Initial Guesses Generation for Fluorescence Intensity Distribution Analysis

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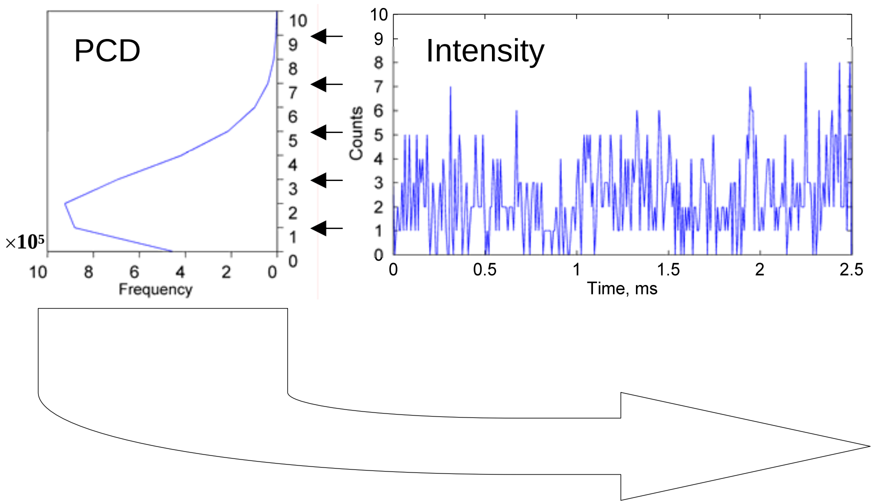
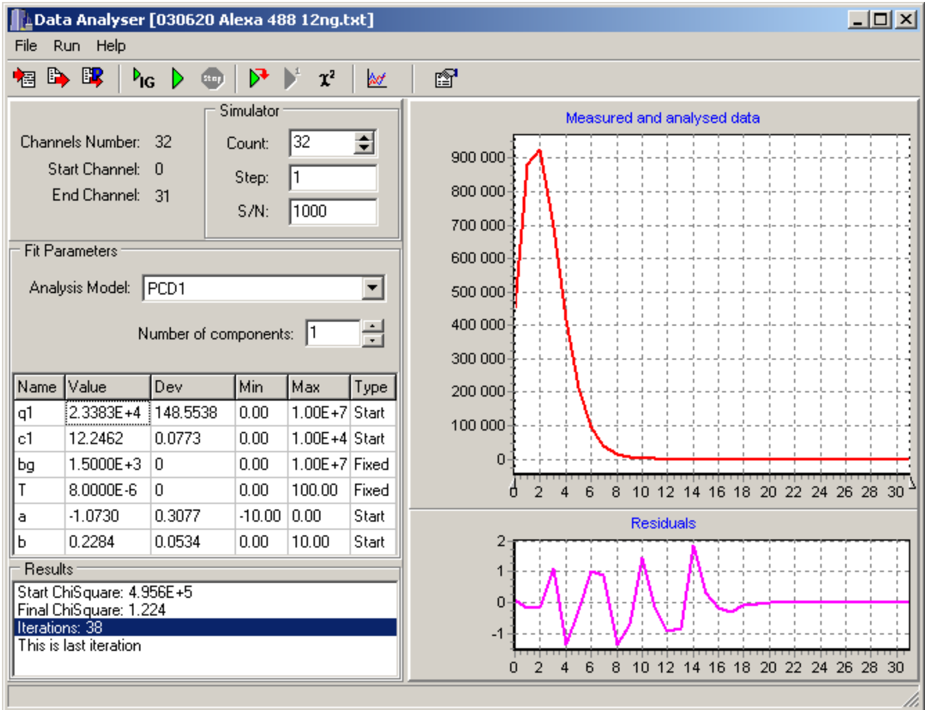
Why IG?





