Two-photon fluorescence and Second-harmonic generation imaging for nondestructive circadian profiling of starch content in fresh intact Arabidopsis leaf

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2023 Annual Meeting of the Physical Society of Taiwan
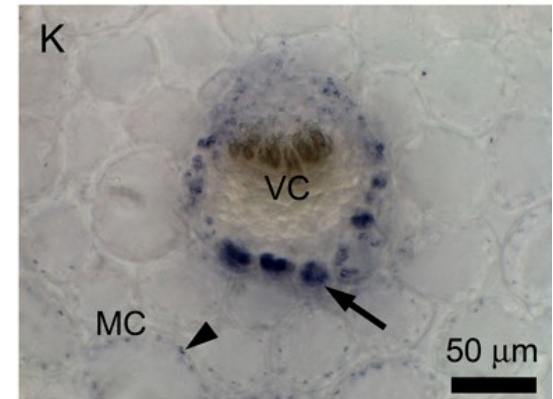
Acknowledgement

Leaf Circadian Rhythm

- Circadian clock: 24 hour rhythm

- Day: photosynthesis convert light to energy, parts stored in starch

- Night: break down of starch for energy

- Tracking variations in leaf starch content allows monitoring of this process
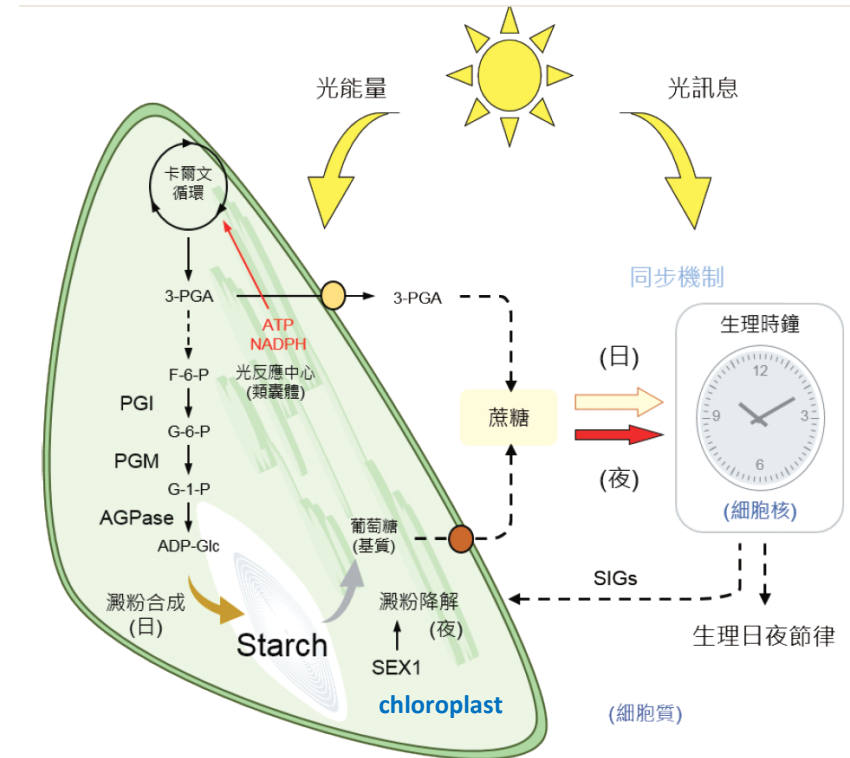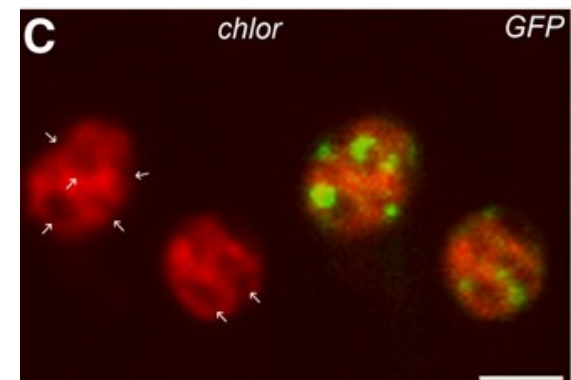
Current Methods to Track Starch

- ## Starch iodine staining
  - Requires dissolving the chlorophyll with ethanol
  - Destroys cells

Ref: Tsai et al. *Plant Physiology*, November 2009, **151**, 1582–1595,

- ## Genetic modification and attachment of fluorescence protein
  - Time consuming and is done for only one ecotype at a time

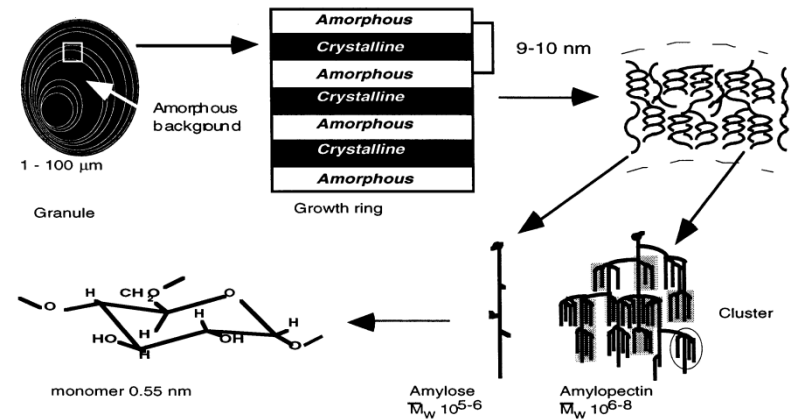Ref: Szydlowski et al. 2009 *The Plant Cell*, **21** 2443–2457

Starch Second Harmonic Generation

- Starch granules are semicrystalline and can generate SHG

- Use SHG as the contrast mechanism for imaging starch

- Use TPF as contrast mechanism for imaging leaf mesophyll cells

Ref: *Buleÿon et al. 1998 International Journal of Biological Macromolecules* **23** *85–112*

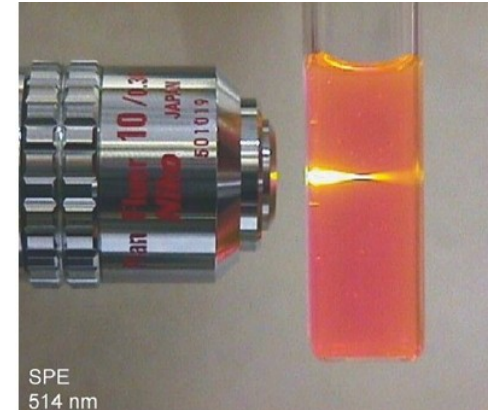SHG image of wheat starch granule
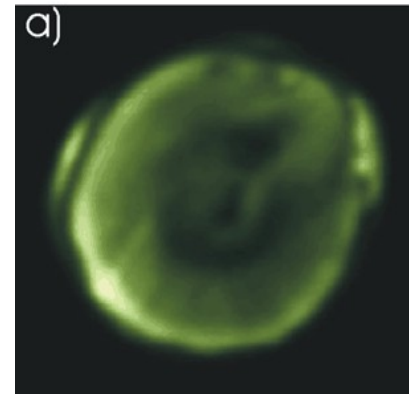
Ref. Psilodimitrakopoulos *et al 2010 J. Opt.* **12 084007**

