

***In vivo* Standard Probe for Fluorescence Cross-correlation Spectroscopy (FCCS)**

A comprehensive manual

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488/633 in vivo standard: order# 5-0000-505
488/547 in vivo standard: order# 5-0000-605

1. Introduction

1.1 General Considerations

Fluorescence correlation spectroscopy (FCS) gives information about processes, which generate temporal fluctuations of the measured fluorescence in a sample of fluorescent molecules. In the easiest case, fluctuations are caused by the Brownian motion of fluorescent molecules in the observation volume giving information about translational diffusion coefficients.

Since the introduction of FCS in the 1970s, ⁽¹⁻³⁾ it has also been used to determine rotational diffusion coefficients, kinetic rate constants, aggregation and molecular weights ⁽⁴⁻¹²⁾. With the implementation of confocal optics and the development of commercial devices, FCS has found a broad practice in biological questions like binding states of biomolecules and enzyme activities up to cellular applications. Unfortunately, detecting binding events of biomolecules by autocorrelation analysis lacks high sensitivity due to dependence on decelerated translational diffusion by increase of the molecular mass.

A significant extension of FCS is the dual-colour cross-correlation analysis (FCCS) ⁽¹³⁻²⁰⁾, which is based on the simultaneous detection of two spectrally separable colours by splitting the emitted fluorescence into two distinct detection channels using adequate dichroic mirrors and filters.

Cross-correlation analysis implies the time correlation of the fluorescent signals in the two detection channels with each other delivering information about molecules which carry both fluorescent dyes. FCCS has been used in various binding studies of biomolecules *in vitro* and *in vivo*. With the IBA *In Vitro* Standard Probes for Fluorescence Cross-correlation Spectroscopy (488/633 standard: order# 5-0000-504; 488/543 standard: order# 5-0000-604) it is now possible to verify the daily adjustment of the optical setup for cross-correlation measurements.

The *In Vivo* Standard Probe for Fluorescence Cross-correlation Spectroscopy allows detection of fluorescence cross-correlation in cultured cells useful for calibration of *in vivo* FCCS measurements.

Necessary Materials for Measurements

2. Starting point methods

2.1. General considerations

The instructions given below represent typical protocols that were applied successfully *in vitro*, in solution using different setups for fluorescence cross-correlation spectroscopy. Optimal conditions depend essentially on accurate adjustment of the confocal detection system. Thus, an optimum may be found in overlap of the laser foci and the respective

detection foci and additionally overlap of the two distinct detection foci. It may be helpful to adjust the optical setup for maximal cross-talk to the second detection channel using the solely green labelled probe excited only by the green laser, at the expense of the overlap of laser and detection foci of the second channel (especially recommended for older Zeiss Confocor systems).

2.2 Standard protocols for *in vitro* cross-correlation measurements

Materials and important notes

- 3 vials containing double labeled Standard
- 6 vials containing single labeled standard (1st and 2nd channel)
- Transfection into cells requires the InfluxTM Pinocytotic Cell-loading Reagent (Order I-14402, Molecular Probes/ Invitrogen, Karlsruhe, Germany) (not included).
- Imaging in cultured cells needs glass bottom culture plates (e.g. MatTek Corp., Part.-No. P35G-0, Ashland, MA, USA) (not included).
- Choose appropriate beam path for the FCCS 488/633 or 488/543 standard probe with excitation wavelengths 488/633 or 488/543, respectively.
- Vortex the cross-correlation standard probe before use.
- Store the cross-correlation standard probe at 4°C or on ice.
- Do not reuse aliquots of the standard probe after cross-correlation measurements.
- Verify the obtained cross-correlation signal by checking the channel separation of the optical setup for cross-talk using the single labelled samples obtained by IBA.