IG generation

Allows to:

- ➢increase efficiency and correctness of the fit
- ➢avoid trapping into local minima
- ➢increase robustness of the analysis
- ➢decrease number of iterations
- ➢reduce user participation and make the whole procedure more standardized

Demands:

- ➢developing of straightforward, noniterative methods

Method of moments

$$M_k(\eta_1, \eta_2, \ldots, \eta_m) = \tilde{M}_k, \quad k = 1, 2, \ldots, m, \quad (1)$$

where $\eta_1, \eta_2, \ldots, \eta_m$ is a set of unknown parameters

$$\tilde{M}_k = \langle n^k \rangle = \sum_{n=1}^{N-1} n^k P^*(n) \quad (2)$$

P^*(n) is a probability to get n photons within a counting time interval T

in application to factorial cumulants

$$K_k(\eta_1, \eta_2, \ldots, \eta_m) = \tilde{K}_k \quad (3)$$

$$K_k = \left. \frac{d^k \ln G(\xi)}{d\xi^k} \right|_{\xi=1} \quad (4) \qquad G(\xi) = \sum_{n=0}^{\infty} \xi^n P(n) \quad (5)$$

$$\tilde{F}_k = \langle n(n-1)\ldots(n-k+1) \rangle = \sum_{n=k}^{N-1} n(n-1)\ldots(n-k+1) P^*(n) \quad (6)$$

$$\tilde{K}_k = \tilde{F}_k - \sum_{i=1}^{k-1} \binom{k-1}{i} \tilde{K}_{k-i} \tilde{F}_i, \quad \text{where} \binom{k-1}{i} = \frac{(k-1)!}{i!(k-i-1)!} \quad (7)$$

Historical background

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Moment analysis of fluorescence fluctuations

$$\begin{cases} <\Phi> = \chi_1 \sum_i q_i c_i \\ <\Delta\Phi^2> = \chi_2 \sum_i q_i^2 c_i \\ <\Delta\Phi^3> = \chi_3 \sum_i q_i^3 c_i \\ <\Delta\Phi^4> - 3 <\Delta\Phi^2>^2 = \chi_4 \sum_i q_i^4 c_i \end{cases} \quad (8)$$

here Φ is the fluorescence intensity, c_i is the number of molecules per observation volume, q_i is the specific brightness expressed in cpm, $B(r)$ is a spatial brightness function, i is the number of molecular species

$$\chi_k = \int B^k(r)dr \quad (9)$$

Qian, H., and E.L. Elson. Biophys. J. 57, 1990
Qian, H., and E.L. Elson. PNAS 87, 1990

Fluorescence Cumulant Analysis

$$K_k = \chi_k \sum_i c_i q_i^k \quad (10)$$

Muller J.D. Biophis. J. 86, 2004

FIDA

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$$G(\xi) = \sum_{n=0}^{\infty} \xi^n P(n) \quad (11)$$

$P(n)$ is photon counting distribution (PCD)

$$G(\xi) = \exp\left((\xi - 1)\lambda T + \sum_j c_j \int_V \left\{ \exp\left[(\xi - 1) q_j T B(r) \right] - 1 \right\} dV \right) \quad (12)$$

here c_j is the mean number of molecules per observation volume, q_j is the specific brightness expressed in cpmt, V is the observation volume, T is the counting time interval, $B(r)$ is brightness profile function which is the product excitation intensity and detection efficiency, j is the number of molecular species and λ is the mean background count rate of detector.

Evotec Biosystems AG. 1998. Int. Patent WO 98/16814.

Kask at al. PNAS 96, 1999.

$$\frac{dV}{dx} = A_0 \, (x + ax^2 + bx^3), \quad x = \ln\left[B_0 \big/ B(r) \right], \quad B(r) = B_0 \, e^{-x} \quad (13)$$

where a, b are instrumental parameters
and A_0, B_0 can be calculated from system of normalization equations:

$$\chi_1 = \int_V B(r) dV = 1, \qquad \chi_2 = \int_V B^2(r) dV = 1. \quad (14)$$

Finally $P(n) = FFT^{-1}(G(e^{i\varphi}))$

Evotec Biosystems AG. 1998. Int. Patent WO 98/16814.