SHG and TPF Microscopy

- SHG and TPF as contrast in a laser scanning microscope

- Nonlinear process → Point excitation -> optical sectioning without a confocal pinhole

- Longer wavelength excitation for greater sample penetration

- Nondescanned detection improves signal collection

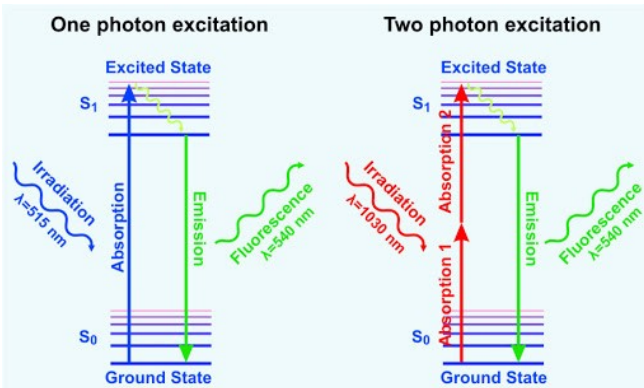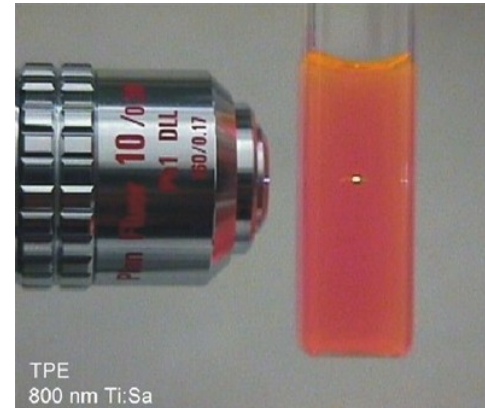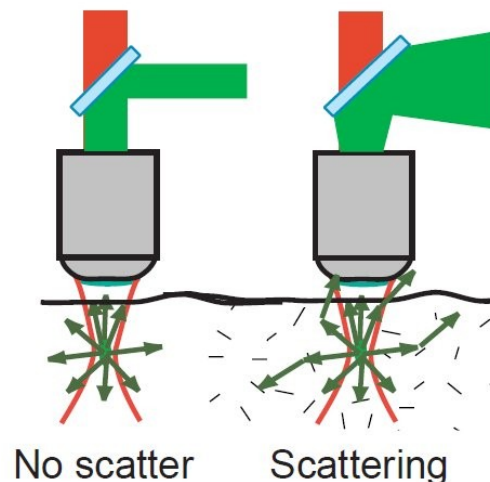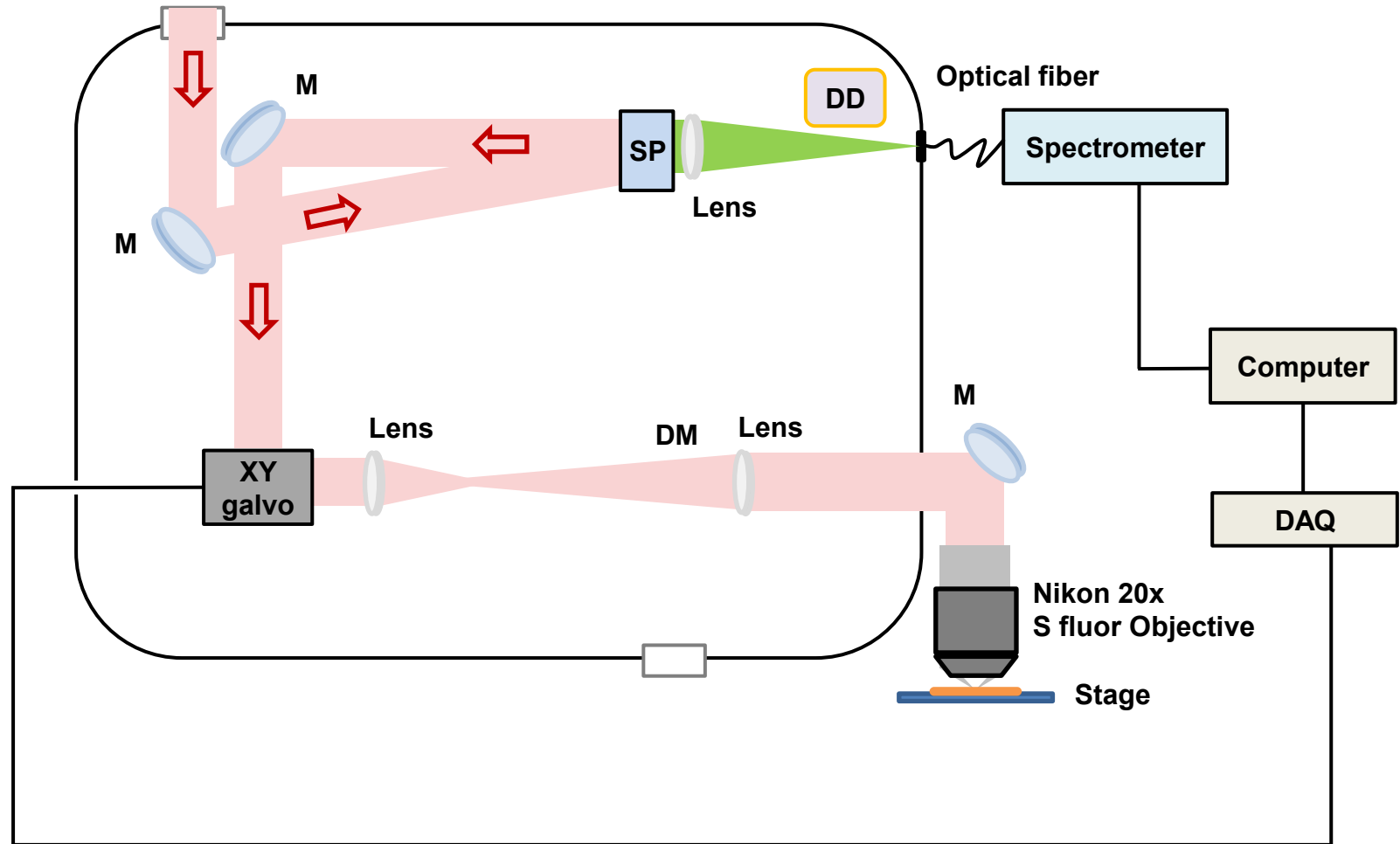One photon Excitation

