



ELSEVIER

Contents lists available at ScienceDirect

MethodsX

journal homepage: www.elsevier.com/locate/mex

Method Article

Methods toward simplification of time resolved fluorescence anisotropy in proteins labeled with NBD (4-chloro-7-nitrobenzofurazan) adducts

Ben J. Glasgow^{*}, Adil R. Abduragimov*Departments of Ophthalmology, Pathology and Laboratory Medicine, Jules Stein Eye Institute, University of California, 100 Stein Plaza Rm. BH 623, Los Angeles, CA 90095, United States*

A B S T R A C T

The analysis of time resolved fluorescence anisotropy for NBD tagged proteins is difficult when multiple exponential components arise from heterogeneous amino acid fluorescent adducts. Two approaches were taken toward simplification. First, N terminal selective labeling of tear lipocalin with NBD-Cl was attempted at pH 7.0. While lysines were predominantly labeled at pH 8.0, selective N terminal labeling was attained at neutral pH. Second, fluorescence anisotropic decay analysis was simplified to recover only the rotational correlation time of the protein not the side chain. The boundaries for analysis of anisotropic decays were limited to the longer lifetimes. A modified tail fit enabled fitting the anisotropic decay to a single exponential. The correlation time for tear lipocalin matched published values. Additionally, a method for normalization of acquisition times of vertically (VV) and horizontally (VH) polarized fluorescence emission decays is presented for time-resolved anisotropy. Here it is applied to PicoHarp software (Picoquant, Berlin). PicoHarp software is programmed with an automatic stop at unequal acquisition times if the fluorescent counts exceeds a default. The method adjusts the intensity decays to the same acquisition time and is applicable to all time-resolved anisotropic decay data collected with time-correlated photon counting.

- NBD labeling at pH 7.0 was not selective for N terminus of LCN1.
- Constraints for range simplifies fittings of anisotropic decays.
- Different acquisition times for decays can be normalized to facilitate fitting in data obtained by PicoHarp.

© 2019 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

A R T I C L E I N F O

Method names: Constrained tailfit for parameter reduction in time resolved fluorescence anisotropy

Keywords: LCN1, Lipocalin 1, Fluorescence lifetime analysis, N terminal labeling of proteins, pH selectivity of NBD-Cl, Fluorescence anisotropy, Normalization of VH, Time correlated single photon counting

Article history: Received 26 November 2018; Accepted 24 April 2019; Available online 29 April 2019

DOI of original article: <http://dx.doi.org/10.1016/j.bbapap.2018.07.001>

^{*} Corresponding author.

E-mail address: bglasgow@mednet.ucla.edu (B.J. Glasgow).

<http://dx.doi.org/10.1016/j.mex.2019.04.025>

2215-0161/© 2019 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Specifications Table

Subject area:	<ul style="list-style-type: none"> • <i>Biochemistry, Genetics and Molecular Biology</i> • <i>Chemistry</i>
Specific subject area:	<i>Fluorescence analysis of NBD adducts</i>
Method names:	<i>Constrained tailfit for parameter reduction in time resolved fluorescence anisotropy</i> <i>Normalization of acquisition times in time resolved fluorescence microscopy</i>
Name and reference of original method:	<i>Ligand binding complexes in lipocalins: underestimation of the stoichiometry parameter (n)</i> <i>Submitted to BBA Proteins and Proteinomics</i>
Resource availability:	<i>PicoHarp and Fluofit software</i>

Method details

Background

Time resolved fluorescence anisotropy is often used to recover the rotational correlation time of biomolecules with fluorescent labels. Analysis of fluorescence anisotropy is useful for the determination of molecular weight of mobile species. Applications include detection of dimerization, identification of protein–ligand binding, and molecular interactions. The analysis of a sample with a fluorophore, freely mobile in solution, which exhibits a single lifetime is the ideal case. However, in the case of a fluorophore in heterogeneous environments with multiple lifetimes, analysis requires additional parameters and raises uncertainty in the analysis. The difficulty is further complicated by fluorophore adducts with side chain rotation in addition to rotation of the molecule. To viate detection of the side chain rotation, site directed mutagenesis of the protein can be performed to place a reactive residue such as cysteine at a buried site of the protein. However, often several mutations are needed to remove other reactive residues, risking the alteration of functional attributes. A simpler procedure is to label the protein directly with NBD-Cl, but this often results in NBD adducts on multiple amino acid residues. The various environments of the labels create multi-exponential fluorescent lifetimes and even multi-exponential anisotropic decays. Fitting of multi-exponential anisotropic decays can be very challenging and require a complicated reconvolution procedure. Additional variables such as the G factor, instrument response function, background scatter, offset parallel and vertically polarized decays can be floating parameters and contribute uncertainty to the analysis. Therefore, 2 methods were studied to simplify the procedure.

The first method follows the report of Bernal-Perez et al. that selective N terminal labeling of the Z domain protein was achieved using NBD-Cl at neutral pH [1]. In their study fluorescence was monitored during labeling. Fluorescence was evident 1 h after NBD-Cl treatment at pH 7 in the unblocked N terminus of the protein. If the N terminus were acetylated, very little fluorescence was observed after treatment. The data suggested the selectively labeling of the N terminus occurred at pH 7, rather than the side chains of other amino acids. The implication was that other susceptible amino acids in the polypeptide chain such as the primary amines of lysine and arginine, the hydroxyl groups of tyrosines and the sulfhydryl groups of cysteines were unlabeled. A potentially exciting application for this method is to limit the heterogeneity of labeled sites. More uniform labeling of a single site would obviate the need for site directed mutagenesis. Compared to current methods of NBD-Cl labeling, N terminal selective labeling would reduce the environmental variability encountered by the fluorophore. A concomitant reduction in both the number of fluorescence lifetimes and their associated exponential parameters would simplify analysis.

