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# Time-resolved fluorescence anisotropy and fluctuation correlation analysis of major histocompatibility complex class I proteins in fibroblast cells

Ahmed A. Heikal *

*Department of Chemistry and Biochemistry, Swenson College of Science and Engineering, University of Minnesota-Duluth, Duluth, MN 55812, USA*
*Department of Pharmacy Practice and Pharmaceutical Sciences, College of Pharmacy, University of Minnesota-Duluth, Duluth, MN 55812, USA*

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ABSTRACT

Major histocompatibility complex class I proteins, MHC(I), are expressed in almost all nucleated cells and synthesized in the endoplasmic reticulum (ER). The orientation and mobility of these complexes are crucial in their biological function in the immune system, i.e., the cytosolic pathogen peptides loading and their presentation to T-cell receptors at the plasma membrane, where cell destruction is triggered. Here, we investigate the structural flexibility and associations of GFP-encoded MHC(I) alleles ($H2L^d$), namely $H2L^dGFP_{in}$ and $H2L^dGFP_{out}$, in cultured mouse fibroblast cells. Time-resolved fluorescence anisotropy of $H2L^dGFP_{in}$ in the ER indicates a dominant overall tumbling motion of 56 ± 7 ns (ER), with a fast conformational flexibility, as compared with a restricted rotation of $H2L^dGFP_{out}$. At the single-molecule level, the diffusion coefficient of $H2L^dGFP_{in}$ and $H2L^dGFP_{out}$ in the ER is $(1.8 \pm 0.5) \times 10^{-9}$ and $(2.1 \pm 0.6) \times 10^{-9}$ $cm^2/s$, respectively, as revealed by fluorescence correlation spectroscopy. A complementary immunoblotting of $H2L^dGFP$ constructs, isolated from mouse fibroblast cells, reveals band at 75 kDa as compared with 29 kDa of the free EGFP. These real-time dynamics provide new insights into the structural flexibility and intracellular associations of GFP-labeled MHC(I) alleles ($H2L^d$) in living cells.


## 1. Introduction

The assembly of major histocompatibility complex (MHC) class I protein in the endoplasmic reticulum (ER) involves the transporter associated with antigen processing (TAP) and other accessory proteins such as calnexin, calreticulin, tapasin, Bap31, and Erp57 [23,32,40]. Proteasomes break down intracellular pathogens into peptides, which bind to MHC(I) via TAP in an ATP-dependent manner. The peptide loading complex (PLC) [28] consists of TAP, the MHC(I) specific chaperone tapasin, CRT, and ERp57. The peptide-loaded MHC(I) protein then dissociates from the TAP complex prior to exocytosis from the ER to be transported to the plasma membrane via the Golgi apparatus. Once on the plasma membrane, MHC(I) presents the peptides to effector T lymphocytes, which trigger the destruction of infected cells [8,31]. Although, most proteins have been identified in the peptide loading pathway, the extent of aggregation, structural flexibility, and orientation of MHC(I) are far from being well understood.

Marguet et al. [28] have used fluorescence recovery after photobleaching (FRAP) on two functional GFP-tagged MHC(I) molecules to characterize their transient associations with various ER proteins. Two different GFP-tagged forms of the MHC(I) allele $H2L^d$ were constructed, one ($H2L^dGFP_{in}$) has a GFP tag at the cytoplasmic tail and another ($H2L^dGFP_{out}$) with the GFP tag between the molecule's transmembrane and the third exodomain regions (Fig. 1). The GFP tag in $H2L^dGFP_{in}$ is at the C-terminus [28], which is separated from the biologically active N-terminus. Using steady-state fluorescence polarization imaging [35], the rigid $H2L^dGFP_{out}$ is believed to be a better reporter of MHC(I) formation as well as the stability of the PLC in the ER than $H2L^dGFP_{in}$. Steady-state polarization-analyzed fluorescence imaging by Rocheleau et al. [35] on membrane blebs, isolated from mouse fibroblast cell line, also indicate that the $H2L^dGFP_{out}$ construct is more rigid compared with the $H2L^dGFP_{in}$ construct. A similar approach, combined with statistical analysis, was used for examining the molecular orientational order of $H2L^dGFP_{out}$ and $H2L^dGFP_{in}$ proteins in the ER and plasma membrane of living COS-7 cells [24]. However, steady-state polarization imaging techniques inherently lack the temporal information [22] that is required to assess the dynamic nature or rigidity of MHC(I) constructs. As a result, real-time measurements

