Editorial

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We would like to take this opportunity and present to our readers in the current issue of our Newsletter some recent results of our research in the area of two-dimensional infrared spectroscopy (2D IR) and single molecule spectroscopy. Both areas have seen tremendous progress over the last few years, based on technological advances in lasers, detectors and data acquisition methods. They exemplify the work in progress as performed at the RLRL, whereby current advances... continued on page 2, left column

Feature Article

TERTIARY HELICAL INTERACTIONS PROBED BY 2D IR

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Introduction

Previous systematic 2D IR studies in our laboratories on water-soluble, isotopically labeled α-helices (1, 2) highlighted the power of 2D IR as a structural tool in biomedical research to obtain novel information and insight on structural constraints and dynamical behavior of proteins in aqueous solution. Major conclusions on those systems in hydrophilic environments led us to believe that the 2D IR technique is suitable for studying more complex proteins and protein aggregates even in the hydrophobic environment of a membrane. Transmembrane (TM) proteins manifest a large variety of biological functions like ion channels, proton pumps, energy transfer facilitators, and molecular receptors (3). We focus our research here on the TM domain of human erythrocyte Glycophorin A (GpA) protein. This helical dimer structure provides a prototype to extend the application of 2D IR from intramolecular to intermolecular interactions, hence revealing its capabilities in dissecting large biomolecules to retrieve residue-level... continued on page 2, right column
and development in the field of laser spectroscopy are combined with the research interests of biochemists and biophysicists to yield methods and tools to answer questions experimentally in a broad range of topics in biomedical research.

Two of our articles, the feature article by C. Fang starting on the first page to the right and a research article by L. Sinks starting on page 8 present recent results obtained in the application of 2D IR spectroscopy to study intermolecular interactions and their fingerprints in 2D IR spectra. This is a further extension of the applicability of 2D IR methods, some of them in combination with isotopic labeling, that we have developed here at the Resource.

The second and fourth article of this issue presents short overviews of results obtained in our ongoing studies of single molecule spectroscopy. Common to both articles is the theme of breaking the diffraction barrier which limits traditional microscopy, by either exploiting the fluctuating fluorescence signal of a molecular beacon as done in the approach presented by E. Mei on page 6, or by localizing fluorescent single molecules through a fitting procedure to within nanometer precision in a digital image as done by A. Sharonov in his article starting on page 10. The latter further applied the technique to study diffusional and directed motion of single proteins in various environments ranging from lipid bilayers to the endo- and exocytosis of living single cells.

As always, the last article is followed by a short summary of our current research activities as well as a list of some recent publications.

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Figure 1: Detailed structure of GpA [75–87] TM helix dimer. The peptide groups of the key glycine residues of the [GxxxxG] motif are represented as respectively colored spheres. The dotted line indicates the proximity between the two Gly79 residues on the dimer interface.

Studies on many helical membrane proteins revealed the importance of helix-helix interactions in the TM domain to facilitate the functional membrane protein folding or formation of higher order structures (4). The coalescence of the preformed TM helices to form the native tertiary structure in the membrane proves to be an effective channel, and a nice example of it is the dimerization of GpA. The framework of this particular helix-helix association was identified as the [GxxxxG] motif (5) and the packing force is provided by van der Waals interactions. Residue Gly79 plays an important role in the dimerization process.
role as revealed by point mutagenesis studies by Engelman and co-workers (6).

In the following article we present some of our results and insight from a detailed 2D IR study of homo- and heterodimers of isotopically labeled 27-residue GpA TM peptide. We put emphases on the 2D IR signature of tertiary interactions, the effects of distinctive local environments on spectral lineshapes, and structural constraints revealed by a careful examination of our data.

Materials and Methods

Sample preparation: The 27-residue GpA TM peptide sequence was the following: KKITLIFG79VMAGVIGTILLISWG94IKK, in which residues 73 to 95 of human GpA were flanked by two Lys residues at each end. Gly79 that participates intimately at the dimer interface was either 13C=16O or 13C=18O isotopically labeled. Gly94 was 13C=16O labeled to be used for the control sample. The typical GpA TM helix dimer structure is illustrated in Figure 1. It possesses a dyad axis indicating a two-fold symmetry between the two dimer helices.

