Data Article

Time-shifted mean-segmented Q data of a luminal protein measured at the nuclear envelope by fluorescence fluctuation microscopy

Siddarth Reddy Karuka, Jared Hennen, Kwang-Ho Hur, Joachim D. Mueller*

School of Physics and Astronomy, University of Minnesota, MN, 55455, United States

**Article info**

**Article history:**
- Received 25 October 2019
- Received in revised form 25 November 2019
- Accepted 5 December 2019
- Available online 20 December 2019

**Keywords:**
- Fluorescence fluctuation spectroscopy
- Nuclear envelope
- Protein mobility
- Protein assembly
- Two-photon microscopy

**Abstract**

Fluorescence fluctuation microscopy is a widely used method to determine the mobility and oligomeric state of proteins in the live cell environment. Existing analysis methods rely on statistical evaluation of data segments with the implicit assumption that no significant signal fluctuations occur on the time scale of a data segment. Recent work on extending fluorescence fluctuation methods to the nuclear envelope of living cells identified a slow fluctuation process that is associated with the undulations of the nuclear membranes, which lead to intensity fluctuations due to local volume changes at the nuclear envelope. This environment violates the above-mentioned assumption and is associated with biased evaluation of fluorescence fluctuation data by traditional analysis methods, such as the autocorrelation function. This challenge was overcome by the introduction of the time-shifted mean-segmented Q function, which relies on a sliding scale of data segment lengths. Here, we share experimental fluorescence fluctuation data taken at the nuclear envelope and demonstrate the calculation of the time-shifted mean-segmented Q function from the raw data. The data and analysis should be valuable for researchers interested in fluorescence fluctuation techniques and provides an opportunity to examine the influence of slow fluctuations on existing data analysis methods. The data is related to the research article titled "Protein oligomerization and mobility within..."
1. Data description

The data presented here are experimental recordings of the fluorescence intensity of EGFP targeted to the lumen of the nuclear envelope (NE) of a U2OS cell (Fig. 1A). A conceptual picture of the NE illustrates the ~40 nm-wide lumen containing EGFP proteins sandwiched between two nuclear membranes (Fig. 1B). Photon count data (Fig. 2A) were collected with the two-photon excitation spot centered on the NE (Fig. 1A). While the conceptual picture suggests that the fluctuations in the fluorescence intensity (Fig. 1C) solely reflect the diffusion of EGFP across the two-photon spot, it was recently found [2] that the distance between the two nuclear membranes undulates (Fig. 1D), leading to local volume fluctuations, which superimpose a slow fluctuation process on top of the diffusional component, as conceptually illustrated in Fig. 1E.

The raw data (Fig. 2A) were analyzed using time-shifted mean-segmented Q (tsMSQ) analysis (Fig. 2B), a recently developed method, to avoid the biases introduced by the slow undulation process.
into conventional autocorrelation analysis of the data [1]. The tsMSQ curve determines the diffusion time $t_D$ and diffusion amplitude $Q$ of luminal EGFP as well as the characteristic time $t_0$ and amplitude $A_0$ of the undulation process (Fig. 2B). The unbiased separation of local volume fluctuations from diffusional fluctuations achieved by tsMSQ was a prerequisite for successful investigation of protein assembly at the NE by fluorescence fluctuation spectroscopy [1].

2. Experimental design, materials, and methods

Extensive details of our experimental setup and data collection process have been described elsewhere [3]. Briefly, a home-built two-photon microscope excites the sample at a wavelength of ~1000 nm at sufficiently low power (0.2–0.4 mW) to avoid photobleaching artifacts. A C-Apochromat water immersion objective with NA = 1.2 (Carl Zeiss AG, Jena, Germany) focuses the excitation light onto the sample and collects the emitted photons, which are detected by a single-photon counting module (SPCM-AQR-14, PerkinElmer, Dumberry, Quebec) and recorded by a Flex04-12D (correlator.com, Bridgewater, NJ) data acquisition card for subsequent analysis.

The U2OS cells (ATCC, Manassas, VA), maintained in DMEM with 10% FBS (Hyclone Laboratories, Logan, UT), were plated in a 24-well glass-bottom slide with #1.5H coverglass (In Vitro Scientific, Logan, UT).
Sunnyvale, CA) approximately 48 hours before measurement. Cells were transiently transfected with GenJet (SignaGen Laboratories, Rockville, MD) 24 hours prior to measurement, according to the manufacturer’s instructions. A plasmid containing EGFP fused with the signal sequence of the luminal protein torsinA was used to ensure efficient localization of EGFP to the shared lumen of the endoplasmic reticulum and the NE [4,5]. On the day of measurement, the medium was replaced with DPBS premixed with calcium and magnesium (130 mg/L calcium chloride dihydrate and 100 mg/L magnesium chloride hexahydrate) by the manufacturer (BioWhittaker, Walkerville, MD) followed by mounting of the 24-well glass-bottom slide onto the stage of the microscope.

