Rate of excitation PER MOLECULE

Increases with the **n—th power of the average power**

Depends **linearly** from the nPE action $\sigma^{(n)}$

$$k_{exc} = \frac{\sigma^{(n)}}{d_c^{n-1}} \left(\frac{< P >}{hc\lambda} \right)^n NA^{2n}$$

It depends **inversely** on the duty cycle

$$d_c = \tau_p f_R$$

It depends on a **high power of the NA**

Fluorecence signal

Increases with the **n—th power of the average power**

- Uniform density of fluorophores, ρ
- $V_{exc} \simeq \pi w_0 z_R = \frac{\pi w_0^4}{\lambda} \propto \frac{\pi \lambda^3}{NA^4}$

Depends **linearly** from the nPE action $\sigma^{(n)}$

$$\mathrm{k}_{exc} = \pi \frac{\Phi_F \sigma^{(n)}}{d_c^{n-1}} \left(\frac{< P >}{hc} \right)^n \left(\frac{NA^{2n-4} \rho}{\lambda^{n-3}} \right)$$

It depends **inversely** on the duty cycle

$$d_c = \tau_p f_R$$

It depends on a **high power of the NA**

For n= 2: it does NOT depend on NA

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FULL ARTICLE

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Two- and three-photon absorption cross-sec for high-brightness, cell-specific multiphoto imaging

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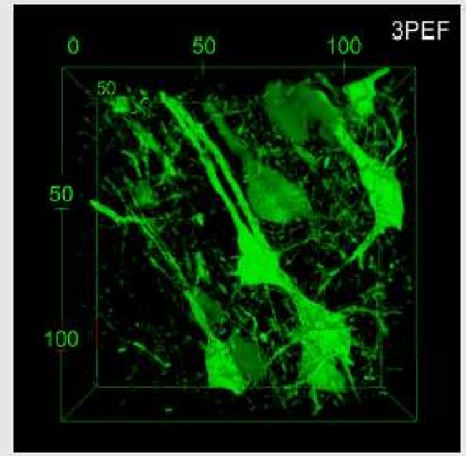
$\Phi\sigma^{(3)} \simeq 3 \cdot 10^{-81} cm^6 s$ for Rhodamine 6G

Abstract

We demonstrate an accurate quantitative characterization of absolute two- and three-photon absorption (2PA and 3PA) action cross sections of a genetically encodable fluorescent marker Sypher3s. Both 2PA and 3PA action cross sections of this marker are found to be remarkably high, enabling high-brightness, cell-specific two- and three-photon fluorescence brain imaging. Brain imaging experiments on sliced samples of rat's cortical areas are presented to demonstrate these imaging modalities. The 2PA action cross section of Sypher3s is shown to be highly sensitive to the level of pH, enabling pH measurements via a ratiometric readout of the two-photon fluorescence with two laser excitation wavelengths, thus paving the way toward fast optical pH sensing in deep-tissue experiments.



KEYWORDS
brain imaging, fluorescent biosensors, three-photon microscopy, two-photon microscopy

3PE efficiency

$$k_{exc}^{(3)} \simeq d_c \sigma^{(n)} \left[\frac{P_{ave}}{hc\lambda d_c}\right]^3$$

$d_c \simeq 1MHz \cdot 20fs \simeq 2 \cdot 10^{-8}$

$\lambda \simeq 1.3\ \mu m$

$P_{ave} \simeq 50mW$

$$k_{exc}^{(3)} \simeq \frac{\sigma^{(3)}}{(d_c\ )^2}\left[\frac{P_{ave}}{hc\lambda}\right]^3$$

$\Phi\sigma^{(3)} \simeq 3 \cdot 10^{-9}\ \ m^6 s$ for Rhodamine 6G

$$\frac{P_{ave}}{hc\lambda d_C} \simeq 10^{34}\left[\frac{\mathrm{ph}}{\mathrm{s} * m^2}\right]$$

$$k_{exc}^{(2)} \simeq \sigma^{(3)} d_c \left[\frac{P_{ave}}{hc\lambda d_C}\right]^3 \simeq 3 \cdot 10^{-93} * 1e^{-5} * [10^{34}]^3$$