A second approach is to constrain the fluorescence lifetime anisotropic decay analysis range to a longer decay lifetime. The short fluorescent anisotropic decay lifetimes (0.2 ns) associated with rapid side chain motion of the NBD compound are intentionally excluded. Both approaches were tested with tear lipocalin in order to leverage published structural information, fluorescence lifetime analysis, fluorescence anisotropy data and correlation times by multiple methodologies [5–7].

An additional methodological tip is presented for users of single photon counting instruments. The analysis of anisotropic decays requires that a large number of counts be obtained so that noise can be distinguished from small differences in the intensity values of vertically (VV) and horizontally (VH) polarized decays. Attempts to obtain a large number of counts may result in count overflow which triggers a default stop in some software (e.g. Picoquant). This situation leads to unequal acquisition times for VV and VH. The instructions manuals often state that data should only be analyzed when the acquisition times are the same. Here, a normalization method is tested in samples with markedly disparate acquisition times. The normalized data proved to be super-imposable over data from the same sample with identical acquisition times. The data indicate that dissimilar acquisition times can be normalized to interpret fluorescence anisotropic analysis.

Method rationale

Native tear lipocalin was chosen as an ideal protein to test this method. Bearing 11 lysines, 5 tyrosines, and 1 free cysteine, tear lipocalin presents many sites for NBD adducts [2]. The native protein has 6 isoforms that present serine, histidine, arginine and leucine for potential N terminal labeling [3]. The molecular weight of the predominant isoform is 17,446 [4]. The correlation time constant has been well studied and reported as 13.5 ns by electron paramagnetic resonance and 9–10 ns by fluorescence anisotropy using a nitroxide or dansyl label respectively [5–7]. In these studies a buried site at residue position 99 in the G strand was chosen because the side chain is motionally restricted [2]. Therefore, a comparison to NBD adducts at the N terminus and/or mobile sites is opportune for this protein.

In the calculation of anisotropy, fluorescence depolarization is linked to rotational diffusion (Perrin equation). A fluorophore rotating faster than its fluorescence lifetime contributes very little anisotropy. A slowly rotating fluorophore relative to its fluorescence lifetime will contribute more to the anisotropy. In the application here the expected fluorescence anisotropic lifetime decays vary by 2 orders of magnitude (e.g. the short lifetime component for side chain rotation versus the longer lifetime for tumbling of the protein). Therefore it seemed reasonable to cull the longer correlation times by eliminating from analysis the first part of the anisotropy decay curve, which contains the information for the fast correlation times. The tail is restricted only to the decay of the longer correlation time, which is sought in this case. If successful the goodness of the fit determined by the autocorrelation calculation for residuals would show a residual trace centered around 0 and χ^2 value close to one. Only this result was considered acceptable in this work.

Experimental

Reagents

CG-NBD ceramide (*N*-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-*D*-erythro-sphingosine) was obtained from Avanti Polar Lipids (Alabaster, AL). 4-Chloro-7-nitrobenzofurazan (NBD-Cl) and dimethyl sulfoxide (DMSO) were obtained from MilliporeSigma (St. Louis, MO).

NBD labeling of tear lipocalin

In all samples labeling was performed with a 10 fold molar excess of 4-chloro-7-nitrobenzofurazan (NBD-Cl), Millipore-Sigma, St Louis, MO) over protein [8]. The final concentration of tear lipocalin were 10 μ M and 100 μ M of NBD-Cl in each reaction mixture. Samples were tested at 3 pH values, 6.2, 7.0 and 8.0 with buffers of 50 mM sodium citrate for pH 6.2, 50 mM sodium phosphate for pH 7.0 and 8.0. A previously used constructed dimer of tear lipocalin was also used for validation [8]. Each reaction was run in duplicate. All mixtures were reacted for 1 h at room temperature in the dark. The unlabeled NBD-Cl was removed by a buffer exchange concentrating the sample (approximately 20 fold) followed by dilution with the original buffer without NBD-Cl. For each sample this protocol was performed five times using an Amicon membrane centrifugal filter with a 10,000 MW cutoff. For the sample at pH 6.2, the procedure was repeated an additional 3 times with 50 mM sodium phosphate

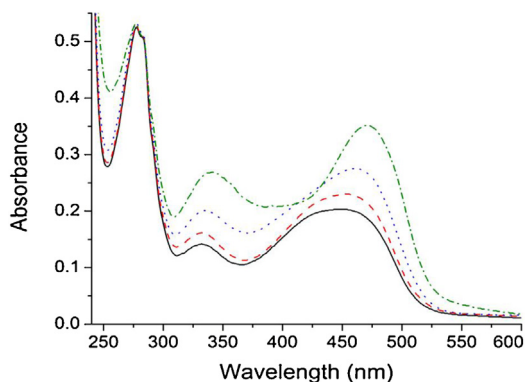


Fig. 1. UV Vis absorbance spectra of NBD labeled tear lipocalin shown at various pH values. Solid black-pH 6.2, red dashed-pH 7.0, blue small dots- pH 8.0, and green dots and dashes- cysteine dimer-NBD.

buffer to change the final pH to 7.0. Labeling efficiency was crudely estimated to be about 25–30% using the molar ratio of NBD label/tear lipocalin. The ratio was estimated by the molar absorptivity, ϵ , for NBD-lysyl residues and TL at $\lambda = 480$ and $\lambda = 280$ nm as $26,000$ and $13,760 \text{ M}^{-1} \text{ cm}^{-1}$ respectively [9–11]. The rationale for this calculation is: 1. the predilection for NBD to label lysines at pH 8.0 is well known, 2. lysines are the predominant amino acid to be labeled in tear lipocalin [3], 3. lysines in tear lipocalin exist in solvent exposed sites to facilitate labeling [2], 4. cysteines and tyrosines are known to exist in buried sites in the protein and have proven resistant to chemical derivatization [2].