SPE
514 nm

Two photon Excitation

TPE
800 nm Ti:Sa

One photon excitation

Two photon excitation

Excited State

Excited State

S_1

S_1

Irradiation λ=515 nm

Absorption

Emission

Fluorescence λ=540 nm

Irradiation λ=1030 nm

Absorption 2

Absorption 1

Emission

Fluorescence λ=540 nm

S_0

S_0

Ground State

Ground State

Image reference: http://candle.am/microscopy/

No scatter   Scattering

Imaging System

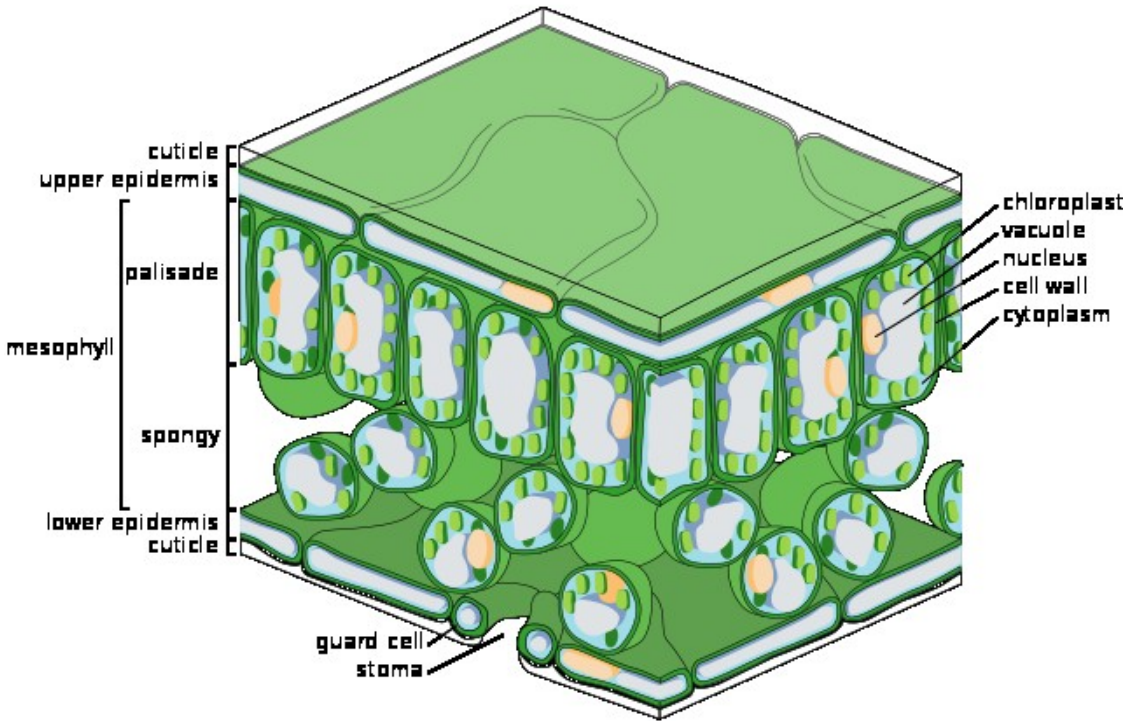• DD: descanned detection (confocal)

Imaging System
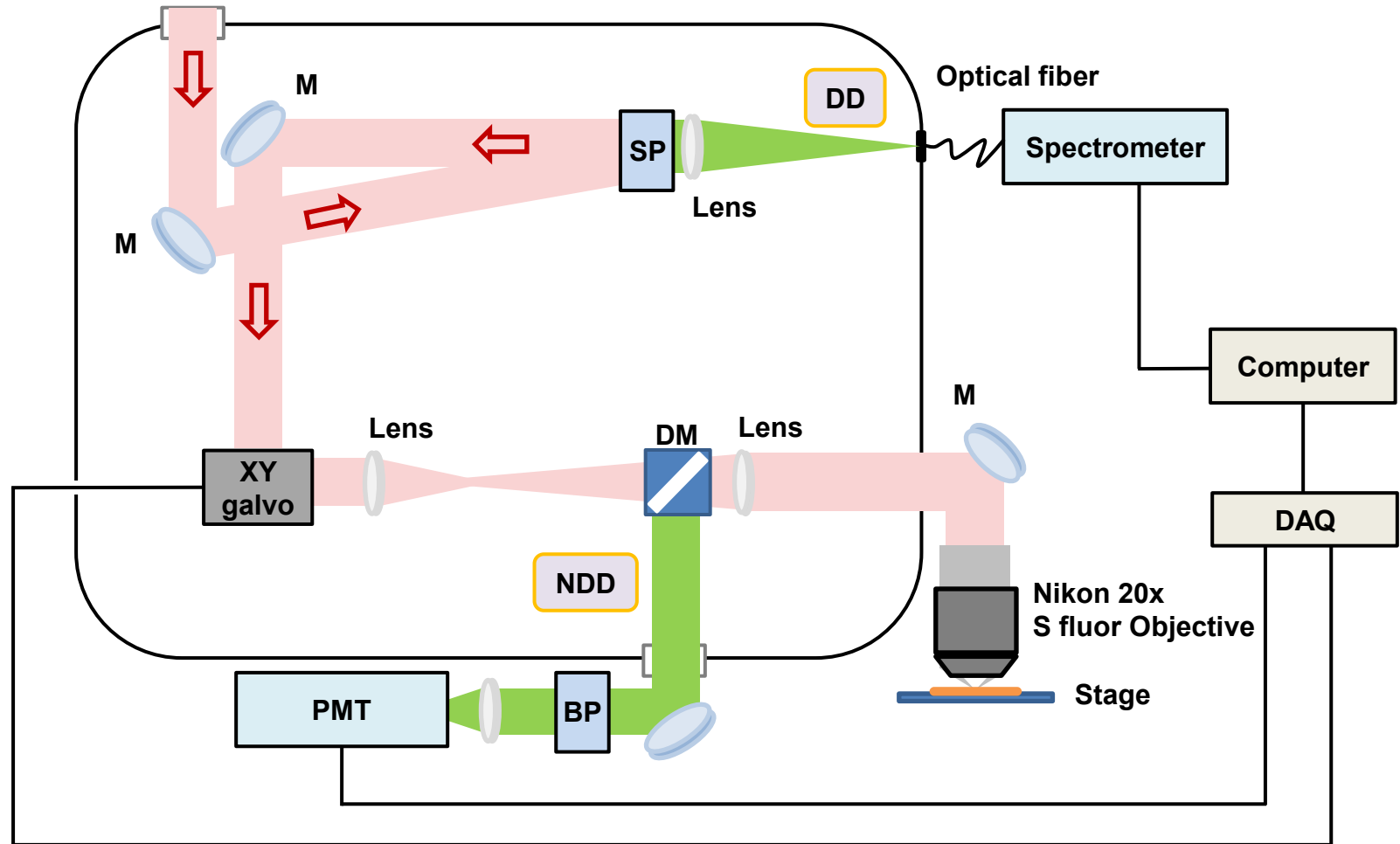
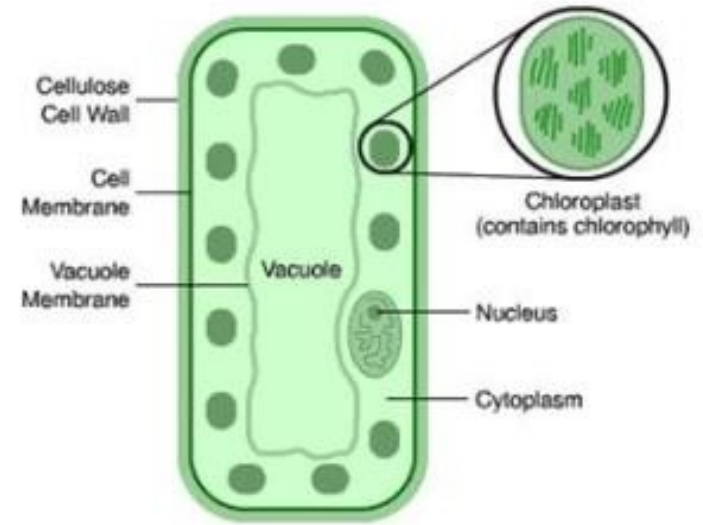- NDD: nondescanned detecton