Labeling notation: Several sample series were prepared with either single peptide species (unlabeled, G7913C=16O and G7913C=18O), called homodimers, or mixed species (G7913C=18O/G7913C=16O) called heterodimers. For the heterodimer case, a 50:50 mixture of G7913C=16O labeled and G7913C=18O labeled GpA TM peptides was examined. In the following text the homodimer of Gly-13C=16O labeled GpA peptide is denoted as G*, and of 13C=18O labeled peptide as G**. Then the above-mentioned 50:50 mixture abbreviated as (G79*+G79**) consists of homodimers G79*/G79* and G79**/G79**, and one each of the heterodimers denoted as G79*/G79** and G79**/G79* that become equivalent if a dyad axis exists for the dimer.

2D IR spectroscopy: Two-dimensional infrared measurements are based on the generation of a vibrational photon echo through nonlinear interactions of the sample with three imposing laser fields. Previous reports from our laboratory (2, 7) will provide the interested readers with more experimental details. All experiments here were performed with intense mid-IR (6 µm) pulses of 75 fs duration. The condensed-phase sample emits an echo signal in the phase-matched direction.

Results and Discussion

Linear IR spectroscopic results: FTIR spectra of homodimers G79 and G79* (see Fig. 2a) show almost identical 13C=16O amide-I’ peaks that can be fitted to two broad transitions at 1655 cm⁻¹ (fwhm: 19.3 cm⁻¹) and 1635 cm⁻¹ (fwhm: 22.0 cm⁻¹), suggesting that the insertion of a single 13C=16O label is not altering the vibrational exciton structure of the whole peptide. The desired 13C=16O isotopomer mode appears at 1613 cm⁻¹. In order to reveal the isotopomer states more clearly, a normalization scheme to bring the optical absorption density of the main membrane helical band of various samples to comparable levels was applied.

![Figure 2](https://example.com/fig2.png)
before subtracting the unlabeled GpA sample (G79) from the isotopically labeled samples to obtain the difference spectra as shown in Figs. 2b & 2c. The line-shape of the G79** isotopomer band is significantly asymmetric suggesting multiple underlying spectral components that can be least-squares fitted (between the two vertical lines in Fig. 2c) to two adjacent modes at 1589 cm\(^{-1}\) and 1598 cm\(^{-1}\), which are separated by ~9 cm\(^{-1}\) and having an integrated intensity ratio of about 1.6 (lower-frequency mode is stronger). The absorption cross section of 13C=16O is ~1.3 times larger than 13C=18O because the 13C=16O states are energetically closer and hence coupled more strongly to the neighboring 12C=16O helical band states. These effects are due to exciton interactions.

### Structural constraints derivation

Quantitative analyses of the observed cross peak as well as the polarization dependence of the 2D IR spectra allow us to establish structural constraints. Based on a two-state quantum mechanical description of the selected pairs of isotopically labeled amide-I modes, the two underlying components of the G79** 13C=18O mode in FTIR (Fig. 2c) indicate the coupling strength to be \(\beta \approx 4.5\) cm\(^{-1}\). The intensity in FTIR gives an angle \(\theta \approx 103^\circ\) between the two transition dipole moments assuming \(\beta > 0\). The cross peaks in Fig. 3b are a direct measure of the mixed-mode anharmonicity of the amide modes on the two electrostatically interacting helices \((\Delta AB \approx 3.8\) cm\(^{-1}\)) and a simple model (8) relates the coupling \(\beta\) to \(\Delta AB\) with the additional input of the diagonal anharmonicity \(\Delta \approx 11.6\) cm\(^{-1}\) and the separation between the two isotopomer excitonic states (~24.5 cm\(^{-1}\)). This led to another estimate of \(|\beta| \approx 6.7\) cm\(^{-1}\). The relative magnitudes of spectra in different polarizations can be related to \(P_2(\cos \theta)\) through the tensor elements (9) and the intensity ratio of <xxxx>/<xyyx> at the cross peak gives the range \(\theta = 108–113^\circ\) when \(P_2(\cos \theta) < 0\), which is consistent but somewhat larger than expected (90–105°) for NMR dimer structures in DPC micelles (10). The dipole approximation predicts the dipolar coupling between two typical amide-I oscillators each having a transition dipole moment of 0.40 Debye to be \(805G/R^3\) (cm\(^{-1}\)) where \(G\) is a geometric parameter and \(R\) is the point dipole separation in Å units. For a typical value of \(G = +0.4\) from the above-mentioned NMR coordinates for GpA and an average measured coupling of ~5.6 cm\(^{-1}\), we obtained \(R = 3.9\) Å that agrees well with the interamide distance reported by Smith and co-workers (11). The 2D IR also sets limits on the range of geometric parameters for the helix dimers from an analysis of the coupling constant variations. We derived a standard deviation in the coupling of \(\sigma_\beta \approx 2.3\) cm\(^{-1}\) which can be attributed to variations in both \(G\) and \(R\).