$k_{exc}^{(3)} \simeq 10^4\ s^{-1}$ = 10 kHz

Per molecule, 100% efficiency

Two-photon excitation fluorescence scaling law

$$< F > = \eta\sigma_2 I_p^2\, d_c = \eta\sigma_2 \frac{<I>^2}{d_C} = \eta\sigma_2 \frac{<P>^2}{d_C (h\nu)^2 A^2} \qquad (1)$$

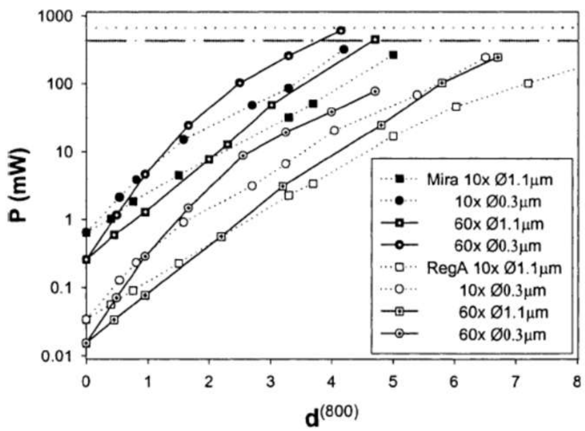


Fig. 4. Average excitation power necessary to detect a TPEF signal of 10^6 photocounts/s, as a function of penetration depth normalized to the medium scattering length $l_s^{(ex)}$. Measurements were obtained with (RegA) and without (Mira) regenerative amplification of the excitation beam, with two different objectives and two scatterer sizes. Dotted and dashed lines correspond to maximum average powers available from Mira and RegA lasers respectively.

$$< P > (z) = P_0 e^{-z/l_{scat}}$$

$$A = \pi w_o^2 \qquad (2)$$

Quantum yield=1

$\eta \simeq 0.05$ collection efficiency

(1)+(2) and logarithm...

$$\boxed{z_{max} = l_{scat} ln\left(\frac{P_0\gamma}{\sqrt{d_C}}\right)}$$

where $\gamma = \frac{1}{A(h\nu)}\sqrt{\frac{\eta\sigma_2}{< F >}}$

Input

$$\sigma_2 \simeq 200\ GM = 200 \times 10^{-50} cm^4 s$$

Theer, Denk. On the fundamental imaging-depth limit in two-photon microscopy. 2006