Standard protocol for cross-correlation measurements *in vivo*

1. Cultivate cells using standard conditions. While cross-correlation measurements can be performed in all cultured cell strains, usage of HEK 293 or HeLa SS6 is recommended even though they show slightly higher autofluorescence.
2. Trypsinize and seed cells 24-48 h before transfection in glass bottom culture plates. It is recommended to use 2 ml cell suspension with 10⁵ cells/ ml in culture medium without phenol red to obtain 60 % confluence.
3. Follow instructions of the InfluxTM pinocytotic cell-loading reagent to prepare hypertonic loading medium, hypotonic lysis medium und recovery medium.
4. Pre-warm all solutions to 37 °C.
5. Centrifuge a lyophilized aliquot of the *In Vivo* FCCS Standard Probe and dissolve in 50 µl loading medium by vortexing. Spin down to recover 50 µl.
6. Adjust the optical setup for optimal overlap of the two detection foci.
7. Perform "Cell Loading Procedure for Adherent Cells on Coverslips" contained in the manual of the InfluxTM pinocytotic cell-loading reagent (I-14402, Molecular Probes, Eugene). The following modifications are suggested.
8. Remove medium with a micropipet and if needed with a sterile Kimwipe ®.

9. Quickly, but gently, add 50 μl pre-warmed hypertonic loading medium containing the cross-correlation sample onto the centre of the glass bottom.
10. Incubate the cell chamber at 37°C for 15 min.
11. Remove the loading medium using a micropipet and if needed with a Kimwipe®.
12. Place the cell chamber *vertically* in a beaker filled with approximately 50 ml pre-warmed hypotonic lysis medium while immediately swaying for 5 times.
13. Incubate for 2 min.
14. Remove hypotonic lysis medium using a micropipet.
15. Place the cell chamber in a beaker filled with 50 ml recovery medium.
16. Incubate for 10 min at 37°C.
17. Additionally, wash the cells for three times with appropriate buffer.
18. Perform cross-correlation measurements.
19. Adjustment of the optical setup for detection of cross-correlation may be disordered due to temperature variations. Check the proper adjustment by performing cross-correlation measurements of a new aliquot of the *In Vitro* FCCS standard probe once in every few hours.

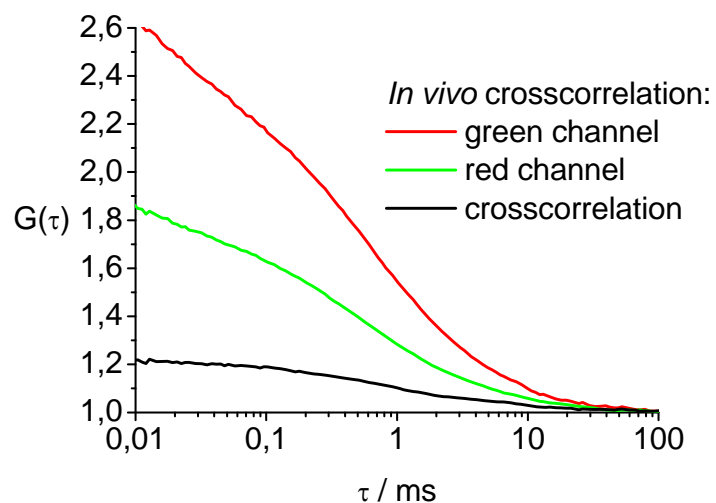
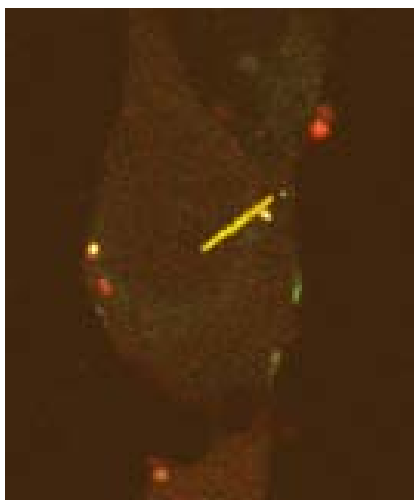


Diagram 1: Exemplary cross-correlation measurement using the IBA *In Vivo* FCCS Standard Probe 488/633 in HEK 293 cells. The cross-correlation signal mainly depends on proper adjustment of the setup.

Related products

***In Vitro* Standard Probe for Fluorescence Cross-correlation Spectroscopy.**

488/633 standard: order# 5-0000-504

488/543 standard: order# 5-0000-604

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