UV visible absorption spectroscopy of NBD labeled products

Absorption spectrophotometric measurements were obtained with a Shimadzu UV-2401PC instrument, (Kyoto, Japan). The spectra of NBD labeled tear lipocalin at v, 6.2, 7.0 and 8.0 show peaks of absorbance at about 450, 453, and 460 nm respectively (Fig. 1).

Primary and secondary amines have absorption maxima at 465 and 485 nm, respectively. Houk demonstrated that cysteines of proteins may be labeled at acidic pH while lysines are preferentially labeled above neutrality [9]. NBD adducts of cysteine and tyrosine produce absorption maxima at 425 nm. NBD lysine adducts show a peak at about 480 nm. Our experimental spectral peaks did not match those predicted for the corresponding pH of the reaction of NBD-Cl and tear lipocalin. The N terminal amine is by nature a primary amine. If the N terminal amine was labeled specifically one would have expected a peak in the samples at pH 6.2 and 7.0 to approach 465 nm not 450 or 453 nm. The expected peak at pH 8.0 was 480 nm. The constructed tear lipocalin dimer labeled at pH 8 showed a broad peak at 470 nm (Fig. 1). The value was much closer to the expected value for lysine adducts indicating a bias for lysine labeling at pH 8.0. This may be due to the fact that the dimer does not contain a free cysteine [8]. However, 470 nm is frequently a peak for proteins labeled with NBD [10,11]. The lack of a clear assignment of peaks that match published specific labeling indicate a mixture of labeled amino acids. Further support is garnered by the shape of the peaks which have broad shoulders and may represent more than one site labeled (Fig. 1). In our hands specific labeling could not be achieved for tear lipocalin as it was for Z domain [1].

Simplification of time resolved fluorescence anisotropy

Despite the lack of selective labeling at neutral pH, we analyzed the time resolved fluorescence of the NBD labeled tear lipocalin. The measured anisotropic decay for tear lipocalin with a published correlation time constant of 9–13 ns is faster than the measured intensity decays (50–75 ns) Fig. 2.

In addition, no residual anisotropy was expected and appears to be zero (Fig. 2). Tail fitting, selecting a range to the right of the instrument response function, would be appropriate choice for

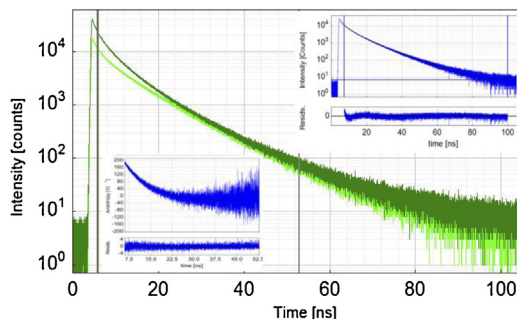


Fig. 2. Example of range restraints for fitting of time resolved fluorescence of NBD labeled tear lipocalin. ●_{ex} = 470, ●_{em} = 530. Central image shows vertically (VV dark green) and horizontally (VH dark green) polarized emission decays. Grey lines are the boundaries for the fit. Upper right inset, fitting for fluorescence lifetime from the magic angle. Decay boundaries are blue vertical bars. Inset, lower left, is the anisotropic decay.

fitting. This avoids: 1. a reconvolution fit for the lifetimes, 2. integrating the emission decays from horizontally polarized excitation, 3. range boundaries that include the IRF and time independent background to both the left and the right of the decay curve and 4. avoiding the components with rapid anisotropic decays. The advantage accrued is to select the components for fitting that were relatively long lifetime decays. The short anisotropic lifetime decays represent the side chain motion [7] whereas the long component reflects the tumbling of the protein as a whole. The boundary shown in Fig. 2, upper inset for the magic angle decay was chosen deliberately to show the irregularity of the residual of the decay close to the IRF value. Normally the fit is chosen with this boundary slightly to the right. The lifetime analysis is minimally impacted but the goodness of the fit improves with this boundary moved to the right.

Therefore, the short range boundary for analysis was selected as is evident by gray lines in the center image, Fig. 2. Beyond about 5 ns after the initial rise, the signals for both the instrument response function and that of rapid side chain mobility (0.2 ns) are excluded from analysis. The long range boundary was extended to a region essentially devoid of residual anisotropy. The constraints rationally limited the region for anisotropic analysis to represent the tumbling rotation of the molecule. The anisotropic decays could be fit to a single exponential parameter, whereas the lifetime decays were generally fit to two exponential parameters. In this case the lifetime decay was fit to two exponentials of 10.6 and 3.1 ns. Because the range for the anisotropic decays was restricted, analysis revealed a single correlation time of about 9 ns with a χ^2 of 1.1. However, a reconvolution analysis performed over the entire range recovered at least one additional component of 0.2 ns that might represent the side chain rotational correlation time. The fractional intensity contribution of this component was <10%. However, the level of uncertainty was high and did not contribute to the goal of recovering the tumbling time of the protein.