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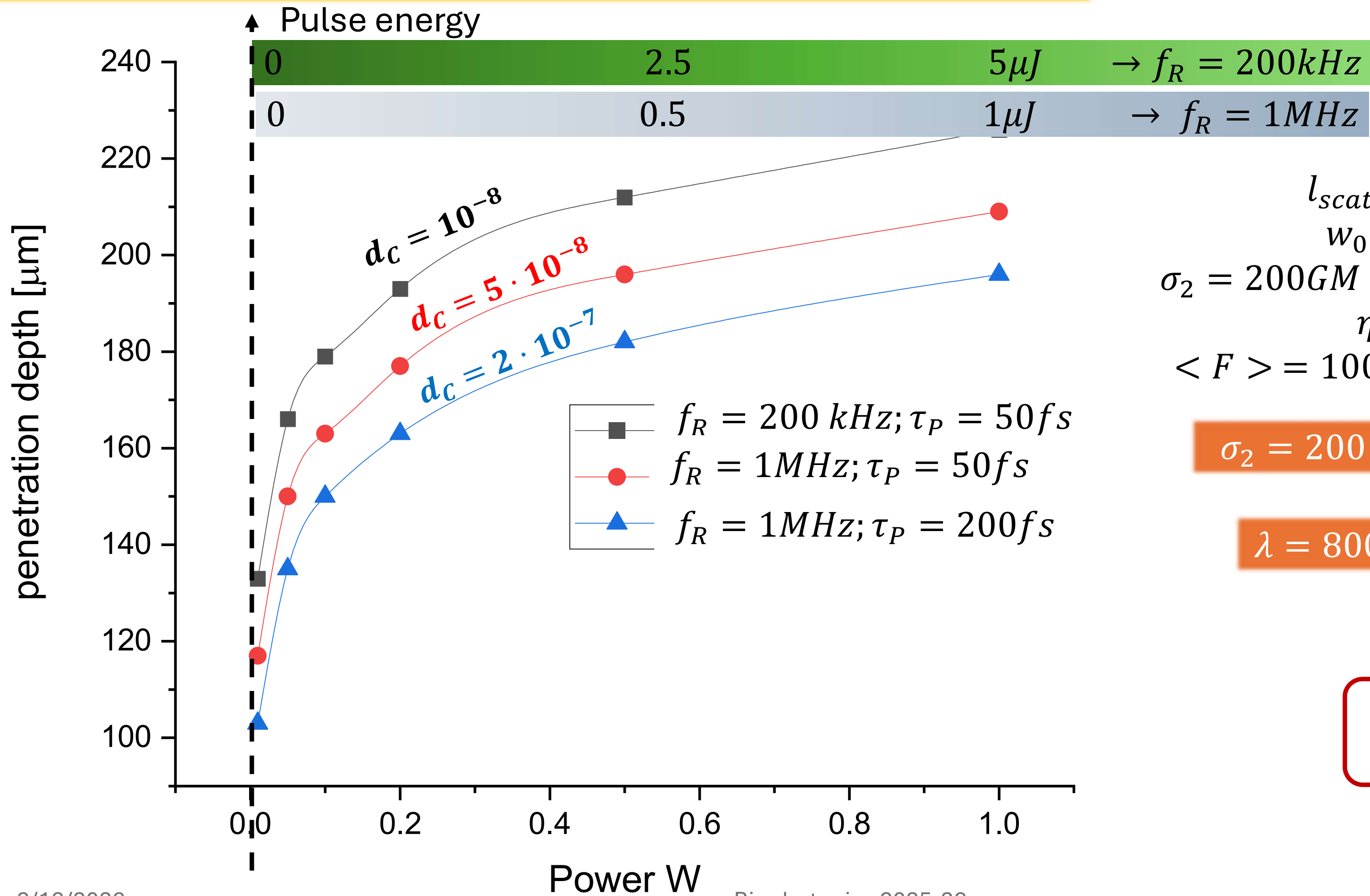
Penetration length as a function of the power at the entrance pupil

$$z_{max} = l_{scat} ln\left(\frac{< P > \gamma}{\sqrt{d_C}}\right)$$



$l_{scat} = 200\ \mu m$

$w_0 = 0.4\ \mu m$

$\sigma_2 = 200GM = 200 \times 10^{-50} cm^4 s$

$\eta = 0.05$

$< F > = 1000\ Hz$ *per molecule*

$\sigma_2 = 200\ GM = 2 \times 10^{-56} m^4 s$

$\lambda = 800nm$

Pulse energy = P / f_R

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Out-of-focus background limitation – 2 photons

$$\frac{S}{B} = \frac{2\pi(\mathrm{NA})^2}{\lambda n l_s} z^2 \exp(-2z/l_s),$$

We want to have at minimum $\frac{S}{B} = 1$

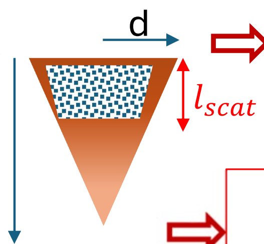
$C_D = dye\ concentration;$
$V = irradiated\ volume$

$V_{out} \simeq a l_{scat}$

Contribution of the scattering of the fluorescence arising from the surface

Illumination area

$a \simeq \frac{\pi d^2}{4} = \frac{\pi z^2 NA^2}{4n^2}$



$$B \approx \frac{P_0^2}{a^2} \sigma^{(2)} C_D V_{out} = P_0^2 \frac{l_{scat}}{a} \sigma^{(2)} C_D$$

Illumination area $\propto w_0^2$

$$S \approx \frac{P_0^2 \sigma^{(2)} C_D e^{-\frac{2z}{l_{scat}}}}{w_0^4} V_{in} = \propto \frac{P_0^2}{\lambda} \sigma^{(2)} C_D \exp\left[-\frac{2z}{l_{scat}}\right]$$

$$V_{in} = \frac{\pi w_0^2}{4} \pi \frac{z_R}{2} = \frac{\pi^2}{8} \frac{w_0^4}{\lambda}$$

