Application Note

Absolute Diffusion Coefficients: Compilation of Reference Data for FCS Calibration

Peter Kapusta, PicoQuant GmbH, July 2010, Rev.1

Conventional FCS can provide absolute diffusion constant and concentration values only when the exact shape and size of the confocal volume is known. In practice, the shape is approximated by a 3D gaussian function. Size and elongation of the volume is obtained by calibration measurement of a reference compound with a known diffusion constant.

In order to get correct confocal volume parameters, the experimental conditions during calibration (wavelengths, excitation power, cover slip thickness, solvent and immersion medium) must be the same as during the measurement.

Precise diffusion coefficients of several reference compounds suitable for calibration purposes are summarized in the following table:

Fluorophore	λ_{Em} maximum in nm	Diffusion coefficient in water **at 25°C (298.15 K)** in 10^{-6} cm^2s^{-1}	Methods and references
Atto655-maleimid	686	4.07 ± 0.10 4.06 ± 0.09 4.09 ± 0.07	2fFCS [1], PFG-NMR [1] 2fFCS [4, 8] pmFCS [4]
Atto655-carboxylic acid	685	4.26 ± 0.08	2fFCS [1,3], PFG-NMR [1,3]
Atto655-NHS esther	685	4.25 ± 0.06	2fFCS [8]
Cy5	670	3.6 ± 0.1	2fFCS [8]
Alexa 647	665	3.3 ± 0.1	2fFCS [8]
Alexa 633	647	3.4 ± 0.1	2fFCS [8]
Rhodamine 6G	550	4.14 ± 0.05 4.3 ± 0.4 4.14 ± 0.01	2fFCS [1, 8] PFG-NMR [6] PB/CF [7]
Rhodamine B	560	4.5 ± 0.4 4.27 ± 0.04	PFG-NMR [6] PB/CF [7]
Rhodamine 123	530	4.6 ± 0.4	PFG-NMR [6]
Rhodamine 110	535	4.7 ± 0.4	PFG-NMR [6]
Fluorescein	520	4.25 ± 0.01	PB/CF [7]
Oregon Green 488	550	4.11 ± 0.06 4.10 ± 0.08	2fFCS [1] 2fFCS [8]
Atto488-carboxylic acid	523	4.0 ± 0.1	2fFCS [5]
TetraSpeck Beads, 0.1µm diameter	430 515 580 680	0.044 ± 0.07	2fFCS [2], DLS [2]

Abbreviations of measurement methods:

2fFCS, Dual Focus Fluorescence Correlation Spectroscopy; PFG-NMR, Pulsed Field Gradient Nuclear Magnetic Resonance; pmFCS, Polarization-Modulation Fluorescence Correlation Spectroscopy; PB/CF, Plug Broadening/Capillary Flow; DLS, Dynamic Light Scattering.

References:

[1] Müller C. B., Loman A., Pacheco V., Koberling F., Willbold D., Richtering W., Enderlein J.: *Precise measurement of diffusion by multi-color dual-focus fluorescence correlation spectroscopy.*
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[8] Loman A., Müller C. B., Koberling F., Richtering W., Enderlein J.: *Absolute and precise measurements of the diffusion of small fluorescent dye molecules across the visible spectrum.*
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Note that the diffusion coefficient is not a constant. It is temperature dependent according to the Stokes-Einstein relationship,

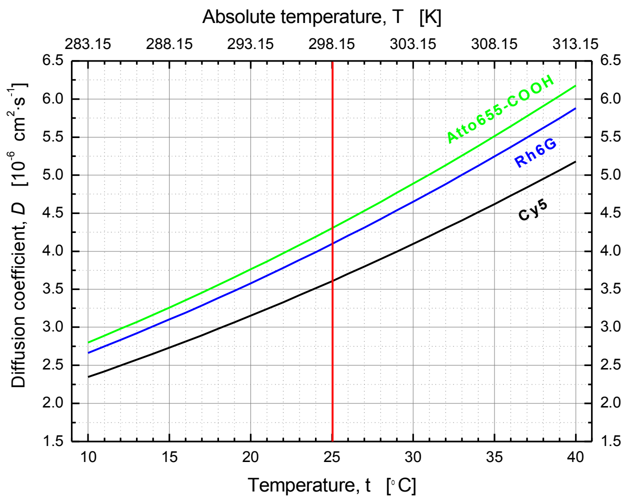
$$D(T) = \frac{kT}{6\pi\,\eta(T)\,r}$$

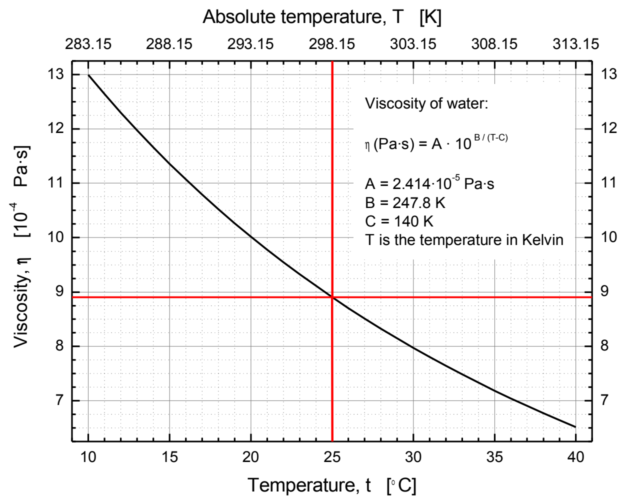
where D is the diffusion coefficient, k is the Boltzmann constant ($1.3807\cdot10^{-23}$ J·K^{-1}), r is the hydrodynamic radius. Moreover, the viscosity, η, of water or solvent in general is also a function of temperature. If the calibration experiment is not performed at T=298.15 K, t=25°C, the tabulated values should be recalculated using this equation:

$$D(T) = D(25\,°C)\cdot\frac{T}{298.15\,K}\cdot\frac{8.9\cdot10^{-4}\,Pa\cdot s}{\eta(T)}$$

$$= D(25\,°C)\cdot\frac{t+273.15}{\eta(t)}\cdot 2.985\cdot10^{-6}\,Pa\cdot s\cdot K^{-1}$$

The temperature dependence is strong and unfortunately often forgotten when comparing results. The effect is illustrated below for three popular fluorophores and the viscosity of water:





Helpful comments and suggestions of Prof. Wajih Al-Soufi, University of Santiago de Compostela, are gratefully acknowledged.

PicoQuant GmbH
Rudower Chaussee 29 (IGZ)
12489 Berlin
Germany

Phone +49-(0)30-6392-6929
Fax +49-(0)30-6392-6561
Email info@picoquant.com
WWW www.picoquant.com