Method validation

Tear lipocalin dimer, circular dichroism, binding of C6-NBD ceramide and anisotropy.

In order to completely validate this method, it was necessary to have a protein that could be used as control. We chose a covalent dimeric construct for tear lipocalin. We designed a mutein lipocalin with a cysteine positioned at the ninth amino acid in methods and molecular weight results detailed elsewhere [8]. The cysteine substituted mutant permitted crosslinking to form a dimer. To validate that the dimer retained secondary structural features similar to the monomer, 1.0 mg/ml of tear lipocalin dimer in 10 mM sodium phosphate, pH 7.4 was scanned in the far UV by circular dichroism (Chiron 100V Applied Photophysics). Typical structural features were seen that are similar to that of the tear lipocalin monomer [12]. There is predominant β structure evident as a trough near 212–218 nm, crossover near 200 nm and a positive band below 198 (Fig. 3).

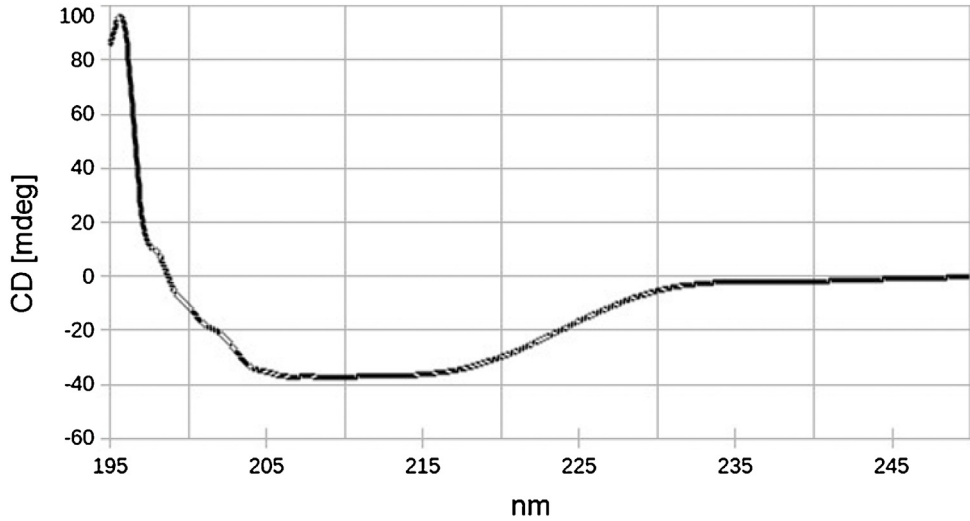


Fig. 3. Far UV circular dichroic spectrum of the cysteine linked dimer of tear lipocalin, 1 mg/ml. There is a trough at 212–218 nm, crossover near 200 nm and a positive band below 198 nm, similar to monomeric tear lipocalin. The spectrum shown is the signal average of four individual spectra.

Next the synthetic dimer of tear lipocalin was functionally tested for lipid binding. In this case a methodological maneuver permitted simultaneously visualizing quenching of native tryptophan fluorescence and C6 ceramide NBD fluorescence. C6 NBD labeled ceramide shows fluorescence when bound inside the lipocalin cavity. Tear lipocalin contains a single tryptophan at position 17, which is deeply buried in the calyx. The excitation wavelength 295 nm was advantageous because tryptophan is the only amino acid with selective absorption at this wavelength. Fortunately the absorbance spectra of NBD-TL shows a marked rise around 295 nm (Fig. 4) so the NBD adduct was also excited at 295 nm.

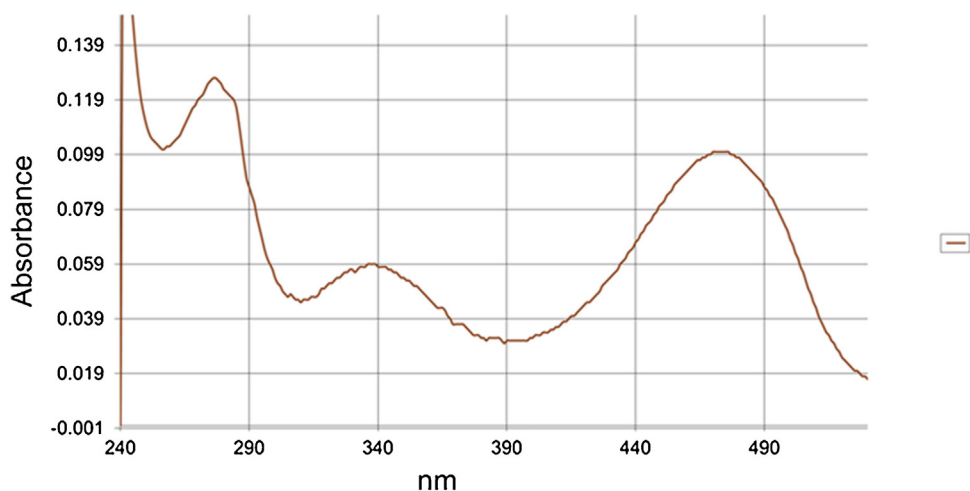


Fig. 4. Absorption spectra of NBD ligand bound to tear lipocalin. The absorption at 295 nm was the basis for simultaneous excitation of tryptophan and the NBD-tear lipocalin adduct (see Fig. 5).