### Local structure probe

A significant lineshape difference in the \(13C=16O\) region was observed between the heterodimer sample \((G79^*+G79^{**})\) and the control sam-
ple (G94*+G79**) in Figs. 3b & 3c. This manifests the distinct local environments sensed by the two $^{13}$C=16O labels either at Gly79 or at Gly94 position. We propose that the rigidity at Gly79 is a reflection of the hydrophobic interior of the SDS micelles, with little water penetration and less fast dynamics, resulting in the smaller inhomogeneous distribution. In sharp contrast, residue Gly94 locates almost at the end of the TM peptide that is in the SDS polar headgroup region. This floppy local environment with higher spatial uncertainties seems to contribute directly to a significant elongation of the Gly94-$^{13}$C=16O diagonal peak in comparison to the Gly79-$^{13}$C=16O peak.

Heterogeneity of different environments: The comparisons between the current GpA TM $\alpha$-helices and the previously reported water-soluble $\alpha$-helices provide some insight regarding the heterogeneity of the respective molecular environments. The rephasing magnitude spectra of the TM dimer and $\alpha$-helix monomer in water (2) are plotted in Figs. 4a & 4b. The environmental effect on the $^{13}$C=18O isotopomer modes is striking: the water-soluble helix (Fig. 4b) shows a much rounder peak at a lower transition frequency than the TM helix (Fig. 4a). Clearly the distribution of amide-I frequencies in the water-soluble helix is much more strongly averaged due to fast water dynamics that cause amide-I frequency fluctuations (12, 13). Recent 2D IR experiments on another TM peptide have reported the residue dependence of this averaging (14).

Conclusion

Our recent 2D IR experiments on GpA TM helices are consistent with a dimeric helix structure in micellar solution derived from NMR. The 2D IR results identify a somewhat larger helix crossing angle (~45°) in SDS from the angular constraints ($\theta \approx 108°$) and also set limits on the range of helix dimer geometrical parameters from the obtained coupling constant distribution ($\sigma_{\beta} \approx 2.3$ cm$^{-1}$). We regard these new experiments as an important step forward to show the power of 2D IR in the study of biologically relevant protein systems in their natural environments. These measurements further confirmed that 2D IR allows visualization of tertiary interactions between various functional groups and they prove that the vibrational energy is delocalized over the two helices of the TM dimer (15).

Acknowledgement

This work was done with R. M. Hochstrasser and supported by NIH Grant P41RR001348.

Literature

As mentioned above, we use a fluorescent single molecule beacon, the dye Nile Red (NR), to “probe”, and thereby image, our object, a lipid vesicle (see ref. 5,6 for more experimental details). The trick is the fact that NR emits brightly only in hydrophobic, but not in hydrophilic environments. Therefore, freely diffusing and continuously laser-illuminated NR molecules in an aqueous solution containing lipid vesicles emit fluorescence bursts only when they collide with the hydrophobic vesicles. Figure 1 shows schematically this process.

The bimolecular kinetics inherent in these bursts is contained in the distribution functions of the on-time \( \tau_{\text{on}} \) and off-time \( \tau_{\text{off}} \) of the observed fluorescence, both

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**Research Article**

**SUB-DIFFRACTION LIMITED MICROSCOPY OF LIPID VESICLES**

*Core Researcher: Erwen Mei*

*Department of Chemistry, University of Pennsylvania*

Part of the RLBL is actively involved in the development of microscopy through the application of single molecule spectroscopy. Out of our endeavors emerged recently the principle of a new microscopy technique that we called *trajectory time distribution optical microscopy* (TTDOM). Instead of measuring light intensities as in conventional imaging techniques, TTDOM uses the on/off-switching of the fluorescence of a single molecule probe, the beacon, colliding with an object of interest to be imaged. The experimentally determined mean of the fluorescence off-rate and the mean of the fluorescence on-time are used to construct an image using a scanning confocal microscope. Experimental results and simulations show that this technique has the capability of achieving optical images beyond the diffraction limit.

