pixels inside of the protoplast but not containing plastid.

With the current model, there is still too much uncertainty to tell if the GFP mutants possess more or equivalent carotenoid compared to untransformed controls.
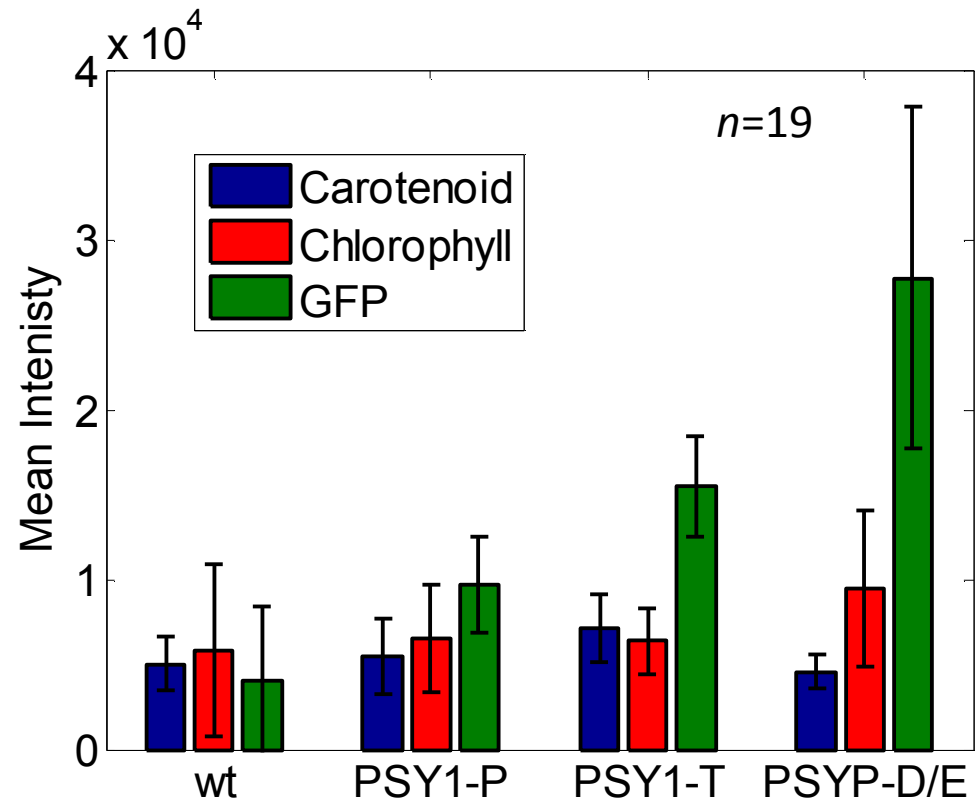
# Thoughts

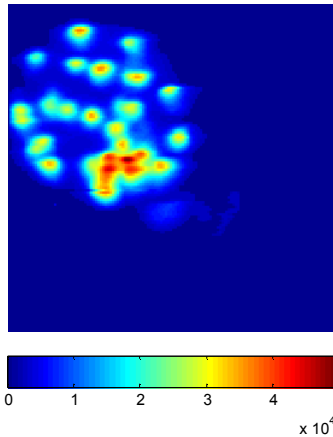The MCR model is not perfect but I wanted to share the working model for discussion. The per-protoplast mean data trends in the correct direction for carotenoid content in that the mean carotenoid value is lowest in wt and PSYP-D/E and slightly higher in PSY1 lines however the error associated with the data says the mean difference is not significant.
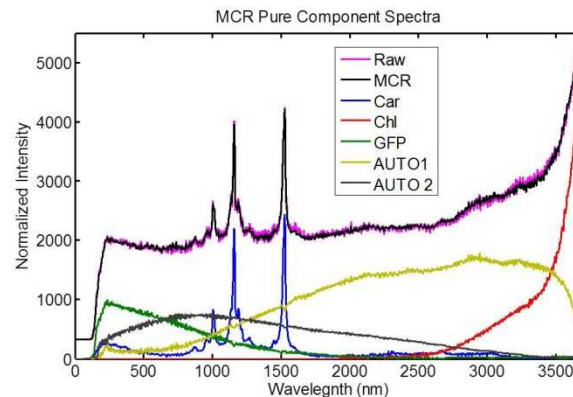
We still "see" the GFP signal in the control (non-transformed) protoplasts and this means there is an autofluorescence signature that is congruent with GFP.
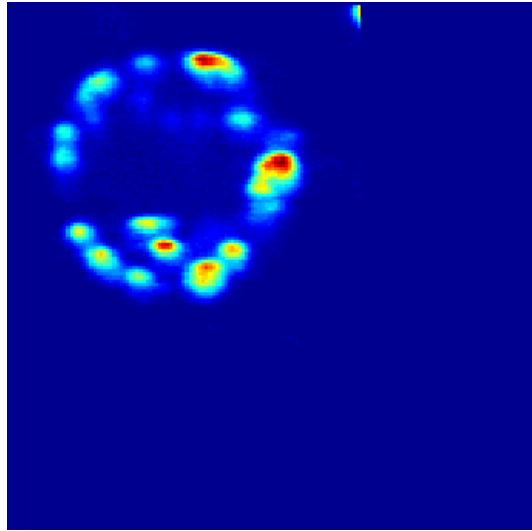
Non-transformed cell