Both tryptophan and NBD bound to a protein are excited simultaneously. Tear lipocalin dimer (21 μM) was incubated in 10 mM NaP with 2.1 and 12 μM of C6-ceramide and continuously monitored between 310–650 nm for tryptophan quenching and NBD fluorescence using a CCD detector (Ocean optics integrated with the Chiron V-100). With an increased dose of C6 ceramide the emission peak at about 330 nm decreases corresponding to quenching of the internally located tryptophan (Fig. 5).

Concomitantly, there is a rise in fluorescence at about 530 nm, which is the emission peak of C6-ceramide when protein bound [13]. The tear lipocalin dimer mirrors the ceramide binding function of the monomer to bind lipid in the calyx.

The dimer served as control to study dimer formation with lipid binding in time resolved fluorescence anisotropy studies. After labeling the dimer with NBD, the free NBD label was removed with a 10 kDa centrifugal membrane filter, as described above. In addition, the retentate was subjected to 5 serial applications in a 30 kDa centrifugal membrane filter (Amicon). This served to further enrich the dimer population for time resolved fluorescence anisotropy.

Fluorescence decays were obtained by time-resolved fluorescence spectroscopy as previously described [8]. Time-correlated single-photon counting was performed with FluoTime 100 (PicoQuant, Berlin, Germany). Samples were excited at 470 nm with a subnanosecond pulsed diode laser, LDH D-C 470 (PicoQuant, Berlin, Germany), repetition rate of 10 MHz, in line with a 475 nm short pass filter (Tech Spec Edmund Optics) and rotatable linear polarizer. An emission OG-530 nm long pass filter and second rotatable linear polarizer and detector were oriented 90° from the excitation path. The fluorescence intensity decay was analyzed using FluoFit iterative-fitting software based on the Marquardt algorithm (PicoQuant, Berlin, Germany). Anisotropic decay ($r(t)$) was measured using the formula:

$$r(t) = \frac{V_V(t) - G V_H(t)}{V_V(t) + 2 G V_H(t)},$$

where $V_V(t)$ and $V_H(t)$ are the parallel and perpendicular polarized time decays respectively. The G factor, is the instrument responsive function/wavelength correction factor and was measured to compensate for polarization bias with the excitation polarizer set in the horizontal position. The

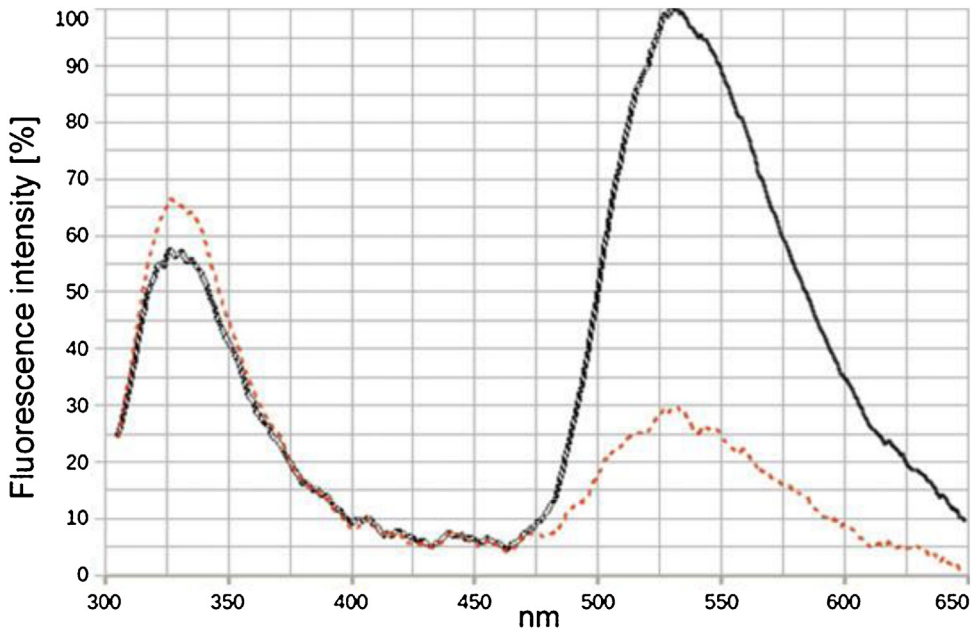


Fig. 5. CCD fluorescence emission spectrum of the tear lipocalin dimer 21 μM incubated with C6-NBD ceramide (2.1 μM orange trace and 12 μM dotted black trace). $\bullet_{\text{ex}} = 295$ nm with the entire emission spectra collected between 305 and 650 nm.

emission polarizer was set at the magic angle to measure lifetime intensity decays. The rotational correlation time (φ) was derived from the fluorescence anisotropic decay $r(t)$ of a sphere by:

$$r(t) = r_0 e^{-t/\varphi}$$

where r_0 is the anisotropy at time (t) 0. Fitting of anisotropic decays was made using both a reconvolution fit and modified tailfit. The goodness of the fit was determined by the autocorrelation of residuals as part of the Fluofit software. Only χ^2 values approaching 1 were considered acceptable. The modified tailfit involved adjusting range to avoid the short lifetimes and the instrument response functions. Fluofit software adds parameters for channel shifts both parallel and perpendicular as well as scatter if necessary. The rationale for the choosing the experimental curve boundaries is the disparity of the expected lifetimes by one to two orders of magnitude. It is known that side chain rotational correlation time is extremely fast about 0.2 ns compared to the expected time for tumbling rotation of the entire molecule 10–20 ns. Therefore, the information contained in the latter portion of the decays represents only the larger molecule rotation.

