Electronic Supplementary Material (ESI) for Lab on a Chip.

High-throughput Time-Correlated Single Photon Counting
Supplementary Information

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1 Instrument response function

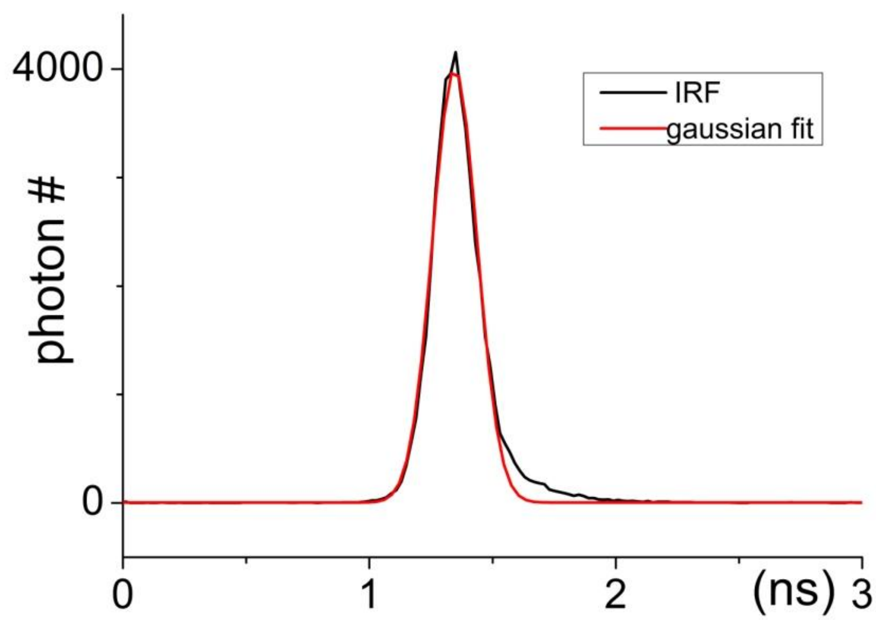

Figure 1: Instrument Response Function of the TCSPC set-up, and fit by a gaussian function yielding a 200-ps FWHM.

Figure 1 displays the instrument response function (IRF) of the TCSPC set-up described in the paper. The IRF is recorded by removing the notch filter, so as to detect the reflection of the laser excitation pulses on the microfluidic chip. The measured IRF results from the convolution of the response functions of the SPAD, specified to be 40 ps FWHM by the manufacturer, of the acquisition system and of the laser pulse duration.

Here, the measured 200-ps FHWM is essentially representative of the laser pulse duration. The low-amplitude wing of the IRF function is attributed to spontaneous emission by the laser diode, following the main laser emission pulse.