In conventional intensity based imaging optical resolution is limited by the wavelength, the numerical aperture (N.A.) and the sample environment, which may perturb the penetrating light on its path through the sample. In the case of microscopy, a successful sub-diffraction approach developed recently is near field scanning optical microscopy \([1,2]\). This technique reported resolution down to a few nanometers. Other known avenues include the 4-Pi confocal microscope \([3]\) and the confocal microscope based on stimulated emission \([4]\). The former uses a second objective, thereby doubling the N.A. to increase the resolution power, and the latter uses a reduced laser spot size through the overlap of two laser sources. Photobleaching combined with point measurements presents still another approach (see *Research Article* by Sharonov).
of which can be obtained experimentally by measuring the fluorescence intensity time records as shown in Figure 2.

Clearly, the single step characteristics of the fluorescence signal for most bursts shows that each burst corresponds to a single NR molecule interacting with a vesicle. Collecting long fluorescence intensity trajectories and binning the observed on- and off-times into a histogram yields a distribution probability function of $\tau_{on}$ and $\tau_{off}$, called $P_{on}$ and $P_{off}$ at a given concentration of NR and vesicles.

The mean on- and off-times of these distributions from many trajectories are then simply obtained by $<\tau_{on}> = \Sigma \tau_{on}P_{on}$ and $<\tau_{off}> = \Sigma \tau_{off}P_{off}$ and plotted in Figure 3. The idea behind TTDOM is now the fact that $<\tau_{on}>$ and $<\tau_{off}>$ are sensitive to the location of the vesicle in the laser focus point spread function. In marked contrast to the usual intensity-based point spread function, which shows a Gaussian-like profile, the point spread function based on $<\tau_{on}>$ and $<\tau_{off}>$ exhibits sharp features and an unusual shape, as shown in Figure 4, as it scans over a lipid vesicle of approximately 100 nm diameter. In this figure, $1/ <\tau_{off}>$, which is directly related to the collision rate $\tau_R$ of NR with the vesicle, is plotted as a function of the relative position of the scanning stage over the laser focus in a confocal microscope, together with the measured (squares) and simulated (dashed) intensity variation. Clearly, the experimentally obtained scans of $1/ <\tau_{off}>$ exhibit distinctive spikes when the vesicle overlaps with the edge of the laser profile, and a plateau when it is centered over the laser profile. This is markedly different from the Gaussian-like intensity distribution. The spikes are caused by the rapid fluctuations of $1/ <\tau_{off}>$ at the edge of the laser profile due to the collision dynamics of NR with the lipid layer. Multiple vesicles present within the laser focus will generate a shape with multiple pairs of spikes, an analysis of which will allow for a measure of the distance between individual vesicles. For example, Figure 5 shows a simulated image of $1/ <\tau_{off}>$ of two 100nm diameter vesicles separated center to center by 230nm, about half of the wavelength of the laser light used to excite the sample. This image clearly resolved the two vesicles, the intensity-based image is shown for comparison. Based

![Figure 3: Experimentally determined mean on- and off-time distributions of NR fluorescence intensity time records in DMPC (a and b) and SOPC (c and d) vesicles. See text for more details.](image1)

![Figure 4: Experimentally determined scan of the reciprocal value of the mean off time as a function of the scan position relative to the laser spot. The dashed line represents a best-fit Gaussian intensity profile.](image2)

![Figure 5: Simulated images of two vesicles either by TTDOM (top) or conventional intensity imaging (bottom).](image3)
on our simulations, two point-like objects with a separation as small as 30 nm should be distinguishable in this way, an improvement over the resolving power of an intensity-based microscopic system. We are currently further developing this technique.

