Dynamic contrast for plant phenotyping

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1 Illumination conditions for imaging plant leaves with Speed OPIOM

Speed OPIOM exploits resonant sinusoidally modulated dual illumination at two wavelengths $\lambda_1$ and $\lambda_2$. At resonance, the average light intensities $I_1^0$ and $I_2^0$ and angular frequency $\omega$
of the modulated lights should fulfill the conditions given in Eqs. (S1, S2)

\[(\sigma_{12,1} + \sigma_{21,1}) I_1^0 = (\sigma_{12,2} + \sigma_{21,2}) I_2^0 \quad (S1)\]

\[\omega = 2 (\sigma_{12,1} + \sigma_{21,1}) I_1^0. \quad (S2)\]

which involve the photoswitching cross sections at \(\lambda_1\) and \(\lambda_2\), \(\sigma_{12,1}\), \(\sigma_{12,2}\), \(\sigma_{21,1}\), and \(\sigma_{21,2}\), between the states 1 and 2 of the reversibly photoswitching fluorophores. The values of \(\sigma_{12,480} + \sigma_{21,480}\) and \(\sigma_{12,405} + \sigma_{21,405}\) for the RSFPs investigated in this work have been already reported.\(^\text{S1}\)

The plant tissues are optically more complicated environment than transparent solutions. Hence the application of Speed OPIOM deserves a preliminary specific radiance analysis.

1.1 Decay of light intensity through living leaves

The interaction of light with plant leaves depends on the chemical and physical properties of the leaves. Generally two main factors contribute to the decay of light through leaves: light absorption of the pigments (chlorophyll and carotenoids) and light diffusion by the tissue structure. To evaluate the light penetration through the leaf, we used the PROSPECT model, which has been developed to analyze the spectral transmission by \textit{Arabidopsis thaliana}.\(^\text{S2}\) Using representative leaf parameters extracted from literature,\(^\text{S3–S6}\) the transmission through the total leaf is 14 % at \(\lambda_{\text{fluo}} = 525\) nm, 1.17 % at \(\lambda_1 = 480\) nm and 0.2 % at \(\lambda_2 = 405\) nm, which are the emission wavelength and the excitation wavelengths to be used to photoswitch the RSFPs.

We then assumed that the light decay through the leaf was exponential as a function of the depth (see Eq. S3)

\[I = I_0 10^{-\frac{z}{\delta}} \quad (S3)\]
where $\delta_\lambda$ is the typical penetration depth at wavelength $\lambda$ and $I_0$ is the light intensity on the leaf surface. Considering that the typical thickness of the leaves is 220 $\mu$m, we could first derive $\delta_{525} = 258 \mu$m, $\delta_{480} = 114 \mu$m and $\delta_{405} = 85 \mu$m. Then we could deduce the length $z_\lambda$ over which the light intensity decays by a factor 10 since $z_\lambda = \delta_\lambda$. These values agree with the measurements reported in literature.$^{S7,S8}$

### 1.2 Fluorescence emission from leaves

We take into account the leaf tissue environment, through which the excitation light decreases rapidly. The light absorption by the fluorescent protein through a thin layer $dz$ is

$$dI_{\text{abs}}(z) = -dI_{\text{ex}}(z) = I_{\text{ex}}(z)(1 - 10^{-\varepsilon c dz}) \approx \varepsilon c I_{\text{ex}}(z) \ln 10 \, dz \quad (S4)$$

The emitted fluorescence from the deepest layers also suffers from light reabsorption by the tissue before being finally detected at the leaf surface. Hence the total detected fluorescence intensity at the leaf surface $I_{\text{em}}$ is:

$$I_{\text{em}} = \int_0^{\delta_{\text{leaf}}} \Phi_F dI_{\text{abs}} 10^{-z/\delta_{525\text{nm}}}$$

$$= \Phi_F \varepsilon c I_0 \ln 10 \int_0^{\delta_{\text{leaf}}} 10^{-z/\delta_{480\text{nm}}} \cdot 10^{-z/\delta_{525\text{nm}}} \, dz \quad (S5)$$

$$= \Phi_F \varepsilon c I_0 \delta_\gamma (1 - 10^{-\frac{\delta_{\text{leaf}}}{\delta_\gamma}}) \quad (S6)$$

When $\delta_{\text{leaf}} \gg \delta_\gamma$, Eq.(S7) yields Eq.(S8)

$$I_{\text{em}} = \Phi_F \varepsilon c I_0 \delta_\gamma = \Phi_F \varepsilon c \delta_{\text{eq}} I_0 \ln 10 \quad (S8)$$

where $\delta_\gamma = \frac{\delta_{480} \delta_{525}}{\delta_{480} + \delta_{525}} = 79 \mu$m is related to the absorption property of the light through the tissue.