Palo at al. Boiphis. J. 79, 2000

General system of equations for IG generation

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$$K_k = \left.\frac{d^k \ln G(\xi)}{d\xi^k}\right|_{\xi=1}$$

$$K_1 = (\lambda + \sum_j c_j q_j)T$$

$$K_2 = \sum_j c_j q_j^2 T^2 \qquad (15)$$

$$K_k = \chi_k \sum_j c_j q_j^k T^k, \quad k = 3, 4, \dots,$$

$$\chi_k = \int_0^\infty (B_0 e^{-x})^k A_0 (x + ax^2 + bx^3) dx \qquad (16)$$

$$B_0 = \frac{8(2a+6b+1)}{2a+3b+2}, \quad A_0 = \frac{2a+3b+2}{8(2a+6b+1)^2} \qquad (17)$$

IG for one component model

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Basic system of equations:

estimated parameters are *c*, *q*, λ, *a*, *b*

$$\begin{cases} K_1 = (\lambda + cq)T \\ K_2 = cq^2T^2 \\ K_3 = cq^3T^3 \dfrac{64(2a+6b+1)(2a+2b+3)}{27(2a+3b+2)^2} \\ K_4 = cq^4T^4 \dfrac{4(2a+6b+1)^2(4a+3b+8)}{(2a+3b+2)^3} \\ K_5 = cq^5T^5 \dfrac{4096(2a+6b+1)^3(10a+6b+25)}{625(2a+3b+2)^4} \end{cases} \tag{18}$$

Solution:

$$\begin{cases} \dfrac{(10a+6b+25)(2a+2b+3)}{(4a+3b+8)^2} = \dfrac{16875\,K_5K_3}{16384\,K_4^2} \\ \dfrac{729(2a+3b+2)(4a+3b+8)}{(2a+2b+3)^2} = \dfrac{1024\,K_4K_2}{729\,K_3^2} \end{cases} \tag{19}$$

$$\lambda = K_1 - \frac{64\,K_1^2}{27\,K_2}\frac{(2a+2b+3)(2a+6b+1)}{(2a+3b+2)^2} \tag{20}$$

$$q = \frac{K_2}{(K_1 - \lambda T)T}, \quad c = \frac{(K_1 - \lambda T)^2}{K_2} \tag{21}$$

Simplifications: background (λ) is known

estimated parameters are *c*, *q*, *a*, *b*

$$\begin{cases} \chi_3 = \dfrac{(K_1 - \lambda T)K_3}{K_2^2} = \dfrac{64(2a+6b+1)(2a+2b+3)}{27(2a+3b+2)^2} \\ \chi_4 = \dfrac{(K_1 - \lambda T)^2K_4}{K_2^3} = \dfrac{4(2a+6b+1)^2(4a+3b+8)}{(2a+3b+2)^3} \end{cases} \tag{22}$$

$$q = \frac{K_2}{(K_1 - \lambda T)T}, \quad c = \frac{(K_1 - \lambda T)^2}{K_2} \tag{21}$$

In general $$\chi_k = \frac{(K_1 - \lambda T)^{k-2}K_k}{K_2^{k-1}} \tag{23}$$

Solution of system 19

Shape of the fourth order polynomial with respect to parameter *a*

$$H_1 a^4 + H_2 a^3 + H_3 a^2 + H_4 a + H_5 = 0$$

$$\alpha^2\beta^2 - 18\alpha\beta + 27\alpha + 16\beta - 27 > 0 \quad - \quad \text{red line}$$

$$\alpha^2\beta^2 - 18\alpha\beta + 27\alpha + 16\beta - 27 \le 0 \quad - \quad \text{blue line}$$

$$\alpha = \frac{16875\, K_5 K_3}{16384\, K_4^2} \qquad \beta = \frac{1024\, K_4 K_2}{729\, K_3^2}$$