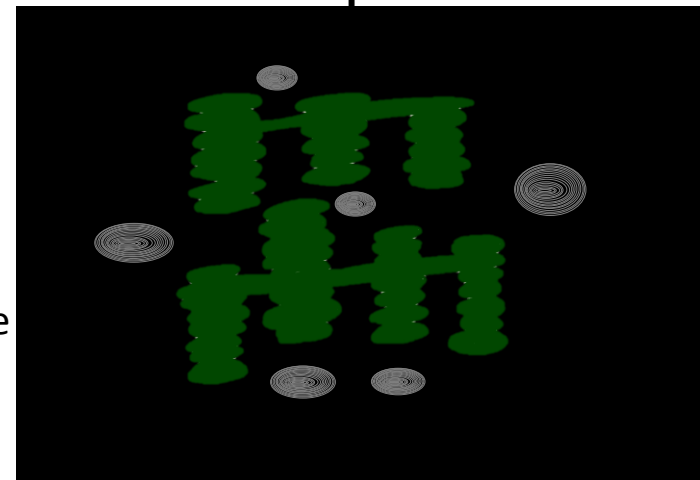# LSCM Photo
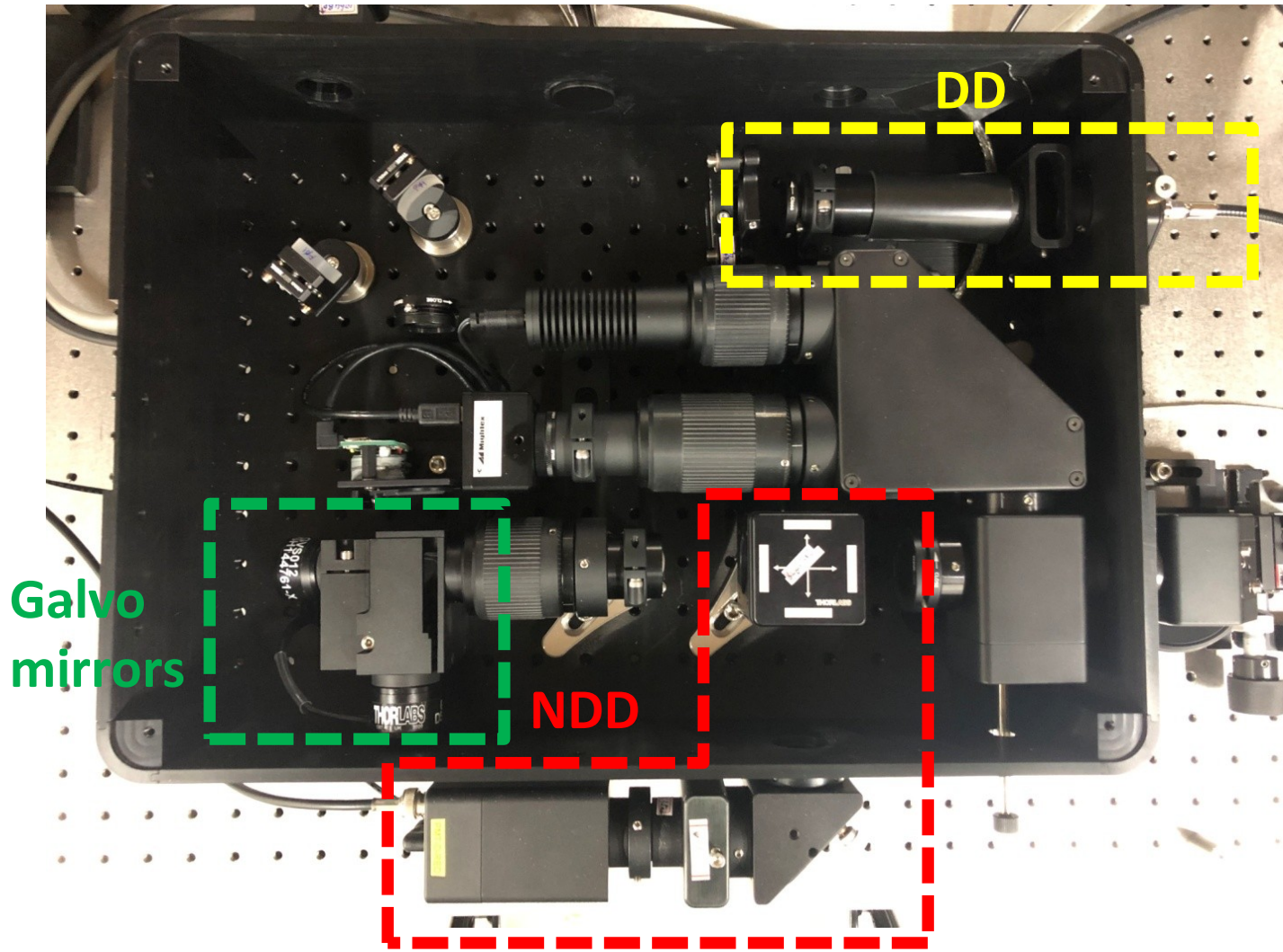
Leaf Structure