The fluorescent lifetime was determined at the magic angle setting, 54.7° for the emission polarizer and the rotational correlation time of the dimer was determined by fitting the horizontal (VH) and vertically (VV) polarized fluorescent intensity decays. The dimer exhibited 2 distinct exponentials in fluorescent lifetime decays similar to the monomer which fit well to 10.6 and 3.7 ns (Fig. 6) with fractional intensity contributions for both monomer and dimer of intensity of about 60–40% of the long and short lifetimes respectively.

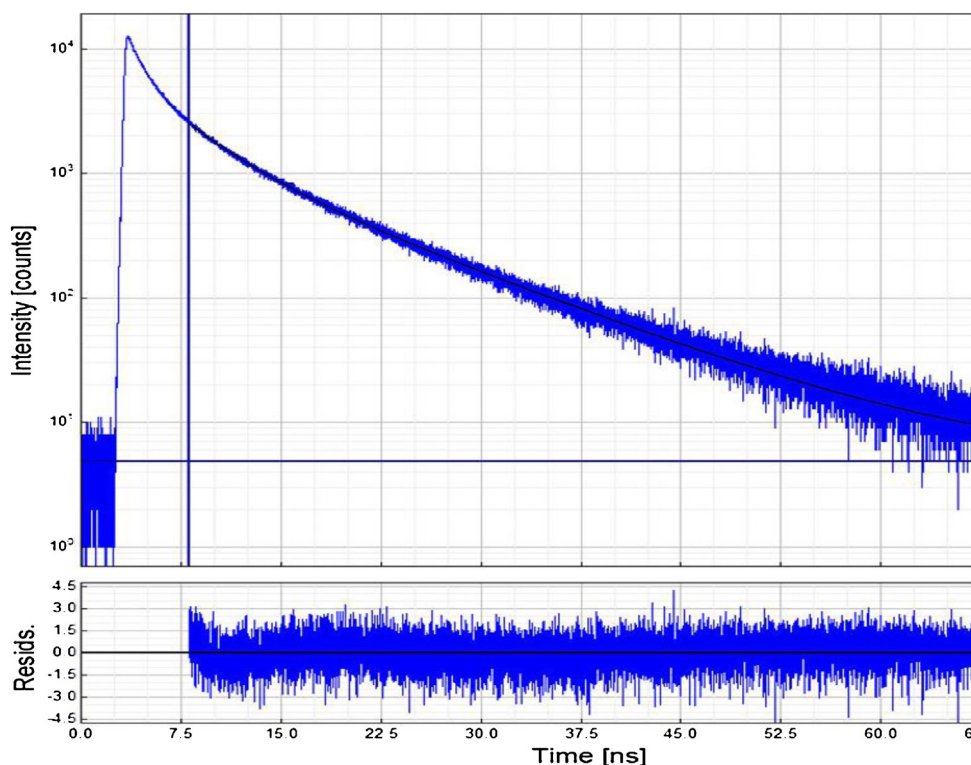


Fig. 6. Time resolved fluorescent decay of NBD labeled dimer, $14 \mu\text{M}$ with emission polarizer oriented at 54.7° from vertical (magic angle), $\bullet_{\text{ex}} = 470$, $\bullet_{\text{em}} = 530$. The curve was fit to two exponentials of lifetimes of 10.6 and 3.7 ns. The range boundaries excluded the instrument response function and short lifetime decays.