2 Reference fluorescence decay kinetics of fluorescein in PBS buffer solution

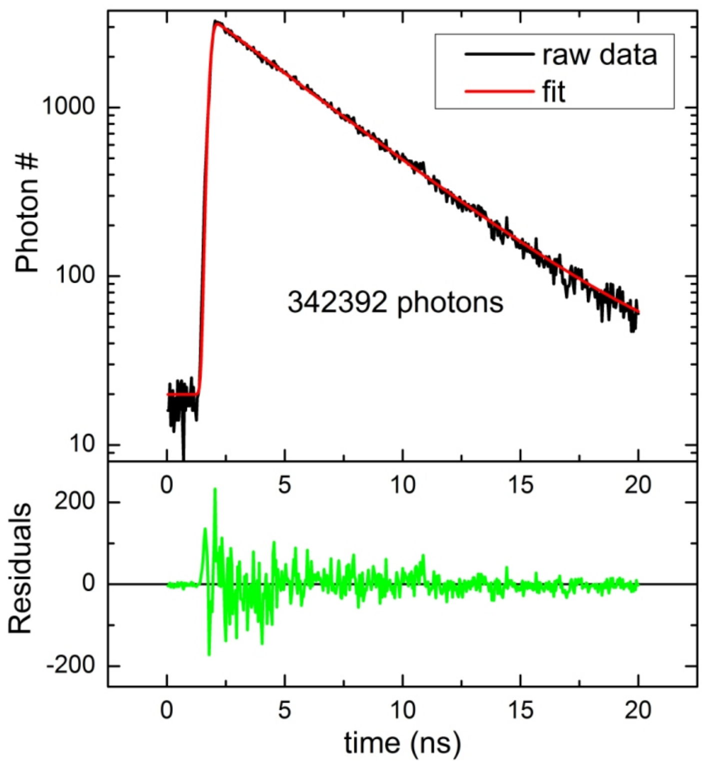

Figure 2: Reference fluorescence decay curve of fluorescein dissolved in PBS, as measured with a streak camera in photon counting mode (black line), with a monoexponential fit (in red) and the fit residuals (in green).

Figure 2 displays the fluorescence decay curve of fluorescein in PBS in a quartz fluorescence cuvette measured with a streak camera (C10627 streak tube from Hamamatsu Photonics) in the photon counting mode. The excitation light source is a femtosecond laser pulse with a low repetition rate of 50 kHz, tuned at 515 nm. The fitting function described below is used and yields a Fluorescence Life Time (FLT) of $\tau = 4.13 \pm 0.02$ ns, with feature-less residuals, evidencing a perfectly monoexponential decay.

3 Fluorescence decay fitting functions

3.1 Multiparameter function

In the above section, as well as in Figure 2 of the paper, we use a non-linear, least-square minimization method to fit the fluorescence decay traces with a monoexponential decaying function starting at $t = t_0$, $H(t - t_0) \times A \exp(-(t - t_0)/\tau)$, and convolved with a normalized gaussian function of standard deviation σ to account for the Instrument Response Function (IRF). Here $H(t - t_0)$ is the Heaviside step function. The convolution may be written analytically, yielding the following fitting function:

$$G(t) = C + \frac{A}{2} \exp\left(\frac{\sigma^2}{2\tau^2}\right) \exp\left(-\frac{t - t_0}{\tau}\right) \times \left[1 + \mathrm{erf}\left(\frac{t - t_0 - \sigma^2/\tau}{\sigma\sqrt{2}}\right)\right], \qquad (1)$$

where erf is the error function resulting from the convolution of the Heaviside function by a gaussian function, and C accounts for a possible constant offset. In total this yields up to 5 fitting parameters among which C, σ, and t_0 may be kept fixed after the prior fitting of the instrument response function, reducing the effective fitting parameters to only 2.

In the case where the observation time window (20 ns $= 1/k_L$) is not large enough compared to the FLT, the signal rises on top of the tail of the fluorescence decay induced by the preceding laser pulse. This is the case for instance in Figure 2A of the paper. We may easily account for that by replacing the constant offset C by the tail of the same exponential function, advanced by 20 ns: $A \exp(-(t - t_0 + 1/k_L)/\tau)$ (with A the same coefficient as in $G(t)$), as was done in the fit of Figure 2A in the paper.

3.2 Single parameter fitting function and Maximum Likelihood estimate

For individual droplets or group of droplets (Figure 3 of the paper), the number of photons in the fluorescence decay histogram is reduced. In order to minimize the uncertainty on the corresponding FLT's, we rather implement the 1-parameter fitting procedure based on the Maximum Likelihood method introduced by Hall & Selinger (1981) (see the References section in the paper).

We define τ the FLT, N the total number of counts in the histogram, b the histogram channel bin width (b=100ps in Figure 3 of the paper), and T the observation time window (i.e. the inverse laser repetition rate, here $T = (50\text{MHz})^{-1} = 20$ ns). Following Hall & Selinger, the Maximum Likelihood (ML) estimator of τ is given by the solution of:

$$1 + (e^{b/\tau} - 1)^{-1} - m(e^{T/\tau} - 1)^{-1} = \frac{\sum_1^m iN_i}{N}, \tag{2}$$

where i is the histogram bin index and $m = T/b$ is the number of channels in the histogram. To solve Eq. 2 we implement a Newton-Raphson algorithm. We note here that as soon as $T >\sim 5\tau$, the left-hand side of Eq. 2 is directly equal to τ.