Leaf cross section

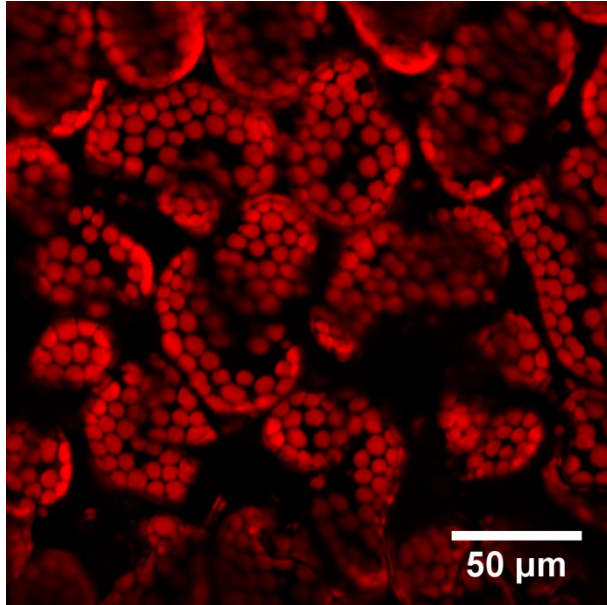**Mesophyll cells**

Chloroplast

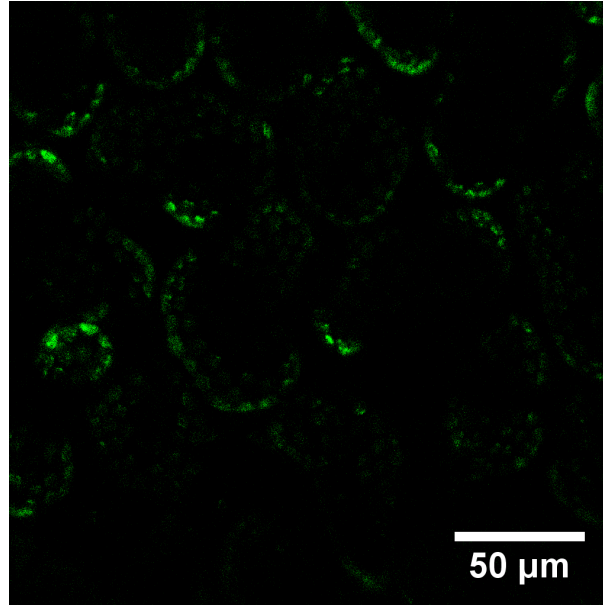- **Goal:** to detect and quantify amount of starch inside chloroplast

starch granule
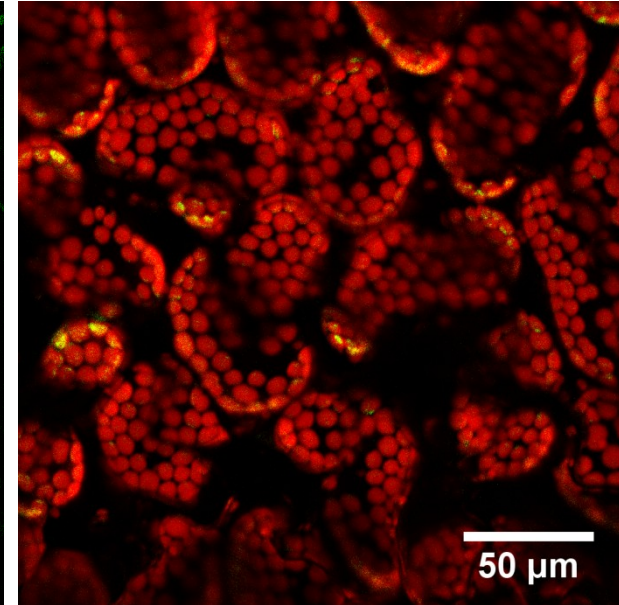
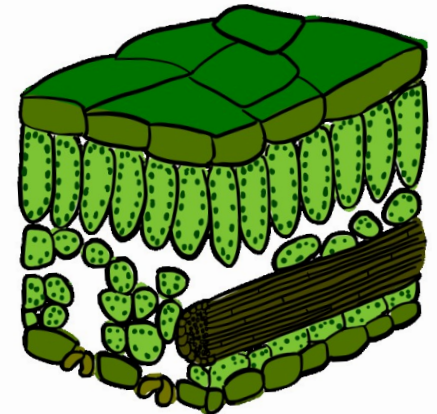thylakoid

TPF+SHG Imaging of Leaf
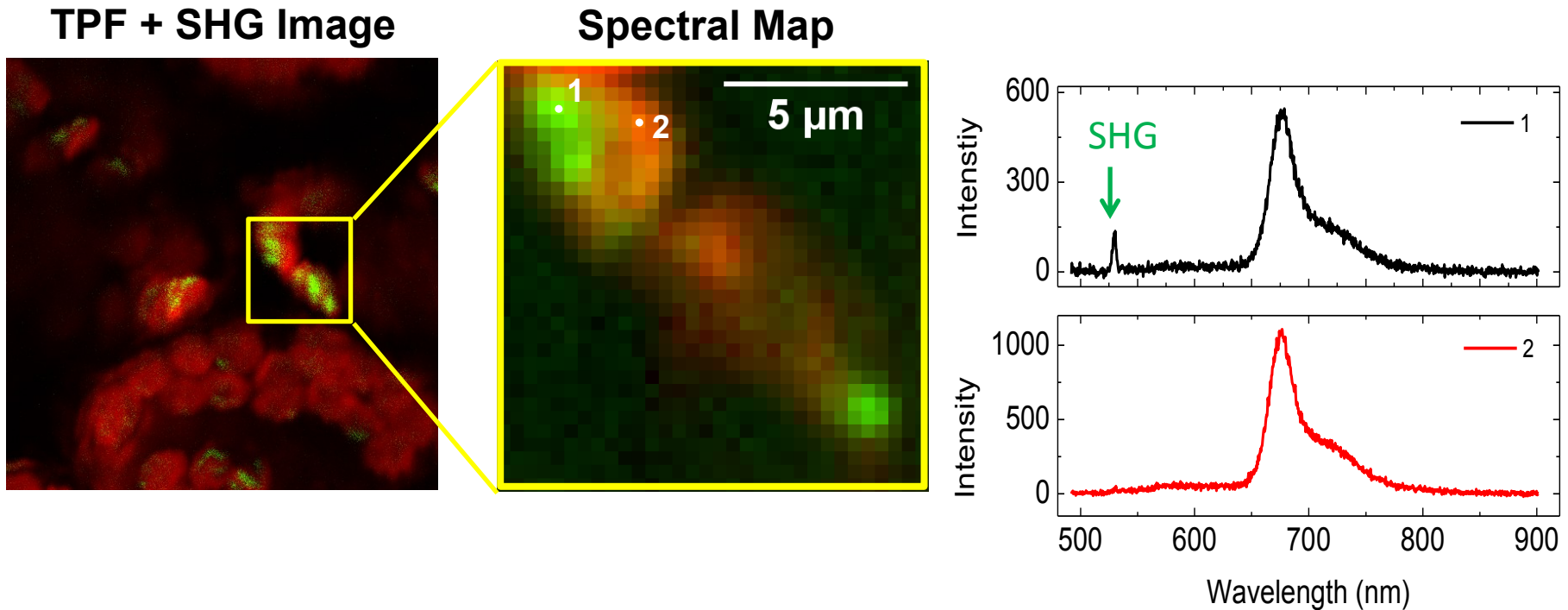
TPF

SHG

TPF + SHG

- **TPF:** 670/30 nm band two-photon fluorescence detection
- **SHG:** 532/3 nm band second harmonic generation detection
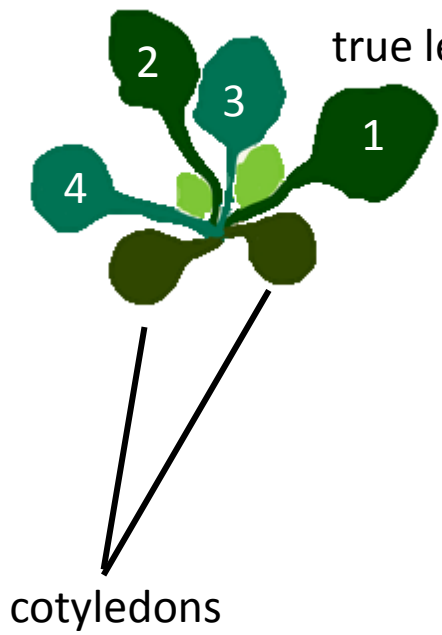- Col-0 ZT15 Image