Root selection:

- setting admissible ranges
- minimization of χ^2 criterion

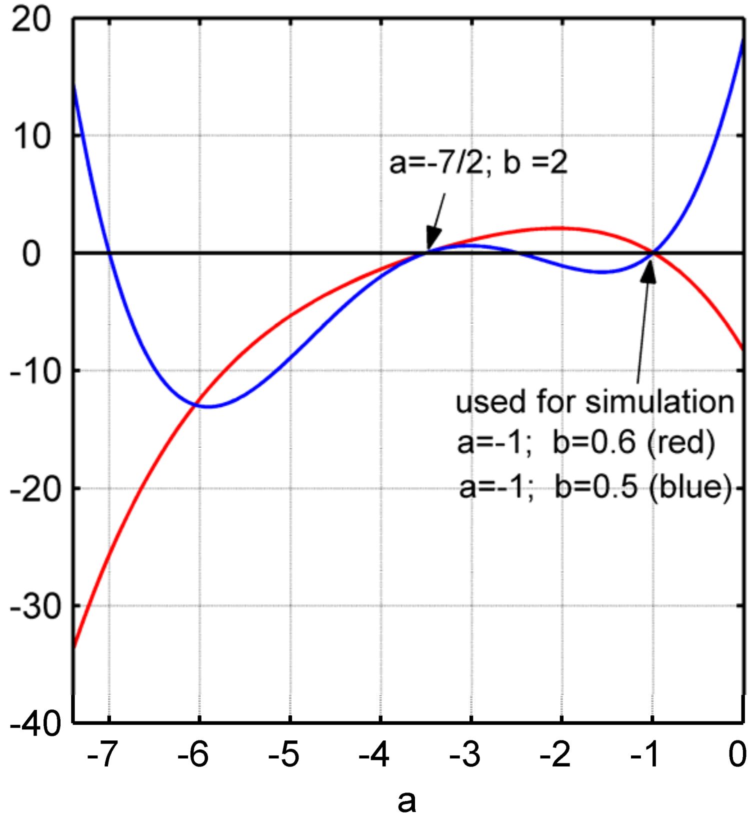

Fig. 1. Roots of polynomial

IG for two component model

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Initial system of equations

estimated parameters are c_1, c_2, q_1, q_2, λ, a, b

$$
\begin{aligned}
K_1 &= (\lambda + c_1 q_1 + c_2 q_2)T \\
K_2 &= (c_1 q_1^2 + c_2 q_2^2)T^2 \\
K_3 &= (c_1 q_1^3 + c_2 q_2^3)T^3 \frac{64(2a+2b+3)(2a+6b+1)}{27(2a+3b+2)^2} \\
K_4 &= (c_1 q_1^4 + c_2 q_2^4)T^4 \frac{4(4a+3b+8)(2a+6b+1)^2}{(2a+3b+2)^3} \\
K_5 &= (c_1 q_1^5 + c_2 q_2^5)T^5 \frac{4096(10a+6b+25)(2a+6b+1)^3}{625(2a+3b+2)^4} \\
K_6 &= (c_1 q_1^6 + c_2 q_2^6)T^6 \frac{4096(2a+b+6)(2a+6b+1)^4}{27(2a+3b+2)^5} \\
K_7 &= (c_1 q_1^7 + c_2 q_2^7)T^7 \frac{8^6(14a+6b+49)(2a+6b+1)^5}{2401(2a+3b+2)^6}
\end{aligned}
\tag{24}
$$

Simplifications:

1. background (λ) and instrumental parameters *a* and *b* are known

$$
\begin{aligned}
K_1 - \lambda T &= (c_1 q_1 + c_2 q_2)T \\
K_2 &= (c_1 q_1^2 + c_2 q_2^2)T^2 \\
K_3 / \chi_3 &= (c_1 q_1^3 + c_2 q_2^3)T^3 \\
K_4 / \chi_4 &= (c_1 q_1^4 + c_2 q_2^4)T^4,
\end{aligned}
\tag{25}
$$

$$
\chi_3 = \frac{64(2a+6b+1)(2a+2b+3)}{27(2a+3b+2)^2} \qquad
\chi_4 = \frac{4(2a+6b+1)^2(4a+3b+8)}{(2a+3b+2)^3}
\tag{26}
$$

2. background (λ) is known

A number of predefined parameters a and b used for solution of the sysmem 25. As result a number of sets of parameters is generated and the set resulting in lowest χ^2 criterion is accepted.