Hall & Selinger further give the expression for the variance σ_τ^2 of the Maximum Likelihood estimator of τ:

$$\sigma_\tau^2 \quad = \quad \frac{1}{N}\frac{\tau^4}{b^2}\left(\frac{e^{b/\tau}}{(e^{b/\tau}-1)^2} - \frac{(T/b)^2\ e^{T/\tau}}{\left(e^{T/\tau}-1\right)^2}\right)^{-1}. \tag{3}$$

Hence, the estimated value of τ is expected to deviate from the exact value by a relative rms standard error of:

$$\frac{\sigma_\tau}{\tau} = \frac{1}{\sqrt{N}}\left(\frac{(b/\tau)^2 e^{b/\tau}}{(e^{b/\tau}-1)^2} - \frac{(T/\tau)^2\ e^{T/\tau}}{\left(e^{T/\tau}-1\right)^2}\right)^{-1/2}, \tag{4}$$

We note that :

$$\text{for } x \to 0 \qquad f(x) = \frac{x^2\ e^x}{(e^x-1)^2} = 1 - \frac{x^2}{12} + o(x^2) \tag{5}$$

$$\text{for } x \to \infty \qquad f(x) = \frac{x^2\ e^x}{(e^x-1)^2} \simeq x^2 e^{-x} \to 0 \tag{6}$$

We conclude that in usual conditions ($b < \tau < T$), we can make the following approximation:

$$\frac{\sigma_\tau}{\tau} \quad \simeq \quad \frac{1}{\sqrt{N}}\left(1 + \frac{(b/\tau)^2}{24} + \frac{(T/\tau)^2\ e^{-T/\tau}}{2}\right), \tag{7}$$

$$\frac{\sigma_\tau}{\tau} \quad \simeq \quad \frac{1}{\sqrt{N}} \quad \text{for} \quad b \ll \tau \ll T. \tag{8}$$

We note that as soon as $b < \tau/2$:

$$\frac{\sigma}{\tau} \quad \simeq \quad \frac{1.5}{\sqrt{N}} \quad \text{for} \quad T = 3\tau, \tag{9}$$

$$\frac{\sigma}{\tau} \quad \simeq \quad \frac{1.1}{\sqrt{N}} \quad \text{for} \quad T = 5\tau. \tag{10}$$

4 Pile-up

4.1 Distortion of the fluorescence decay signal

Upon excitation by a single ultrashort laser pulse, the instantaneous photon emission rate $F(t)$ of a solution may be written:

$$F(t) = k_r N^* f(t),$$

with N^* the number of chromophores initially promoted in the excited state, k_r the chromophore radiative decay rate, and $f(t)$ the population in the excited state normalized to unity at t=0.

In a TCSPC experiment, a single detection event is followed by a deadtime which is much longer than the fluorescence decay kinetics $F(t)$. Hence one photon at maximum may be detected per excitation laser pulse. Therefore, the probability to detect 1 photon during the infinitesimal time interval δt at time t after the impulsive laser pulse excitation, is conditioned to the probability that no photon was detected before that time t.