Spectral Confirmations

TPF + SHG Image

Spectral Map

5 µm

SHG

600

300

0

Intensity

1

1000

500

0

Intensity

2

500 600 700 800 900

Wavelength (nm)
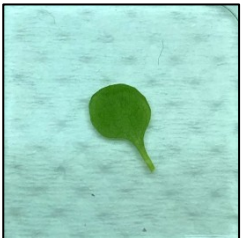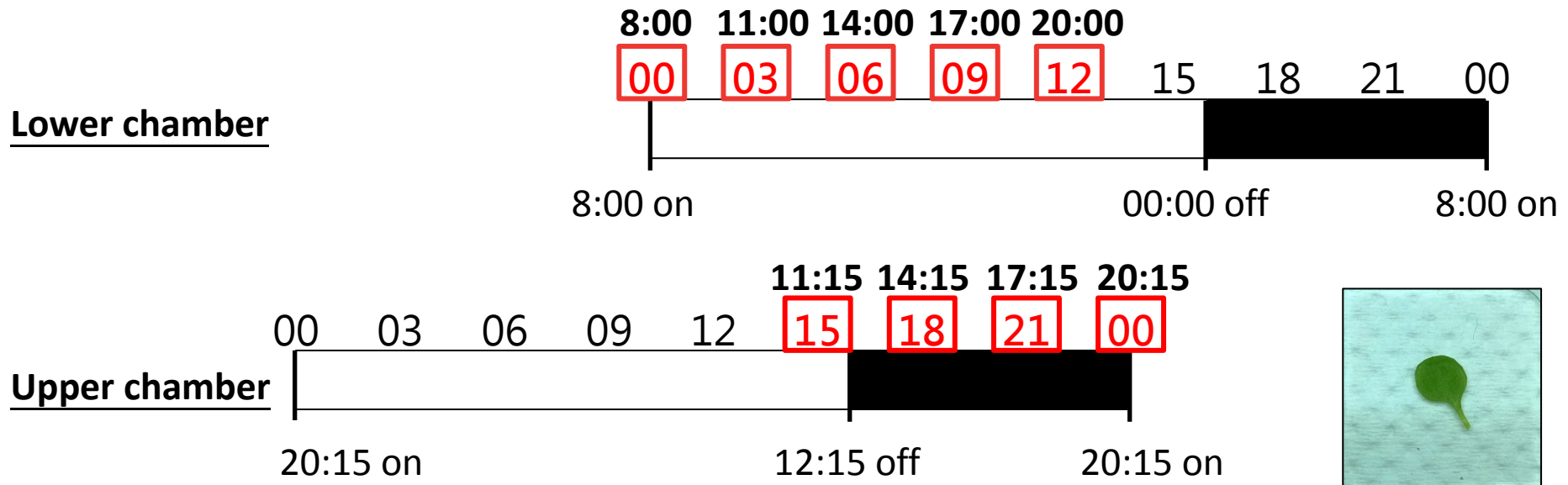
- Excitation wavelength: 1064nm
- Spectral map representation: Red 670/30, Green 532/3
- Spectral confirmation of SHG and TPF signal from starch granule and chlorophyll

24 Hour Study of Starch in Leaves

Lower chamber

8:00 11:00 14:00 17:00 20:00
00 03 06 09 12 15 18 21 00

8:00 on   00:00 off   8:00 on

Upper chamber

11:15 14:15 17:15 20:15
00 03 06 09 12 15 18 21 00

20:15 on   12:15 off   20:15 on
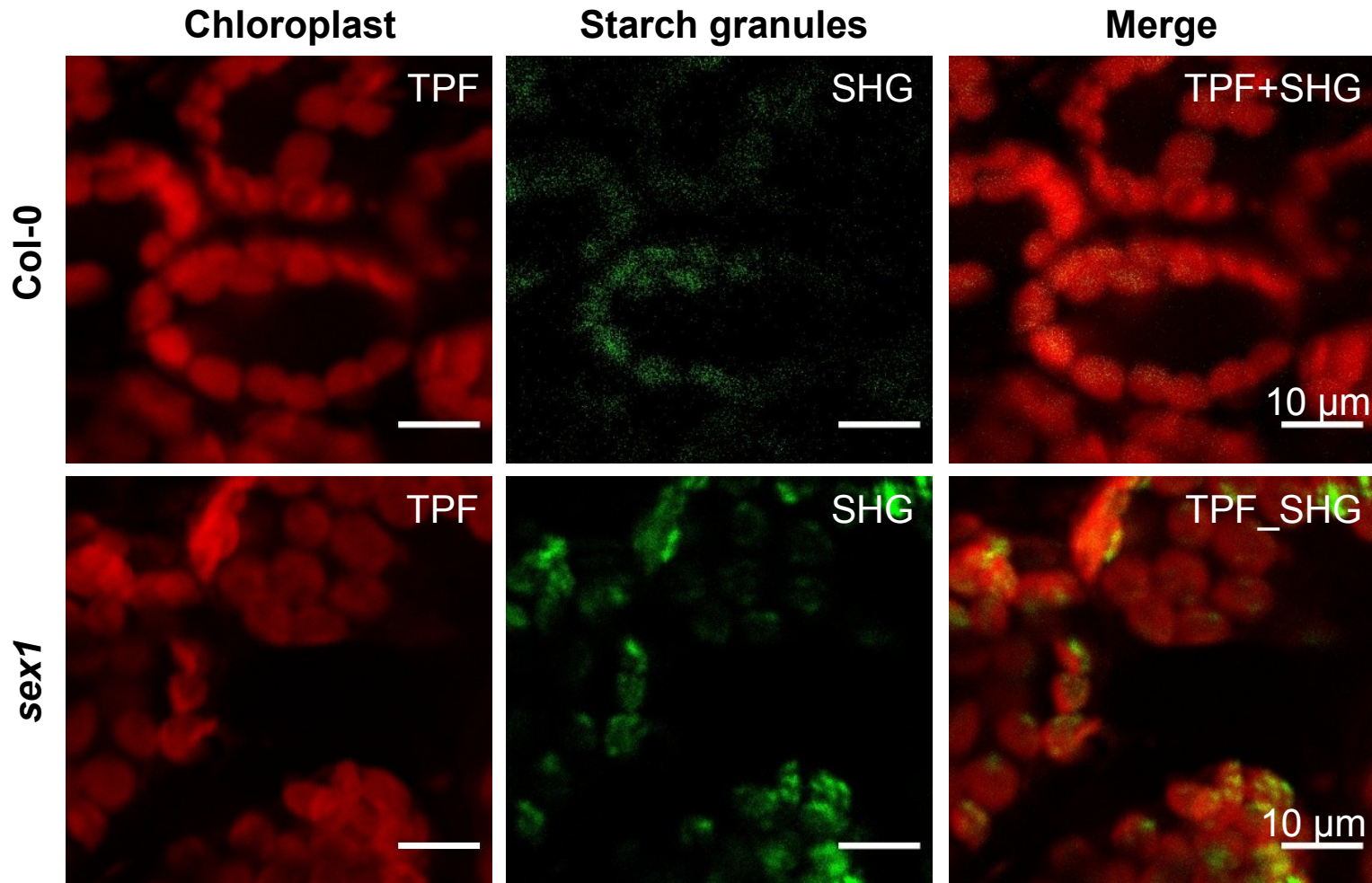
true leaves