Mean image intensity                                    and MCR fit.
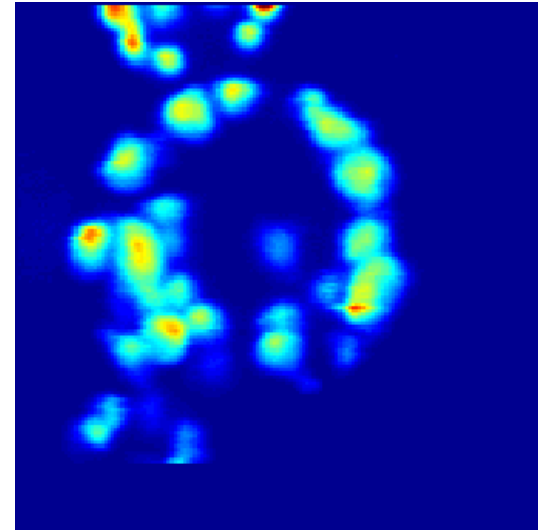


GFP component needed to fit data

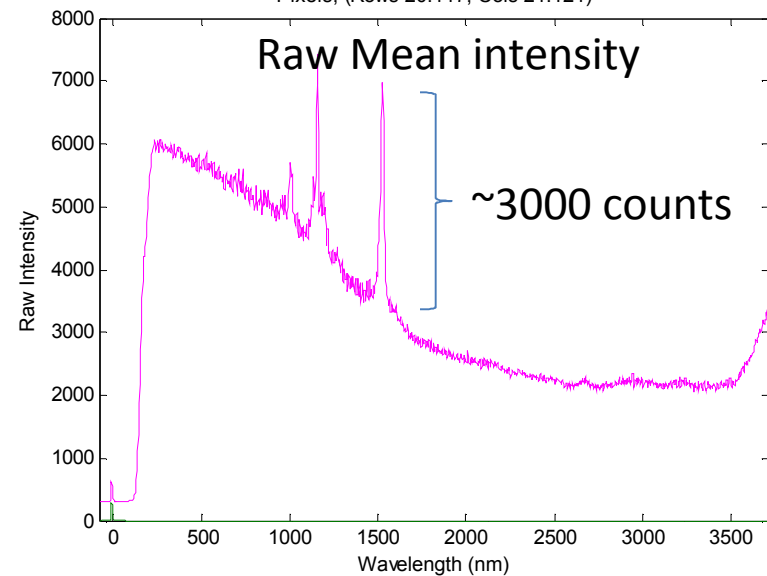# Perhaps MCR is not the most appropriate analysis tool for this study?
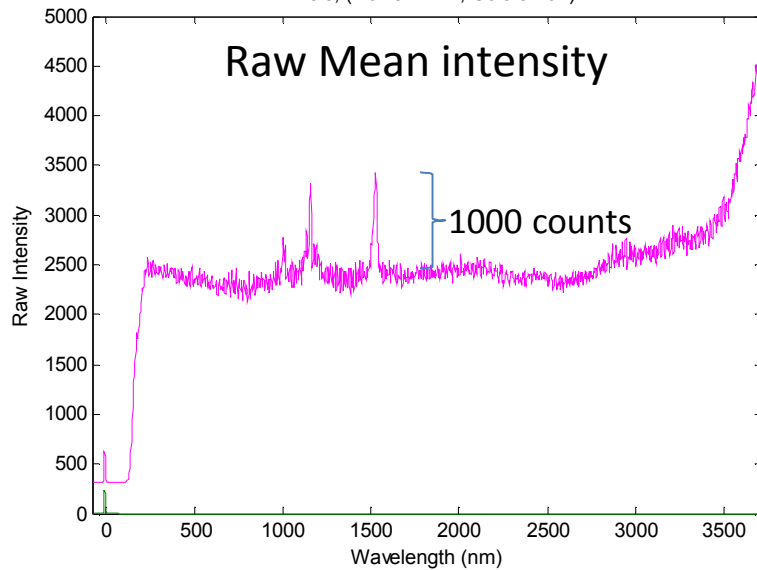


Analysis of the Raw data does show obvious greater Carotenoid intensity in GFP mutant. Because MCR tries to fit every wiggle and bump in the data, we might be downweighting the carotenoid contribution with other spectral components.

# Alternative analysis

We could consider using a "Rolling circle filter" to remove the fluorescence.

http://www.opticsinfobase.org/as/abstract.cfm?uri=as-60-3-288

Here, a post-processing filter is applied to remove the contributions of fluorescence from Raman data. This might let us get at the carotenoid difference because we already identified the protoplast of interest using the fluorescence microscope.