When δt is sufficiently small, the probability to detect 1 photon during δt is $\eta F(t)\delta t$ and the probability to detect more than one photon vanishes, such that the probability to detect no photon during δt is $1 - \eta F(t)\delta t$. Here η is the product of the photon collection efficiency and of the photodetector quantum yield. We now introduce $P_0(t)$, the probability to detect 0 photon during the finite time interval $[0; t]$, after impulsive excitation at time $t = 0$. Provided successive detection events are uncorrelated, we may write:

$$\begin{aligned} P_0(t + \delta t) &= P_0(t) \times (1 - \eta F(t) \times \delta t) && (11) \\ \text{that is} \quad \frac{P_0(t + \delta t) - P_0(t)}{\delta t} &= -P_0(t)\eta F(t) \quad \text{with } \delta t \to 0 && (12) \\ \text{hence} \quad \frac{dP_0(t)}{dt} &= -P_0(t)\eta F(t) && (13) \\ P_0(t) &= \exp\left(-\eta \int_0^t F(u)du\right) && (14) \end{aligned}$$

Thus, the probability to detect 1 photon during the very small time interval b (binning) at the delay time t after excitation is:

$$P_0(t) \times \eta F(t) b = s(t) \times b,$$

where s(t) is the probability density to detect a photon at time t after a single laser pulse:

$$\boxed{s(t) = k_c f(t) \times \exp\left(-k_c \int_0^t f(u)du\right)} \qquad (15)$$

with $k_c = \eta k_r N^*$ the maximum (at $t = 0$) photon collection rate, as introduced in the paper.

In conditions where the detection dead time is much larger than τ but smaller than the interval between two successive laser pulses: $\tau \ll T_D \ll 1/k_L$, then the TCSPC signal $S(t)$ accumulated after N excitation laser pulses is given by:

$$S(t) = Ns(t). \tag{16}$$

Let us now consider the case of a monoexponential decay, $f(t) = \exp(-t/\tau)$. We may thus write:

$$\int_0^t f(u)du = \int_0^t \exp(-u/\tau)du = \tau(1 - \exp(-t/\tau)), \tag{17}$$

and obtain the result initially derived by Hopzapfel (1974) (see the References section in the paper):

$$s(t) = k_c \exp(-t/\tau) \times \exp\left(-k_c\tau(1 - \exp(-t/\tau))\right) \tag{18}$$

In the case $\alpha = k_c\tau \ll 1$, Eq. 18 may be expanded according to:

$$\begin{aligned} s(t) &= k_c \exp(-t/\tau) \times \exp\left(-\alpha(1 - \exp(-t/\tau))\right) && (19)\\ &\simeq k_c \exp(-t/\tau)\left(1 - \alpha(1 - \exp(-t/\tau))\right) && (20)\\ &= k_c\left((1-\alpha)\exp(-t/\tau) + \alpha\exp(-2t/\tau)\right) && (21) \end{aligned}$$

At the lowest order in α, the pile-up effect thus distorts a monoexponential decay of time constant τ into a biexponential decay with time constants τ (weight $1 - \alpha$) and $\tau/2$ (weight α), such that the average decay time constant τ_d of the detected signal appears to be:

$$\boxed{\tau_d \simeq \tau(1 - \alpha/2)} \tag{22}$$

As an illustration, the function $s(t)/k_c$ given by Eq. 18 is plotted in Figure 3 for $\alpha = 0.2$, together with its monoexponential fit which yields a time constant $\tau_d = 0.912\tau$. The real FLT is thus obtained from the monoexponential fit of $s(t)$ by $\tau = \tau_d/0.912 = 1.096\tau_d$ while the formula 22 predicts $\tau \simeq \tau_d(1 + \alpha/2) = 1.10\tau_d$, thus showing an accuracy of better than 1%.

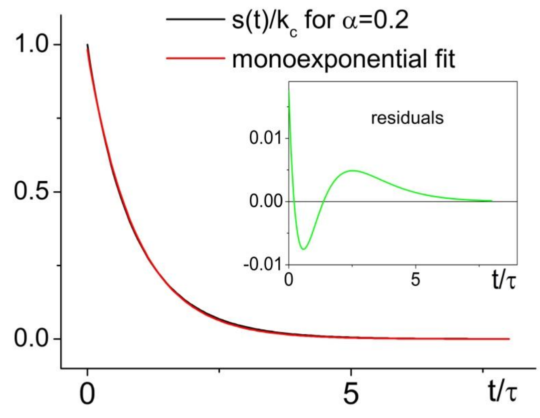

Figure 3: Function $s(t)/k_c$ from Eq. 18 for $\alpha = 0.2$ (black), monoexponential fit (red) and residuals (inset). The result of the fit yields the detected time constant $\tau_d = 0.912\tau$.