1 2 3 4

cotyledons

14-d-old seedling
Two Arabidopsis lines:
Wild type: Columbia-0 (**Col-0**)
Mutant: Starch Excess (*sex1*)

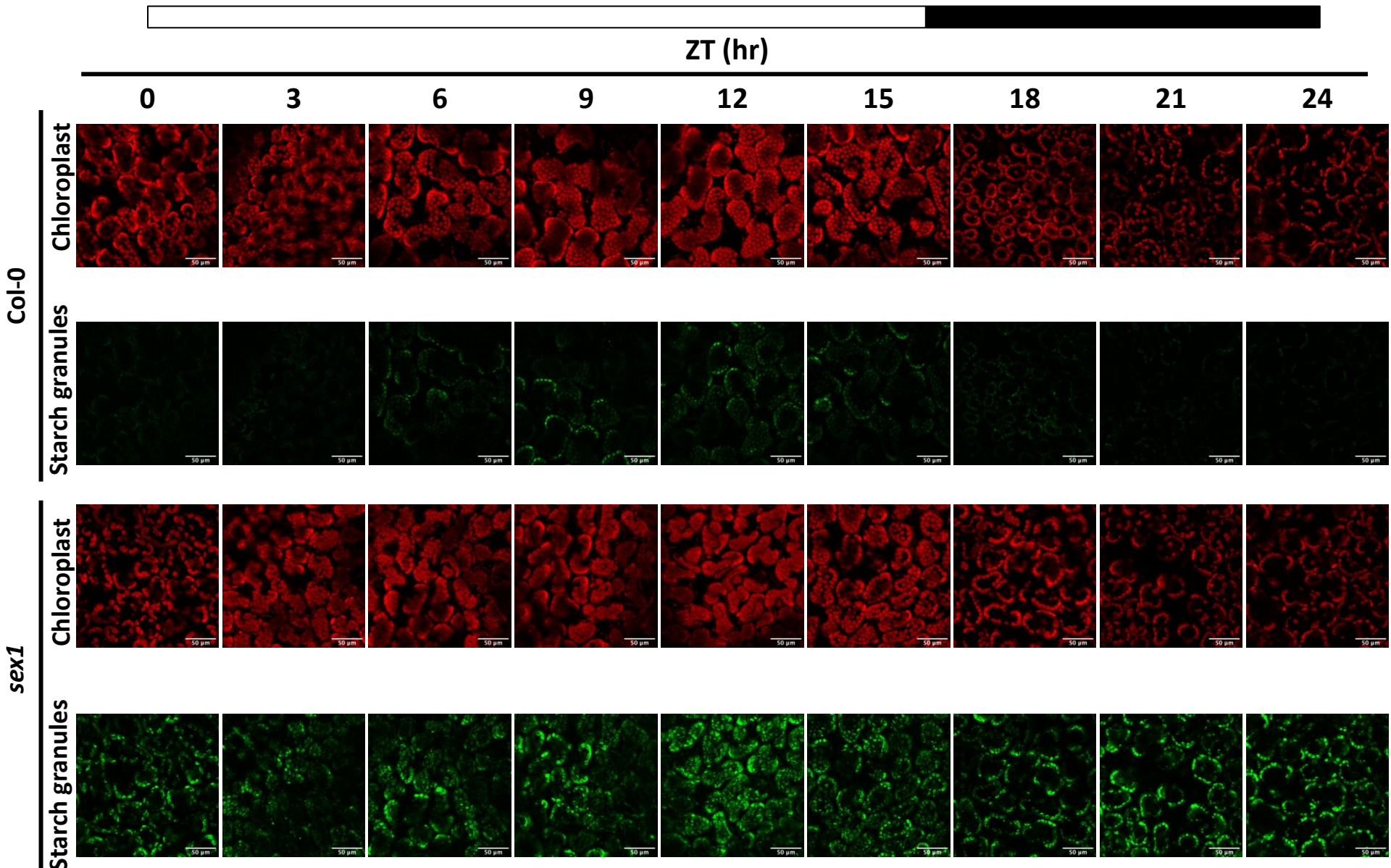-two leaves of the seedling
-different positions of the leaf

A
B C

D E
F

13

Col-0 vs *sex1* Leaf

	Chloroplast	Starch granules	Merge
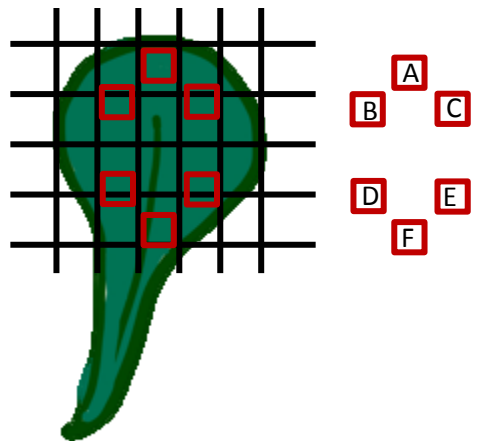
- *sex1* : *starch excess 1* is a mutant type with starch processing gene removed
- Col-0: Columbia-0 wild-type

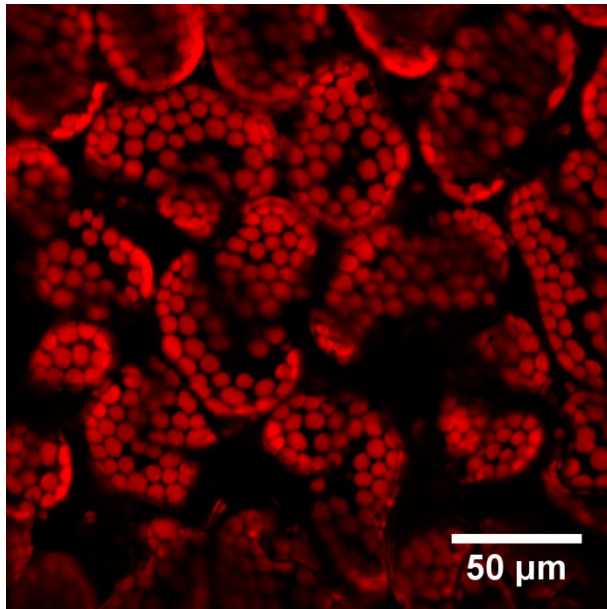# Diurnal variations of starch granule in Arabidopsis