Hence when the thickness of the leaf $\delta_{\text{leaf}}$ is higher than $\delta_\gamma$ (which is always the case),
the actual leaf thickness has little influence on the fluorescence intensity detected at the surface. In another word, the surface fluorescence intensity is exclusively related to the surface concentration of the fluorophore in the leaf.

1.3 Speed OPIOM signal from living leaves upon modulated resonant dual illumination

In Speed OPIOM on green RSFPs, 480 and 405 nm excitation wavelengths are used (respectively denoted by the subscript 1 and 2 in the following). During this work, we fixed illumination to fulfill the resonance conditions at the leaf surface. However, since the light intensity decreases through penetrating the leaf, the resonance conditions cannot be met anymore below the leaf surface.

Since the transmission of UV and blue lights by leaves are very close (vide supra), we assumed that the first resonance condition (S1) was satisfied within $\delta_\gamma$. In contrast, the second resonance condition would be violated when going deep into the leaf due to the decays of the light intensities. This violation results in a drop of the Speed OPIOM signal, which can be evaluated from the expression of the Speed OPIOM response given in Eqs.(97) and (100) of the Supporting Information from reference.\textsuperscript{S1} It yields

$$I_{F}^{OPIOM} = \int_{0}^{\delta_{\text{leaf}}} \Phi_F dI_{abs} 10^{-z/\delta_{25nm}} \frac{I_0/I(z)}{1 + (I_0/I(z))^2}$$

$$= \Phi_F \varepsilon c I_0 \ln 10 \int_{0}^{\delta_{\text{leaf}}} 10^{-\frac{z}{\delta_{480nm}}} \frac{1}{10^{\delta_{480nm}} + 10^{-\delta_{480nm}}} dz$$

The term $\frac{1}{10^{\delta_{480nm}} + 10^{-\delta_{480nm}}}$ decreases from 0.5 to around 0.2 from the surface to the depth $\delta_\gamma$ beyond which essentially no further fluorescence signal is collected. Hence Eq.(S10) shows that the Speed OPIOM is between 0.2 and 0.5 times lower than the Pre-OPIOM signal (instead of 0.5 times lower in the case of a transparent solution).
1.4 Implementation of Speed OPIOM to observe leaves under the sunlight

The fluorescence emission from the leaf surface being isotropic, the illuminated leaf surface can be considered as a lambertian source: $L_{em} = \frac{F_{em}}{\pi S_{em}} = \frac{I_{em}}{\pi}$. According to Eq. (S7), we estimate the fluorescence emission from the leaf surface to be in the $I_{em} \sim 1 \mu W/mm^2$ range at typical $c = 10^{-6}$M RSFP concentration. The typical irradiation of the sunlight on Earth surface is $I_\lambda = 1.3W/m^2/nm$ around 525 nm, which is the wavelength of the fluorescence emission. We take the bandwidth of the fluorescence emission as $\Delta \lambda = 40$ nm. Thus the solar energy reflected by the leaf surface within the bandwidth of fluorescence emission is estimated as $I_{sun} = R \cdot I_\lambda \Delta \lambda \sim 6 \mu W/mm^2$, where $R=12\%$ is the reflectance of the leaf for the green light. From this derivation, we conclude that the signal from the reflection of sunlight on the leaf is higher than the fluorescence signal from the leaf. However it remains at the same level, which makes it possible to extract the fluorescence signal even under sunlight by means of Speed OPIOM.\textsuperscript{S1}

1.5 Speed OPIOM acquisition parameters used for the images
Table S1: Acquisition parameters used for all the images shown in this article. $f_s$ and $f_m$ respectively refer to the sampling rate and to the modulation frequency of the excitation lights.