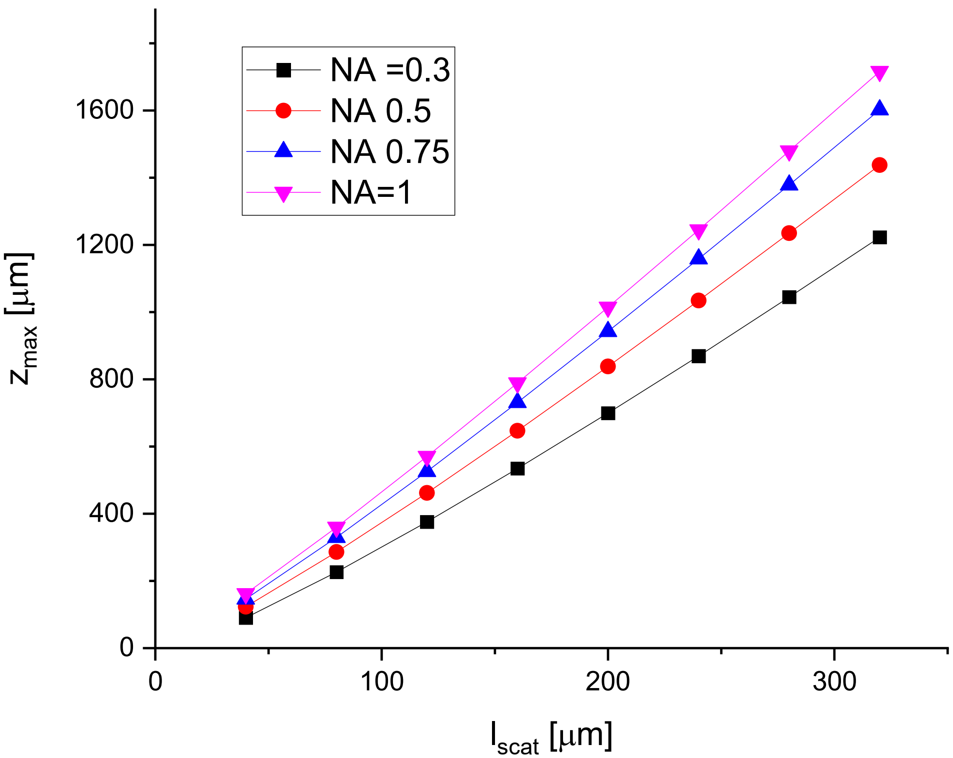
$$\frac{S}{B} \approx \frac{P_0^2 e^{-\frac{2z}{l_{scat}}} \sigma^{(2)} C_D}{\lambda P_0^2 l_{scat} \sigma^{(2)} C_D} a$$

$$\boxed{\frac{S}{B} \approx \frac{e^{-\frac{2z}{l_{scat}}}}{\lambda l_{scat}} \frac{z^2 NA^2}{n^2}}$$

Numerical inversion

Out-of-focus background limitation – 2 photons

Assume $\frac{S}{B} = 1$



$$\frac{S}{B} = \frac{2\pi(\mathrm{NA})^2}{\lambda n l_s} z^2 \exp(-2z/l_s),$$

Contribution of the scattering of the fluorescence arising from the surface with respect to the perifocal fluorescence