Testing of IG for one component model on simulated data

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1. IG for all parameters *c*, *q*, *λ*, *a*, *b*

Table 1. IG for one component model on noisy PCD at different S/N. IG were rejected when either *λ* was negative or *a* and *b* exceed bounds (-2, 0); (0, 2) respectively. $T = 5\times10^{-5}$. 50 simulations in each series.

Parameter	*Used for simulation*	*Recovered*		
		$S/N_i = 7000$	$S/N_i = 3000$	$S/N_i = 1000$
c	5	4.994±0.126	4.882±0.205	4.327±0.542
q	20000	20016±252	20253±436	21633±1462
λ	2000	2064±1260	3208±2086	9170±5948
a	-1	-0.999±0.019	-0.980±0.034	-0.853±0.152
b	0.5	0.500±0.002	0.499±0.003	0.495±0.009

$$S/N = \sqrt{mp_{\max}/(1-p_{max})}$$

$$S/N_{initial} = \sqrt{mp_{\max}} = $$
$$= \sqrt{\text{Value at Maximum}}$$

here $p_{max} = \max_n(P(n))$,

m is total number of photons

2. IG for parameters *c*, *q*, *a*, *b* (*λ* is known)

Table 2. IG calculated for one component model on noisy PCD at different S/N. *λ* fixed to 2000. $T = 5\times10^{-5}$.

Parameter	*Used for simulation*	*Recovered*		
		$S/N_i = 1000$	$S/N_i = 300$	$S/N_i = 50$
c	5	5.000±0.006	4.999±0.025	4.999±0.107
q	20000	20002±24	20003±107	20029±455
a	-1	-1.000±0.004	-1.006±0.015	-1.048±0.106
b	0.5	0.500±0.008	0.514±0.033	0.522±0.183

Testing of IG for two component model on simulated data

IG for parameters c_1, c_2, q_1, q_2 (λ, a, b are known)

Table 3. IG calculated for two component model on noisy PCD at different S/N. λ = 1000. a = -1; b = 0.5; $T = 2\times10^{-5}$. 50 simulations in each series.

Parameter	*Used for simulation*	*Recovered*	
		S/N_i = 1000	S/N_i = 100
c_1	10	9.99±0.13	10.11±1.70
q_2	20000	19883±635	18282±5013
c_1	2	2.04±0.23	2.59±1.64
q_2	50000	49847±1404	50931±11676

Testing of IG for one component model on measured data

1. IG for all parameters *c*, *q*, λ, *a*, *b*

Table 4. IG calculated for one component model on measured data (Alexa 488). $T = 8\times10^{-6}$.

Parameters	IG	Fit starting from IG
χ^2	0.781	0.760
c	23.12	25.08±4.07
q	19192	18426±1495
λ	18424	27±37465
a	-4.41	-4.58±0.47
b	3.22	3.47±0.63

Confidential intervals are calculated as Asymptotic Standard Errors.

2. IG for parameters *c*, *q*, *a*, *b* (λ is estimated from additional measurement)

Table 5. IG calculated for one component model on measured data (Alexa 488). λ fixed to 1500. $T = 8\times10^{-6}$

Parameters	IG	Best fit = fit starting from IG
χ^2	0.668	0.666
c	24.916	24.923±0.189
q	18500	18500±140.5
a	-1.463	-1.472±0.0014
b	0.321	0.324±0.0004

Confidential intervals are calculated as Asymptotic Standard Errors.