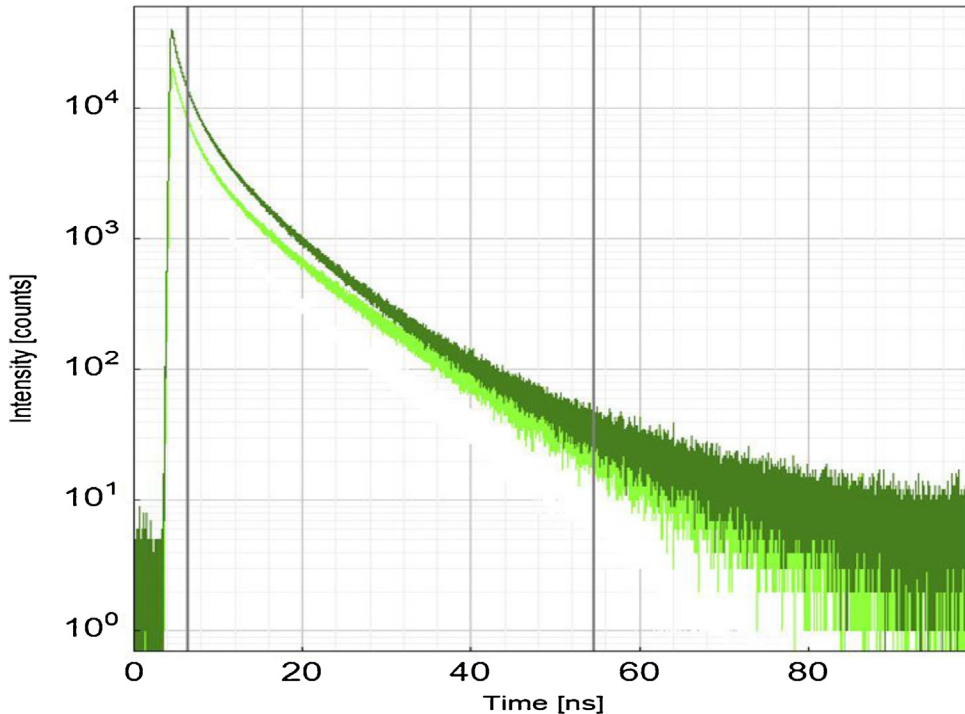


Fig. 7. Time resolved fluorescence decays of NBD labeled dimer, 14 μM , with a vertically polarized decay (VV), dark green curve. The light green trace is the horizontally polarized decay (VH). The fitting range was constrained to avoid the instrument response function and short lifetimes. The G factor was measured at 1.194. The corresponding fit resulted in a calculated correlation time of 19 ns. ●_{ex} = 470, ●_{em} = 530.

Analysis of the vertically and horizontally polarized emission decays revealed a correlation time of about 19 ns, twice that of the monomer (Fig. 7).

Fitting time resolved fluorescence anisotropic decays with dissimilar acquisition times for vertically and horizontally polarized decays.

The sample used for this experiment was that shown in Fig. 2, NBD-tear lipocalin. The method involved reaction of NBD-Cl in 10x molar excess with native tear lipocalin for one hour at pH 8.0, followed by removal of the free label with spin columns [8]. Anisotropic decays, VV and VH were run at exactly the same acquisition time and then the VH was rerun at the same number of counts as VV. This availed the direct comparison of the decay normalized for dissimilar acquisition times versus that obtained for the same acquisition time. For comparison of data, the intensities of individual decays at each time point (over 65,000) were exported from the PicoHarp in a DAT file. The time gated information is embedded in the beginning of these files and must be retained and a time scale constructed in another program (e.g. Matlab, Mathworks). The intensity counts from the horizontally polarized decay at each time point in the decay were normalized to the ratio of the total time of acquisition for vertically polarized decay (t_{VVacq}) and horizontally polarized decay (t_{VHacq}):

$$I_{\text{VHnormalized}} = I_{\text{VH}} * (t_{\text{VVacq}}/t_{\text{VHacq}})$$

The normalized VH result can be re-analysed with fitting software. In our case the normalized VH data replaced the horizontal decay intensities in the PicoHarp dat file. This then was pasted to Fluofit software for data analysis. At this time the G factor was used in the fitting process.

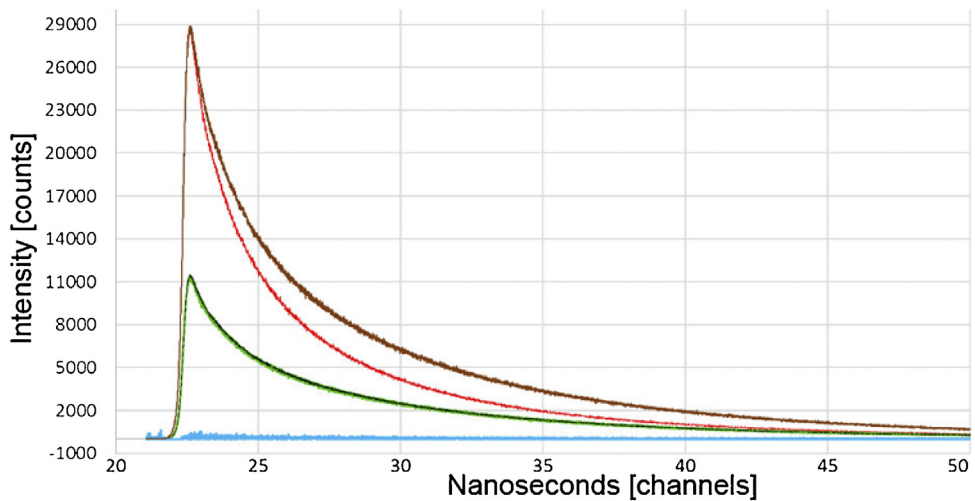


Fig. 8. Normalization of (VH) decay taken at unequal acquisition times compared to (VV) for NBD-TL. Intensity on the y axis is shown on a linear scale to avoid compression of the time-dependent anisotropy. Red (VV) and brown (VH) traces were acquired at the same peak intensity counts but different acquisition times. The green trace (VH) was taken over the same acquisition time as the red trace (VV). The black intensity trace is the result of normalization of the brown trace and overlaps the green trace. The error or difference trace (blue) between the normalized VH trace (black) and the VH trace (green) taken over the same total acquisition time as the VV trace (red). Each channel represent a gated collection at 4 ps. ●_{ex} = 470, ●_{em} = 530.

In order to analyze the resulting normalization data, the anisotropic fluorescence decays for NBD-TL were analyzed. The normalized data set from disparate VH and VV acquisition times was compared to that with the same acquisition times, Fig. 8.