4.2 Saturation of the photon detection rate

4.2.1 The case of low laser repetition rate

In the above conditions ($\tau \ll T_D \ll 1/k_L$), the total number N_d of photons effectively detected after N laser pulses is given by:

$$N_d = \int_0^\infty S(t)dt = N \int_0^\infty s(t)dt \tag{23}$$

In the case of a monoexponential fluorescence decay kinetics, we can write (with $\alpha = k_c\tau$):

$$\int_0^\infty s(t)dt = k_c \exp(-\alpha) \int_0^\infty \exp(-t/\tau) \exp(\alpha\ e^{-t/\tau})\ dt \tag{24}$$

$$= k_c \exp(-\alpha) \int_1^0 X\ \exp(\alpha X) \left(-\frac{\tau dX}{X}\right) \tag{25}$$

$$= \alpha \exp(-\alpha) \int_0^1 \exp(\alpha X) dX \tag{26}$$

$$= \exp(-\alpha)[\exp(\alpha X)]_0^1 \tag{27}$$

$$\int_0^\infty s(t)dt = 1 - e^{-\alpha} \tag{28}$$

Finally, the average photon detection rate is given by:

$$\boxed{k_d = k_L \frac{N_d}{N} = k_L \left(1 - e^{-\alpha}\right)} \tag{29}$$

Hence, for increasing values of k_c (e.g. increasing incident laser power, see Beer-Lambert's law below), the effective photon detection rate saturates at $k_d = k_L$, meaning one photon at maximum per laser pulse, due to the photodetector deadtime (pile-up effect).

4.2.2 Beer-Lambert's law

The number of photons in a laser pulse of wavelength λ, average power P, repetition rate k_L is:

$$\frac{P}{k_L} \frac{\lambda}{hc}$$

where P/k_L is the energy per pulse and hc/λ the energy of a single photon. The number N^* of chromophores excited by this pulse equals the number of photons absorbed by the droplet. The Beer-Lambert law gives the fraction of photons which are absorbed in the incident pulse by the droplet as being:

$$1 - 10^{-A} \sim A \ln(10)$$

in the limit where the absorbance of the droplets $A = \varepsilon C_0 L \ll 1$ is very low. Here, C_0 is the chromophore concentration in the droplet, ε the chromophore extinction coefficient at the excitation wavelength, and L the droplet thickness. Because the typical droplet absorbance is very low, $A = \varepsilon C_0 L \ll 1$, the number of absorbed photons is simply proportional to the chromophore concentration.
Hence the photon collection rate which enters in Eq. 15 or 18 is given by:

$$k_c = \eta k_r N^* = \eta k_r \frac{P}{k_L} \frac{\lambda}{hc} A \ln(10). \tag{30}$$

Let us now write $\alpha = k_c \tau$. Eq. 30 gives:

$$\boxed{\alpha = \eta\, Q\, \frac{P}{k_L} \frac{\lambda}{hc} A \ln(10),} \tag{31}$$

where $\tau k_r = Q$ is the fluorescence quantum yield, by definition. Notice, that α is directly proportional to the collection efficiency η, the laser power P, and the chromophore concentration C_0 (since $A = \varepsilon C_0 L$).

In the case where α is sufficiently small, Eq. 29 gives the photon detection rate as:

$$k_d = k_L(1 - e^{-\alpha}) \simeq \alpha k_L = \eta\, Q\, P\frac{\lambda}{hc} A \ln(10). \tag{32}$$

Here we note that in the case of continuous wave ("cw") excitation with same average power P, the number of photons absorbed per time unit would be:

$$P\frac{\lambda}{hc} A \ln(10),$$

such that the number of fluorescence photons detected per time unit would be given by the same formula 32.