* Address: Department Chemistry and Biochemistry, 1039 University Drive, Minnesota University Duluth, Duluth, MN 55811, USA. Fax: +1 218 726 7394.
*E-mail address:* aaheikal@d.umn.edu

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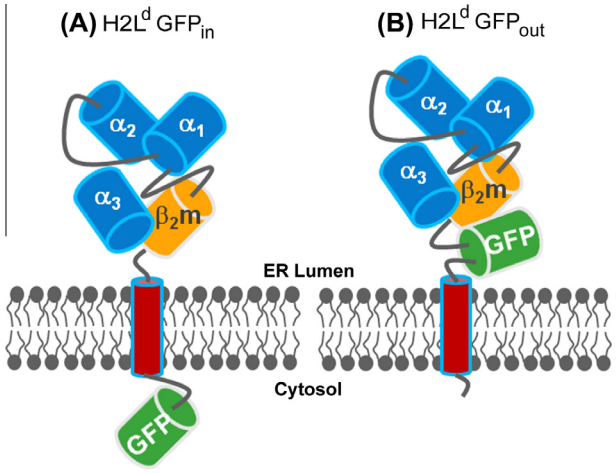


**Fig. 1.** A schematic molecular structure of the H2L$^d$GFP constructs where the GFP gene was inserted either on the cytoplasmic tail of the C-terminal of the transmembrane domain (H2L$^d$GFP$_{in}$) or in between the $\alpha_3$-domain sequence and the tranmembrane domain sequence of the native H2L$^d$ gene (H2L$^d$GFP$_{out}$) [24,28]. The MHC-encoded polypeptide is approximately 350 amino acids long (~45 kDa) and folds into three separate domains ($\alpha_1$, $\alpha_2$ and $\alpha_3$). $\beta_2$-microglobulin ($\beta_2$m) is a 12 kDa polypeptide that is non-covalently associated with the $\alpha_3$ domain. The groove between the $\alpha_1$ and $\alpha_2$ domains is capable of non-covalent binding with small peptides (~ 10 amino acids) to be presented to the T-cell receptor.

of such conformational changes and sizes of these MHC(I) complexes are needed and would require a multiscale approach.

In this report, a multimodal fluorescence micro-spectroscopy approach was used to examine the structural heterogeneity and conformational changes of MHC(I) alleles in mouse fibroblast L cells using H2L$^d$GFP$_{in}$ and H2L$^d$GFP$_{out}$ constructs. The inherently complex dynamics of cellular proteins, with spatial and temporal heterogeneity, dictates the need for multimodal fluorescence methods. The spatial distribution of GFP-tagged MHC(I) proteins in mouse fibroblast cells was imaged using differential interference contrast (DIC), confocal, and two-photon (2P) fluorescence life imaging microscopy (FLIM) to examine the variation of both concentrations and local environment of these constructs. Ultrafast conformational changes and rotational diffusion of H2L$^d$GFP$_{in}$ and H2L$^d$GFP$_{out}$ in fibroblast cells are investigated using time-resolved fluorescence polarization anisotropy. The hydrodynamic volumes of H2L$^d$GFP$_{in}$ and H2L$^d$GFP$_{out}$ aggregates were quantified at the single-molecule level using fluorescence correlation spectroscopy. The results are compared with measured sizes using conventional immunoblotting approach in order to elucidate the restrictive nature of cell environment to these complexes.

## 2. Materials, methods, and data analysis

### 2.1. Cell culture and labeling

Mouse fibroblast cell line, L-M(tk−) (H2k) (ATCC CCL 1.3), was stably transfected with GFP gene to express H2L$^d$GFP$_{in}$ and H2L$^d$GFP$_{out}$ (a generous gift from Dr. Michael Edidin, John Hopkins University). Cells were maintained in RPMI medium (Gibco) and supplemented with L-glutamine (2 mM), HEPES (10 mM), fetal bovine serum (10% v/v), and G418 (300 μg/mL) at 37 °C and 5% $CO_2$. Cells were passed (at 70–90% confluence) into 35 mm Mattek plates the night before experiment. Cells were washed out three times prior to imaging in a buffer that contains 125 mM NaCl, 5.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 10 mM HEPES (pH 7.4). To isolate H2L$^d$GFP$_{in}$ and H2L$^d$GFP$_{out}$ proteins in the plasma membrane, the cells were incubated overnight with 100 μM of *cycloheximide* (YPHFMPTNL) peptides (Sigma Aldrich) [35]. The peptide-loaded cells were then incubated with $H_2O_2$ (1 mM, 1 h) in order to induce membrane blebing.