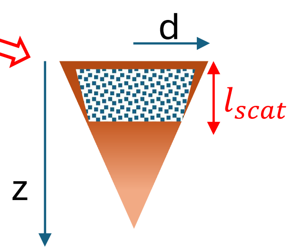
Out of focus contribution

changes with the order

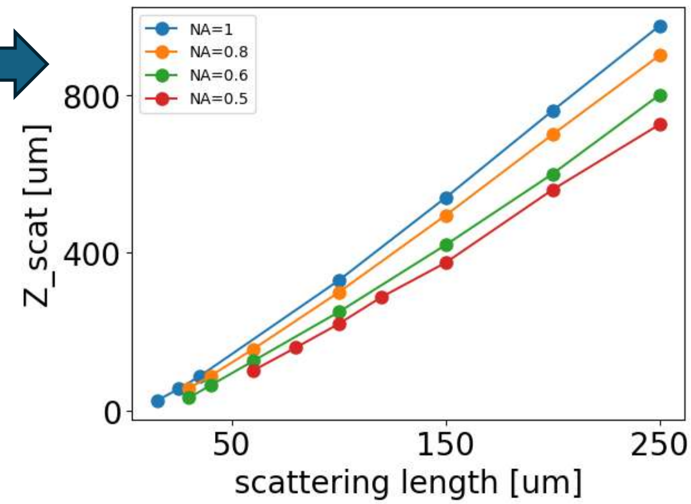
of non-linearity – two and three photons exc.

Illumination area

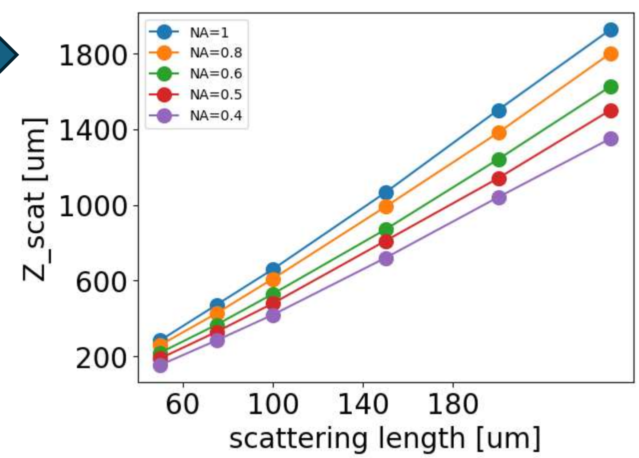
$$a \simeq \frac{\pi d^2}{4} = \frac{\pi z^2 NA^2}{4n^2}$$