A cell expressing luminal EGFP is identified by brief epifluorescence exposure of the sample. After centering the selected cell with the stage, the axial localization of the fluorophore is checked by scanning the two-photon excitation spot along the optical axis as previously described [6]. Next, the two-photon excitation spot is focused on the ventral NE and data is collected for ~60 seconds. The same procedure is then repeated with the focus shifted to the dorsal NE, before moving on to the next cell. The recorded photon data were analyzed with the tsMSQ algorithm implemented in IDL.

The algorithm used to construct the tsMSQ curve from experimental photon count data is depicted in Fig. 3 using a very short photon count record for illustrative purposes. The steps for calculating the tsMSQ value for segment time $T_x$ are as follows:

1. The photon counts $k$ recorded with a sampling time $T_s$ are divided into segments of length $T_x$ (Fig. 3A).
2. The time-shifted Q value ($tsQ$) of the segment is calculated using the equation $tsQ = \frac{\langle (k_{i+1} - \langle k_i \rangle) (k_{i+1} - \langle k_i \rangle) \rangle}{\langle k_{i+1} \rangle}$, where $k_i$ and $k_{i+1}$ are the values at the $i^{th}$ and $(i + 1)^{th}$ bin of the photon record, respectively. $\langle k_i \rangle$ denotes the average value of the photon record excluding the last bin in the segment. $\langle k_{i+1} \rangle$ denotes the average value of the photon record excluding the first bin in the segment. Pictorially the time shift can be visualized as creating a copy of the photon count data that is shifted by $T_s$, as shown for the first segment in Fig. 3B. The $tsQ$ is calculated from the red-shaded portion of the segment.
3. The $tsQ$ values of all segments are calculated and their average $\langle tsQ(T_x) \rangle$ is determined (Fig. 3C).

\[ \langle tsQ(T_x) \rangle = \frac{\langle (k_{i+1} - \langle k_i \rangle) (k_{i+1} - \langle k_i \rangle) \rangle}{\langle k_{i+1} \rangle} \]

![Fig. 3. Illustration of the tsMSQ algorithm.](image-url) (A) Photon count data recorded with sampling time $T_s$ are divided into segments of length $T_x$. (B) The photon counts of the first segment are shown together with a time-shifted copy, which are used to calculate the $tsQ$ value of the segment. (C) The $tsQ$ values from all segments are averaged (dashed line). (D) The tsMSQ value (black circle) for segment time $T_x$ is determined by adding the shot noise term $S$ to the averaged $tsQ$ value. Repeating this process for a range of segment times yields the complete tsMSQ curve (gray circles).
4. The $tsMSQ$ value for segment time $T_x$ is determined by adding the term $S = \frac{(T_y-T_x)T_x}{(T_y-T_z)}$ to $\langle tsQ(T_x) \rangle$ (Fig. 3D).

Steps 1 to 4 are repeated over a range of segment times to determine the complete $tsMSQ$ curve. Typical segment times range from 10 ms to several seconds (Fig. 3D). The experimental $tsMSQ$ curve is fitted to a model accounting for diffusion and NE undulation, as described in detail elsewhere [1]. For the data described in this manuscript the following values were obtained from the $tsMSQ$ fit: $A_0 = 0.017$, $\tau_0 = 523$ ms, $Q = 0.022$, $\tau_D = 1.5$ ms.

**Acknowledgments**

This work was supported by the National Institutes of Health (GM064589).

**Conflict of Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**References**

[1] J. Hennen, K.H. Hur, S.R. Karuka, G.W.G. Luxton, J.D. Mueller, Protein oligomerization and mobility within the nuclear envelope evaluated by the time-shifted mean-segmented Q factor, Methods 157 (2019) 28–41, https://doi.org/10.1016/j.ymeth.2018.09.008.

[2] J. Hennen, K.H. Hur, C.A. Saunders, G.W.G. Luxton, J.D. Mueller, Quantitative brightness analysis of protein oligomerization in the nuclear envelope, Biophys. J. 113 (2017) 138–147, https://doi.org/10.1016/j.bpj.2017.05.044.

[3] J. Hennen, I. Angert, K.–H. Hur, G.W. Gant Luxton, J.D. Mueller, Investigating LINC complex protein homo-oligomerization in the nuclear envelopes of living cells using fluorescence fluctuation spectroscopy, Methods Mol. Biol. 1840 (2018) 121–135, https://doi.org/10.1007/978-1-4939-8691-0_11.

[4] J. Hennen, C.A. Saunders, J.D. Mueller, G.W.G. Luxton, Fluorescence fluctuation spectroscopy reveals differential SUN protein oligomerization in living cells, Mol. Biol. Cell 29 (2018) 1003–1011, https://doi.org/10.1091/mbc.E17-04-0233.

[5] R.E. Goodchild, W.T. Dauer, Mislocalization to the nuclear envelope: an effect of the dystonia-causing torsinA mutation, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 847–852, https://doi.org/10.1073/pnas.0304375101.

[6] E.M. Smith, J. Hennen, Y. Chen, J.D. Mueller, In situ quantification of protein binding to the plasma membrane, Biophys. J. 108 (2015) 2648–2657, https://doi.org/10.1016/j.bpj.2015.04.021.