| Figure  | Periods | $f_s$ (Hz) | $f_m$ (Hz) | $I_1^u(\lambda_{480nm})$ Ein.m$^{-2}.s^{-1}$ | $I_2^u(\lambda_{405nm})$ Ein.m$^{-2}.s^{-1}$ | $I_3^u(\lambda_{550nm})$ Ein.m$^{-2}.s^{-1}$ | Lighting condition |
|---------|---------|------------|------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-------------------|
| 1a–c    | 8       | 50         | 2.5        | -                                             | -                                             | -                                             | On Outdoor        |
| 1d–f    | 8       | 50         | 2.5        | $4.0 \times 10^{-2}$                         | $1.9 \times 10^{-2}$                         | -                                             | Outdoor           |
| 1g–l    | 8       | 10         | 1          | $1.5 \times 10^{-2}$                         | $8.8 \times 10^{-3}$                         | -                                             | dark              |
| 2a,b,d,e,g,h | 8       | 50         | 2.5        | $4.0 \times 10^{-2}$                         | $1.9 \times 10^{-2}$                         | -                                             | dark              |
| 2c,f,i  | 8       | 50         | 2.5        | -                                             | -                                             | -                                             | On dark           |
| 5a–l    | 8       | 50         | 2.5        | $4.0 \times 10^{-2}$                         | $1.9 \times 10^{-2}$                         | -                                             | lab light         |
| S1a–f   | 8       | 10         | 1          | $1.5 \times 10^{-2}$                         | $8.8 \times 10^{-3}$                         | -                                             | dark              |
| S2a,e   | 8       | 50         | 2.5        | $4.0 \times 10^{-2}$                         | $1.9 \times 10^{-2}$                         | -                                             | dark              |
| S2b,f   | 3       | 50         | 0.5        | $5.3 \times 10^{-2}$                         | $4.7 \times 10^{-3}$                         | -                                             | dark              |
| S2c,g   | 4       | 40         | 0.5        | $4.4 \times 10^{-2}$                         | $2.4 \times 10^{-3}$                         | -                                             | dark              |
| S2d,h   | 4       | 20         | 0.5        | $4.3 \times 10^{-2}$                         | $1.6 \times 10^{-2}$                         | -                                             | dark              |
| S3a,b,d,e,g,h | 8       | 50         | 2.5        | $4.0 \times 10^{-2}$                         | $1.9 \times 10^{-2}$                         | -                                             | dark              |
| S3c,f,i | 8       | 50         | 2.5        | -                                             | -                                             | -                                             | On dark           |
2 Supplementary figures

Figure S1: Selective Speed OPIOM epifluorescence microscopy image of Dronpa-2 against EGFP in *Nicotiana benthamiana* leaves. Pre-OPIOM (a–c) and Speed OPIOM (d–f) images of constructs expressing Dronpa-2 and/or EGFP (a,d: PAS1-EGFP and Fbr-Dronpa-2; b,e: Fbr-Dronpa-2; c,f: PAS1-EGFP). *Scaling bars*: 50 µm.
Figure S2: Selective Speed OPIOM imaging of RSFPs against autofluorescence in *Nicotiana benthamiana* leaves. Pre-OPIOM (a-d) and Speed OPIOM (e-h) images of constructs expressing RSFPs under the control of the 35S promoter (a,e: Dronpa-2; b,f: Dronpa-3; c,g: rsFastlime; d,h: Padron). The plant leaves have been imaged in the dark with the Speed OPIOM macroscope by setting the parameters of excitation to resonance for each RSFP (see Table S1). As expected, Speed OPIOM could selectively retrieve the fluorescence emission of the four RSFPs without any interference from autofluorescence. Interestingly, Dronpa-2, Dronpa-3, and rsFastlime on one hand, and Padron on the other hand exhibit Speed OPIOM observables of opposite signs due to their opposite change of brightness upon light-induced photoswitching. Images acquired in the dark. *Scaling bars*: 1 mm.
Figure S3: Fluorescence imaging of *Nicotiana benthamiana* leaves transiently expressing Dronpa-2 (imaged with Pre-OPIOM and Speed OPIOM in the 480+405 nm/525 nm channel) and DsRed (imaged with in-phase fluorescence imaging in the 550 nm/585 nm channel) constructs. Promoters: p35S (a-c), pACTIN2 (d-f), pNOS (g-i). Beyond evidencing that Speed OPIOM efficiently overcomes the interference of autofluorescence, the main conclusion of this investigation is that the level of fluorescence is significantly higher with the p35S:DRONPA2 construct than with the pACTIN2:DRONPA-2 and pNOS:DRONPA2 ones as expected from the drop of the promoter strength from p35S to pNOS. Images acquired in the dark. *Scaling bars*: 1 mm. Illumination conditions: see Table S1.
Figure S4: Correlations of the Speed OPIOM signals of Dronpa-2 and in phase fluorescence signals of DsRed collected at each pixel of the image of a Camelina sativa leaf permanently expressing Dronpa-2 (a) and DsRed (b) under the control of the p35S promoter (c). The slope of the linear fitting (0.0325) is close to the ratio of the integrated signals over the whole leaf surface, which equals to 0.0317. Images acquired in the dark. Scaling bars: 1 mm. Illumination conditions: see Table S1.
Figure S5: Signals of *Nicotiana benthamiana* leaves from different imaging channels. In-phase DsRed signal (a) from the images collected at 585 nm with 550 nm illumination, and Pre-OPIOM (b) and Speed OPIOM (c) signals from the images collected at 525 nm with 480/405 nm illumination, for the three constructs p35S, pACTIN2 and pNOS. Signals have been extracted from integration over the whole surface of the leaf. 16 samples have been measured for each promoter construct.

References

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