4.2.3 The case of high laser repetition rate

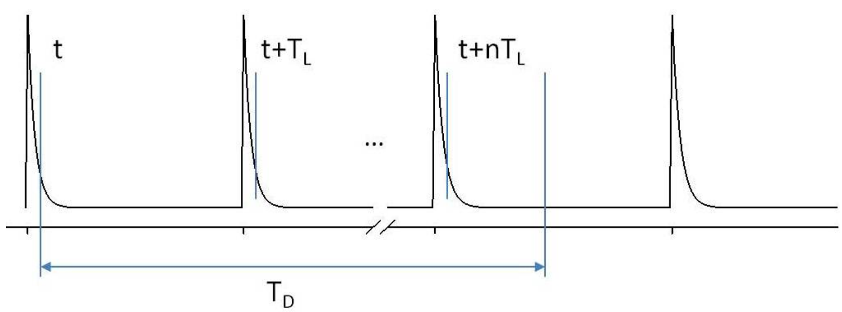

Figure 4: The instantaneous photon emission rate F(t) is periodic with the laser repetition period. A detection event at time t is followed by a detector deadtime of $T_D = n \times T_L + \Delta T$ during which the following n laser pulses cannot yield any detection event.

Consider now the case where the laser repetition rate is increased such that $\tau \ll T_L \leq T_D = nT_L + \Delta T$ (see Fig. 4), as it is in the experimental conditions of the paper.Whenever a photon is detected, the detector becomes blind to any other photon which would be collected as a result of the n following laser pulses occurring during the deadtime. Therefore, if the overall experiment lasts for a period of time T, during which $N = k_L T$ laser pulses have occurred and N_d photons have been detected, there has been N_d periods of time T_D during which the detector was blind, meaning that a number of $N_d \times n$ laser pulses certainly yielded no detection event. Hence the N_d detected photons have been produced by an effective $N - nN_d$ number of laser pulses each yielding an average $\int_0^\infty s(t)dt$ number of detection events, such that:

$$N_d = (N - n\, N_d) \times \int_0^\infty s(t)dt \tag{33}$$

In the end the average photon detection rate is:

$$k_d = \frac{N_d}{T} = (k_L - nk_d) \times \int_0^\infty s(t)dt \tag{34}$$

$$\boxed{k_d = k_L \frac{1 - e^{-\alpha}}{1 + n(1 - e^{-\alpha})}} \tag{35}$$

Using the Beer-Lambert law (Eq. 31), this formula may be rewritten as:

$$\boxed{k_d = k_L \frac{1 - e^{-\beta P}}{1 + n(1 - e^{-\beta P})}} \tag{36}$$

where P is the excitation laser average power. Eq. (36) was used to approximate and fit the dependence of the experimental average photon detection rate k_d with the laser power P, plotted in Figure 4A of the paper. A single-parameter (least-square minimization) fit is performed to infer the value of β (since k_L=50 MHz and $n = 2$ are known). We thus obtain the quantitative relation between α and the laser excitation power P ($\alpha = \beta P$).

5 Application to high-throughput screening

From the above considerations on the Maximum Likelihood estimate of the FLT, we conclude that the minimal coefficient of variation CV one may achieve is:

$$\mathrm{CV}_{\min} = \frac{1}{\sqrt{N}},$$

where N is the number of photons detected in a single fluorescence decay histogram. We also note that N itself is limited by the trade off between achieving the maximum photon detection rate k_d and avoiding significant pile-up distortion ($\alpha \ll 1$).