The normalized VH curve originally taken from different acquisitions times overlap precisely to the VH curve obtained from the same acquisition times. Note that this is the raw intensity data from time-correlated photon counting. The IRF data are included and there is no restriction of tail fitting. To analyze the error the individual counts at each of over 65,000 time gates were compared in 0.004 ns intervals for the normalized VH. This included regions with background counts and the IRF signal. The mean absolute percentage error (M) was determined for all of the individual data points (n) according to:

$$M = 100/n \sum |(I_{VH} - I_{VH\text{normalized}})/I_{VH}|$$

The mean absolute percentage error was 3%. If confined only to the data set where there was significant anisotropic information, the error was about 1% as the error straddled the zero baseline. The error is shown at the bottom of the trace in Fig. 8. Fitting of the data, using the same method as described above for Fig. 7, using a standard Levenberg-Marquardt analysis provided in the Fluofit software revealed the same correlation time of 9.5 ns and matched closely the published correlation times obtained for tear lipocalin [6–8].

Although applicable in our situation where the overflow trigger in PicoHarp was activated, the normalization method appears effective to normalize disparate acquisition times. This may be extremely useful when the signal intensity is low in the horizontal decay and a longer acquisition time is needed to collect enough data points for better analysis.

Uniformity of NBD labeling of proteins can be problematic. The methods presented here call into question pH selectivity for N terminal labeling. However, simple NBD labeling of native proteins may be useful for time resolved fluorescence analysis with reduction of fitting parameters and exponential decays. In our case, the consistent lifetimes and correlation times for tear lipocalin are reassuring.

Acknowledgements

This work was supported by Public Health Service grants EY 11224 (BG) and EY 00331 (Institute Core Grant) from the National Eye Institute as well as the Edith and Lew Wasserman Endowed Professorship (BG). The authors are grateful to Volker Buschmann and colleagues from Picoquant, Berlin, Germany who provided valuable theoretical insight and advice regarding the details of the Fluofit and PicoHarp software, use of range constraints for fitting and the analysis for normalization for disparate acquisition times.

References

- [1] L.F. Bernal-Perez, L. Prokai, Y. Ryu, Selective N-terminal fluorescent labeling of proteins using 4-chloro-7-nitrobenzofurazan: a method to distinguish protein N-terminal acetylation, *Anal. Biochem.* 428 (2012) 13–15, doi: <http://dx.doi.org/10.1016/j.ab.2012.05.026>.
- [2] O.K. Gasyimov, A.R. Abduragimov, T.N. Yusifov, B.J. Glasgow, Site-directed tryptophan fluorescence reveals the solution structure of tear lipocalin: evidence for features that confer promiscuity in ligand binding, *Biochemistry* 40 (2001) 14754–14762.
- [3] B.J. Glasgow, Tissue expression of lipocalins in human lacrimal and von Ebner's glands: colocalization with lysozyme, *Graefe's Arch. Clin. Exp. Ophthalmol.* 233 (1995) 513–522.
- [4] B.J. Glasgow, A.R. Abduragimov, T.N. Yusifov, O.K. Gasyimov, J. Horwitz, W.L. Hubbell, K.F. Faull, A conserved disulfide motif in human tear lipocalins influences ligand binding, *Biochemistry* 37 (1998) 2215–2225, doi: <http://dx.doi.org/10.1021/bi9720888>.
- [5] B.J. Glasgow, O.K. Gasyimov, A.R. Abduragimov, T.N. Yusifov, C. Altenbach, W.L. Hubbell, Side chain mobility and ligand interactions of the G strand of tear lipocalins by site-directed spin labeling, *Biochemistry* 38 (1999) 13707–13716.
- [6] O.K. Gasyimov, A.R. Abduragimov, P. Merschak, B. Redl, B.J. Glasgow, Oligomeric state of lipocalin-1 (LCN1) by multiangle laser light scattering and fluorescence anisotropy decay, *Biochim. Biophys. Acta—Proteins Proteomics* 1774 (2007) 1307–1315, doi: <http://dx.doi.org/10.1016/j.bbapap.2007.07.014>.
- [7] O.K. Gasyimov, A.R. Abduragimov, T.N. Yusifov, B.J. Glasgow, Interaction of tear lipocalin with lysozyme and lactoferrin, *Biochem. Biophys. Res. Commun.* 265 (1999) 322–325, doi: <http://dx.doi.org/10.1006/bbrc.1999.1668>.
- [8] B.J. Glasgow, A.R. Abduragimov, Ligand binding complexes in lipocalins: underestimation of the stoichiometry parameter (n), *BBA Proteins Proteomics* 1866 (2018) 1001–1007, doi: <http://dx.doi.org/10.1016/j.bbapap.2018.07.001>.
- [9] T.W. Houk, M. Ovnicek, S. Karipides, pH and polymerization dependence of the site of labeling of actin by 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole, *J. Biol. Chem.* 258 (1983) 5419–5423.
- [10] M.S.T. Gonçalves, Fluorescent labeling of biomolecules with organic probes, *Chem. Rev.* 109 (2009) 190–212, doi: <http://dx.doi.org/10.1021/cr0783840>.
- [11] Y. Doi, T. Hashimoto, H. Yamaguchi, A. Vertut-Doi, Modification of gelsolin with 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole, *Eur. J. Biochem.* 199 (1991) 277–283.
- [12] B.J. Glasgow, A.R. Abduragimov, T.N. Yusifov, O.K. Gasyimov, Studies of ligand binding and CD analysis with apo- and holo-tear lipocalins, *Adv. Exp. Med. Biol.* 438 (1998) 105–112.
- [13] B.J. Glasgow, A.R. Abduragimov, Interaction of ceramides and tear lipocalin, *Biochim. Biophys. Acta—Mol. Cell Biol. Lipids* 1863 (2018) 399–408, doi: <http://dx.doi.org/10.1016/j.bbapap.2018.01.004>.