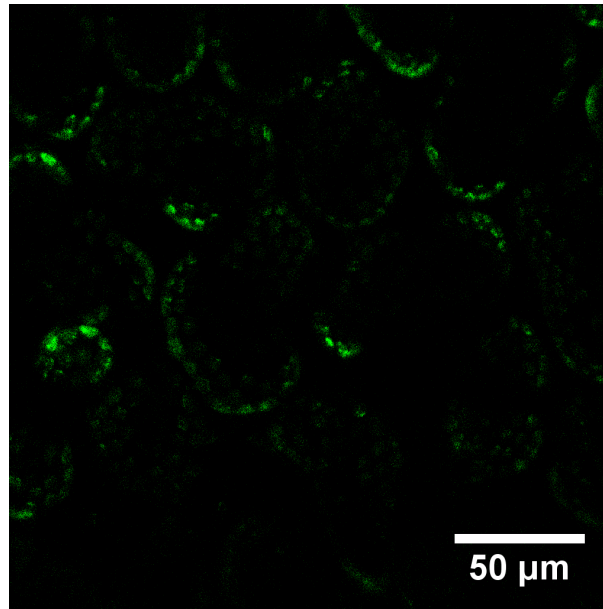ZT0: lights on, ZT16: lights off; scale bar, 50 μm

Starch Content Determination

$$\text{starch content} \propto \frac{I_{SHG}}{Cnt_{TPF}} = \frac{\text{Summed SHG intensity}}{\text{Number of TPF Pixels above threshold}}$$

TPF

SHG

TPF + SHG
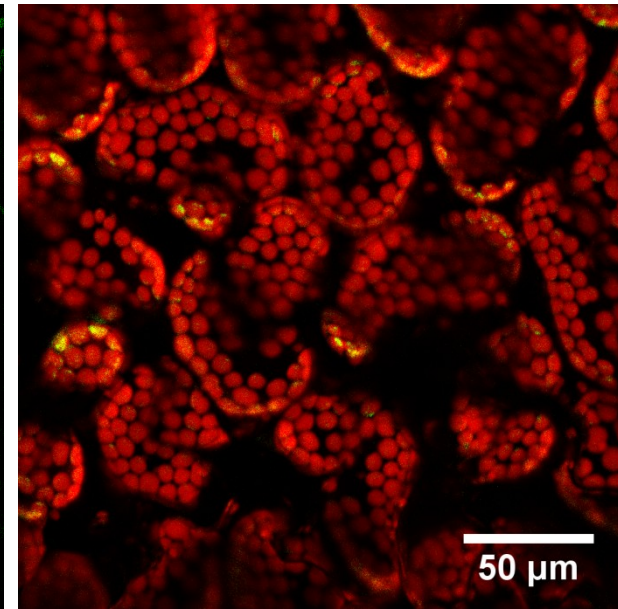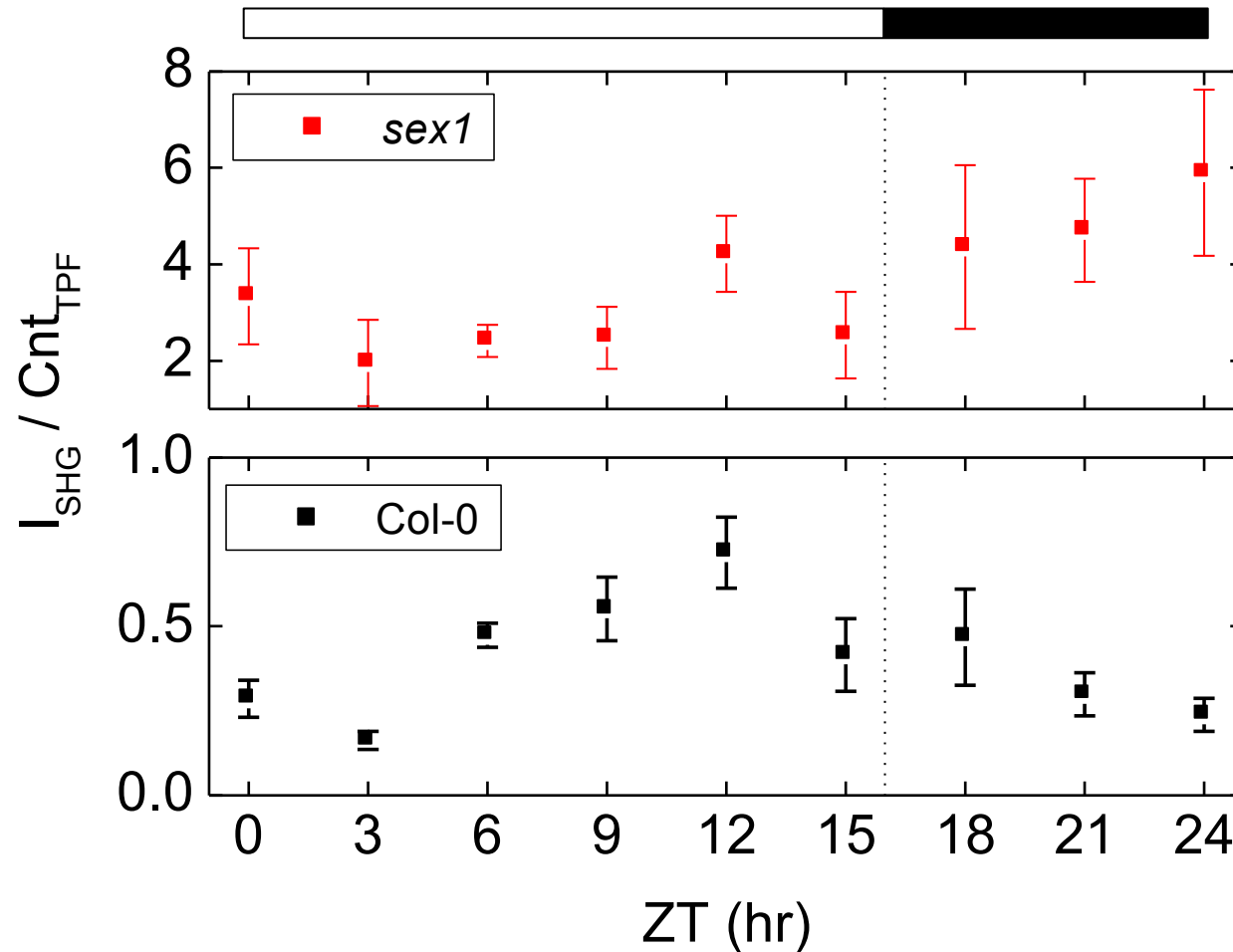
24 Hr Starch Content Variation

$$\frac{I_{SHG}}{Cnt_{TPF}} = \frac{Summed\ SHG\ intensity}{Number\ of\ TPF\ Pixels\ above\ threshold}$$

Conclusion

- Two photon fluorescence (TPF) and second harmonic generation (SHG) imaging allows quantification of leaf starch content

- Application to two lines of Arabidopsis leaf over 24 hours show similar results as previous methods

- The method provide a nondestructive method to monitor leaf starch content