Testing of IG for two component model on measured data

Table 8. IG calculated for two component model on measured data (mixture of IgG labeled with Alexa 488 and pure dye). λ fixed to 1000. $T = 2\times10^{-5}$

Parameters	IG	Best fit
χ^2	1.26	0.78
c_1	5.477	5.166±0.089
q_1	32099	32768±872
c_2	0.387	0.528±0.052
q_2	90770	78824±2511
λ	1000 (fixed)	1000 (fixed)
a	-0.85	-0.769±0.014
b	0.25	0.296±0.018

Confidential intervals are calculated as Asymptotic Standard Errors.

minima in χ^2 space

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Table 6. Parameters used for calculation (obtained from best fit of Alexa 488). $T = 8\times10^{-6}$.

Parameter	Value
c	24.923
q	18500
λ	1500
a	varied
b	varied

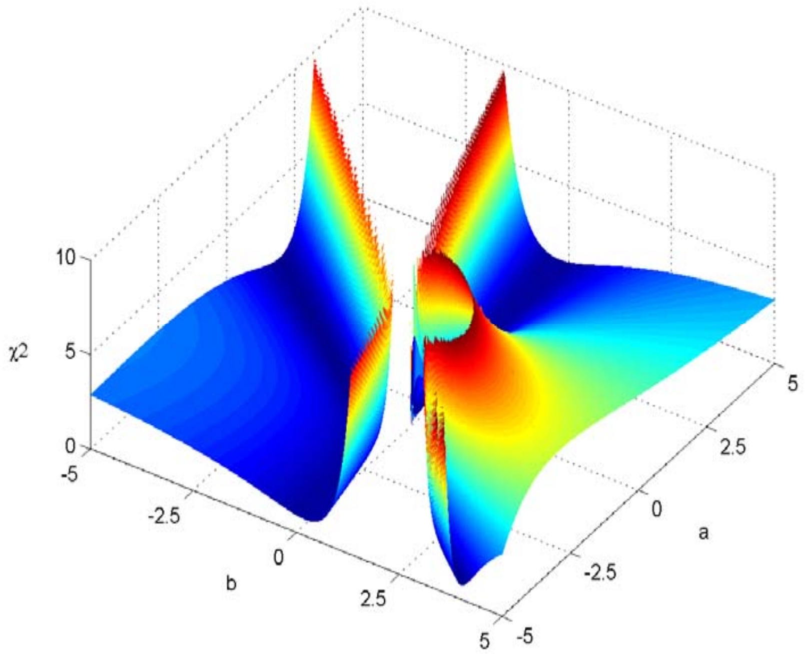

Fig. 2. χ^2 surface plotted versus *a* and *b*.

Table 7. Calculation of χ^2 for all three roots of the system 22.

Parameter	set 1	set 2	set 3
a	-1.393	-1.463	-4.643
b	-0.861	0.321	3.539
χ^2	0.732	0.668	0.711

$$\begin{cases} \dfrac{64(2a+6b+1)(2a+2b+3)}{27(2a+3b+2)^2} = \dfrac{(K_1-\lambda T)K_3}{K_2^2} = \chi_3 \\ \dfrac{4(2a+6b+1)^2(4a+3b+8)}{(2a+3b+2)^3} = \dfrac{(K_1-\lambda T)^2 K_4}{K_2^3} = \chi_4 \end{cases} \quad (22)$$

Conclusions

1. In theory, if we have molecular system with n-components, IG can be obtained as solution of system of equations 15.
2. A non iterative, straightforward method of IG calculation for one and two-component system is proposed.
3. Applicability of method was verified with testing on simulated and measured data.
4. A rule of selecting of IG from possible results is suggested.
5. Impact of possible discontinuity points in model domain and consequently in χ^2 space is discussed
6. developed IG allows to increase the speed of analysis in most cases at least into 5 times.