For the application to HTS, we may estimate how this fundamental limit affects the quality of an assay based on a change in fluorescence quantum yield Q measured by FLT detection. To that end, we can express the Z' factor as a function of the number of photons detected in two control samples yielding two different FLT's τ^+ and τ^- , with $\tau^+ > \tau^-$. By definition, the two FLT's correspond to two fluorescence quantum yields Q^+ and Q^-, respectively. All other experimental conditions being constant, the change in quantum yield results in a change in α (see Eq. 31) and in the total number of photons detected. Therefore we shall define correspondingly α^+, α^- and N^+, N^-. Let us also define $r = \tau^-/\tau^+$ ($0 < r < 1$) the ratio between both FLT's. We thus can write

$r = Q^-/Q^+ = N^-/N^+ = \alpha^-/\alpha^+$. By plugging Eq. 8 into the definition of Z' given by Zhang et al. (1999) (see the References section in the paper), we get:

$$
\begin{aligned}
Z' &= 1 - \frac{3\sigma^+ + 3\sigma^-}{\tau^+ - \tau^-} && (37)\\
&= 1 - 3\frac{\frac{\tau^+}{\sqrt{N^+}} + \frac{\tau^-}{\sqrt{N^-}}}{\tau^+ - \tau^-} && (38)\\
&= 1 - \frac{3}{\sqrt{N^+}} \frac{1 + r\frac{\sqrt{N^+}}{\sqrt{N^-}}}{1-r} && (39)
\end{aligned}
$$

The number of photons detected in a sample is given by the photon detection rate k_d and the exposure time, the latter being presumably the same for all samples.

In the case we use expression (29) for k_d as a function of α, we get (hereafter we write $\alpha^+ = \alpha$ and $\alpha^- = r\alpha$):

$$
\begin{aligned}
\sqrt{\frac{N^+}{N^-}} &= \sqrt{\frac{1-e^{-\alpha}}{1-e^{-r\alpha}}} \simeq \left(\frac{\alpha - \alpha^2/2}{r\alpha - r^2\alpha^2/2}\right)^{1/2} && (40)\\
&\simeq \frac{1}{\sqrt{r}}(1-\alpha/4)(1+r\alpha/4) \simeq \frac{1}{\sqrt{r}}\left(1 - \frac{\alpha}{4}(1-r)\right) \simeq \frac{1}{\sqrt{r}} && (41)
\end{aligned}
$$

Now if we are in conditions where k_d is given by expression (35), we rather get:

$$
\sqrt{\frac{N^+}{N^-}} = \sqrt{\frac{1-e^{-\alpha}}{1-e^{-r\alpha}} \times \frac{1+n(1-e^{-r\alpha})}{1+n(1-e^{-\alpha})}}, \quad (42)
$$

$$
\text{with} \quad \frac{1+n(1-e^{-r\alpha})}{1+n(1-e^{-\alpha})} \simeq \frac{1+nr\alpha}{1+n\alpha} \simeq (1+nr\alpha)(1-n\alpha) \simeq 1 + n(r-1)\alpha, \quad (43)
$$

$$
\begin{aligned}
\text{such that} \quad \sqrt{\frac{N^+}{N^-}} &\simeq \frac{1}{\sqrt{r}}\left(1 - \frac{\alpha}{4}(1-r)\right) \times \left(1 + n(r-1)\frac{\alpha}{2}\right) && (44)\\
&\simeq \frac{1}{\sqrt{r}}\left(1 - \alpha(1-r)\frac{2n+1}{4}\right) \simeq \frac{1}{\sqrt{r}} && (45)
\end{aligned}
$$

Finally, we conclude that in both cases, for small values of α, the Z' factor is expressed as:

$$
\begin{aligned}
Z' &= 1 - \frac{3}{\sqrt{N^+}} \frac{1 + r\sqrt{\frac{N^+}{N^-}}}{1-r} && (46)\\
&\simeq 1 - \frac{3}{\sqrt{N^+}} \frac{1+\sqrt{r}}{1-r} = \boxed{1 - \frac{3}{(1-\sqrt{r})\sqrt{N^+}} \simeq Z'} && (47)
\end{aligned}
$$