Illumination area $\propto w_0^2$

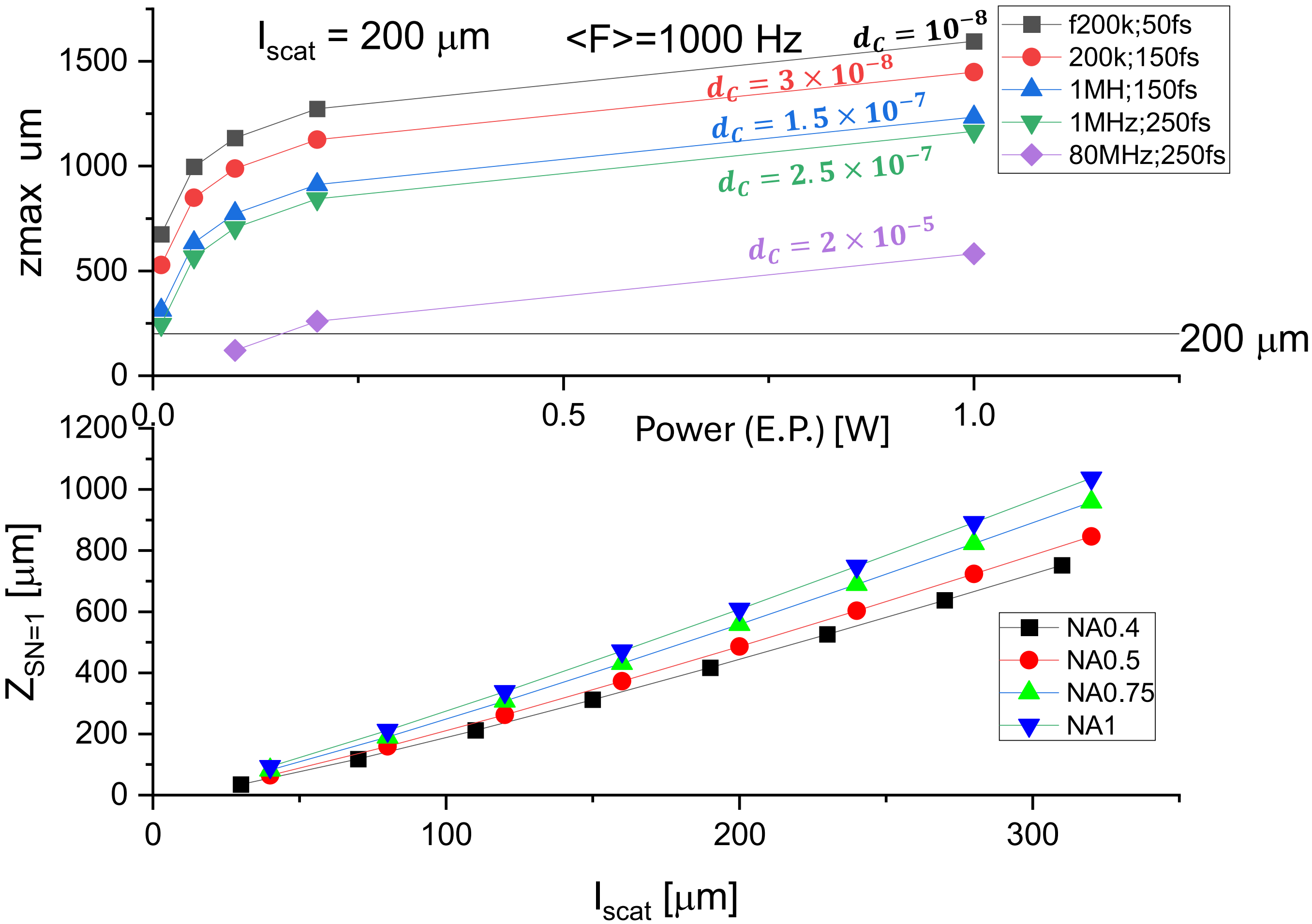


2PE



3PE

3 photons excitation, penetration length



$\sigma_3 = 10^{-94} m^6 s^2$

$\lambda = 1300nm$

$$z_{max} = l_{scat} ln\left(\frac{< P > \gamma}{(d_c)^{2/3}}\right)$$

where $\gamma = \frac{1}{A(h\nu)}\left(\frac{\eta\sigma_3}{< F >}\right)^{1/3}$

$A = \pi w_0^2$

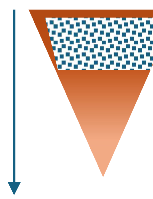
Out-of-focus background limitation – 3 photons

We want to have at minimum $\frac{S}{B} = 1$

$C_D = dye\ concentration;$
$V = irradiated\ volume$

Illumination area

$$a \simeq \frac{\pi d^2}{4} = \frac{\pi z^2 NA^2}{4n^2}$$

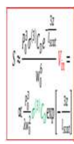


$$V_{out} \simeq a l_{scat}$$

$$B \approx \frac{P_0^3}{a^3}\sigma^{(3)}\, C_D V_{out} = P_0^3 \frac{l_{scat}}{a^2}\sigma^{(3)}\, C_D$$

Contribution of the scattering of the fluorescence arising from the surface

Illumination area $\propto w_0^2$



$$S \approx \frac{P_0^3 \sigma^{(3)} C_D e^{-\frac{3z}{l_{scat}}}}{w_0^6} V_{in} = \propto \frac{P_0^3}{\lambda w_0^2}\sigma^{(3)} C_D \exp\left[-\frac{3z}{l_{scat}}\right]$$

$$V_{in} = \frac{\pi w_0^2}{4}\pi\frac{z_R}{2} = \frac{\pi^2}{8}\frac{w_0^4}{\lambda}$$

$$\frac{S}{B} \approx \frac{P_0^3 e^{-\frac{3z}{l_{scat}}}\sigma^{(3)}\, C_D}{\lambda w_0^2 P_0^3 l_{scat}\sigma^{(3)}\, C_D} a^2$$

$$\boxed{\frac{S}{B} \approx \frac{e^{-\frac{3z}{l_{scat}}}}{\lambda l_{scat}}\left(\frac{a}{w_0}\right)^2 = \frac{e^{-\frac{3z}{l_{scat}}}}{\lambda l_{scat}}\left(\frac{\pi z^2 NA^2}{4n^2 w_0}\right)^2}$$

Numerical inversion