### 2.2. Western blot

Anti-GFP antibodies were used with either transiently or stably-transfected mouse fibroblast cells that express H2L$^d$GFP$_{in}$ or H2L$^d$GFP$_{out}$. Comparison with RBL mast cells, expressing cytosolic EGFP, was also made as a control. Cell equivalents of lysate were electrophoresed on 12% non-reduced SDS–polyacrylamide gels, transferred to BioTrace polyvinylidene difluoride membrane (PALL Gelman Laboratory), and blotted with horseradish peroxidase-conjugated anti-GFP (Jackson Immunological). Enhanced chemiluminescence (Pierce Biotechnology) was used to detect GFP constructs.

### 2.3. FLIM imaging

The setup of confocal and FLIM used in these studies has been described in detail elsewhere [1,2,9,48]. Briefly, a confocal system consists of an inverted microscope (IX81), a scanner (FV300, Olympus), and fiber-coupled cw laser systems [1,10,48]. The samples were excited using a microscope objective (1.2NA, water immersion, infinity corrected, 60x, Olympus). Excitation wavelengths (488 nm) and emission filters (525/30 band pass) were optimized for EGFP. The confocal microscope was modified to allow for 2P-fluorescence lifetime and polarization imaging using a titanium–sapphire femtosecond laser system (Mira 900-F, Coherent) [2,10,15,16,47,48]. The 2P-microscopy and FLIM measurements (690-nm short pass filter) were carried out using 960-nm excitation, while the second harmonic (480 nm) was used for 1P single-point excitation (~120 fs pulses at 4.2 MHz repetition rate) experiments. The epi-fluorescence at magic-angle polarization was then detected using a microchannel plate photomultiplier tube, MCP-PMT, (R3809U, Hamamatsu) for fluorescence lifetime measurements based on time-correlated single-photon counting approach [30]. A histogram of fluorescence photon arrival times were recorded using a SPC-830 module (Becker and Hickl, Germany) for FLIM analysis [6]. In these 2P-FLIM images (256 × 256 pixels), each pixel had 64 time bins (i.e., at 259 ps/bin) with a data acquisition time of 3–5 min. In order to enhance the temporal resolution (1024 time bins at 24.4 ps/bin), complementary single-point time-resolved fluorescence measurements were carried out where the 1P laser pulses were focused on regions-of-interests inside the cells (i.e., without laser scanning as in FLIM).

The excited-state lifetime of cellular H2L$^d$GFP$_{in}$ and H2L$^d$GFP$_{out}$ fluorescence was detected at magic angle ($I_{54.7}$) and generally described as [1,2,9,25]:

$$I_{54.7}(x,y,t) = \sum_{i=1}^{3} \alpha_i(x,y) \cdot \exp[-t/\tau_i(x,y)]. \quad (1)$$

The average fluorescence lifetime was calculated, $\langle \tau_{fl} \rangle = \sum \alpha_i \tau_i / \sum \alpha_i$, using the measured fluorescence decay times ($\tau_i$) and amplitudes ($\alpha_i$). The nonlinear fitting of both FLIM and single-point time-resolved fluorescence was performed using the SPCImage (Becker and Hickl) and the goodness of the fit was judged by both $\chi^2$–value ($\leqslant$1.2) and the residual [6,48]. Using a computer-generated system response function for deconvolution and an incomplete multiexponential decay model [6,7], single-exponential fluorescence decay was adequate to describe the majority of pixels (binning of 3) in FLIM images [6,7]. In contrast, a multi-exponential function was required to fit the single-point fluorescence decays collected at high temporal resolution and high signal-to-noise ratio. In these single-point measurements, the 1P laser pulses were randomly focused on H2L$^d$GFP$_{in}$ and H2L$^d$GFP$_{out}$ in cultured mouse fibroblast cells.