Acknowledgement

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References


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**Research Article**

**2D IR Spectroscopy of a Weak Hydrogen Bond**

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**Introduction:** Recently, it has been found that numerous helical dimers have multiple Cα-H…O contacts that may be involved in weak hydrogen bonds.² Properties of these bonds have been deduced from FTIR spectroscopy.³ In addition 2D IR work on the C=O and C-D modes in liquid formamide have revealed that the hydrogen bond environment is very different for the C=O and C-D modes⁴ and the C-D frequency correlation function decays in agreement with quantum simulations in a manner consistent with weak hydrogen bonding with the C=O. One of the goals of RLBL is to further characterize these unusual H-bonds by the methods of 2D IR.

The dual-frequency method: The two dimensional infrared (2D IR) methods introduced for the study of individual modes of polypeptides¹ ² have found frequent recent application in which the IR pulse sequences mimic some three pulse NMR techniques. The general method of 2D IR experiments performed in the RLBL are based upon on three-pulse laser experiments. An IR laser pulse (k₁) at time zero prepares a vibrational coherent state, which subsequently evolves during a time interval τ (called the coherence time) until the second IR pulse (k₂) is applied. This second pulse creates a new population distribution of the various vibrational states involved. This distribution decays during the called waiting time interval T until the third IR laser pulse (k₃) is applied. Like the first pulse, the third pulse drives the system into a new vibrational coherent state from which a signal field is detected at a time t after the third pulse. The time interval t is correspondingly called the detection time. The signal is heterodyned with the local oscillator field. The raw data collected using this method are in the form of a two-dimensional array of heterodyned signal versus τ and t. The 2D IR spectrum in the (ωτ, ωt) was then obtained by Fourier transformation along the two time axis. As in NMR, variations in the pulse sequence or type can be used to obtain various types of information. The dual-frequency method, akin to heteronuclear NMR, measures coupling between vibrational modes separated by more than the frequency bandwidth of the pulses. It employs a pair of pulses having center frequencies coinciding with one of the vibrators and a third pulse at the frequency of the other.³⁵

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![Figure 1: The amide-I (C=O) and the C-D mode FTIR of D₃-formamide. Insert: DFT optimized structure as discussed in the text. The arrows are transition dipoles.](image-url)
**Formamide spectra:** Formamide has structural motifs found in peptides, so is an important prototype with which to face the experimental challenge of determining the C-D...O=C interaction that will be essential in applying 2D IR to proteins. The weakness of the C-D absorption peak and the necessary high optical density in the C=O region make such experiments less straightforward than those with transitions of nearly the same strength. Both pump-probe and the heterodyned dual-frequency photon echoes of the C-D, C=O and combined C-D/C=O modes have been measured for d₃-formamide in solution.

We performed a calculation of COND₃ at the level of B3LYP/6-311++G** with Gaussian03 that yielded frequencies $\omega_{\text{C-D}} = 2097.8$ cm⁻¹ and $\omega_{\text{C=O}} = 1742.1$ cm⁻¹, with anharmonicities of 75.1 cm⁻¹. The transition dipole of the amide-I mode is tilted 27.4º to the C=O bond (see Figure 1). The C-D mode is localized (99%) on the C-D bond, but its computed transition dipole is tilted by 16.8º. The computed value of $\Delta_{\text{CD/CO}} = \omega_{\text{C-D}} + \omega_{\text{C=O}} - \omega_{\text{C-O}, \text{C-D}}$ is -0.6 cm⁻¹. The computation also predicts that the C-D and C=O mode frequencies are anticorrelated for fluctuations around the optimized structure.

**2D IR of formamide C-D...C=O interaction:** In the heterodyned 2D experiment $k₁$ is used to create a C-D 01-coherence which becomes a C-D population after $k₂$. The third pulse creates the (C-D+C=O), C-D coherence or the C=O coherence which cause radiation at different frequencies if there is coupling, otherwise they will cancel one another in the 2D IR spectrum if they relax similarly. In Figure 2, the y-axis ($\omega_τ$) corresponds to the C-D mode frequency, while the x-axis ($\omega_t$) corresponds to the C=O frequency; the peak shown is termed the cross peak, and its presence indicated that the two modes (C-D/C=O) are coupled. From the evolution of this spectrum with waiting time (T), we can learn about the distribution of structures. The 2D IR spectral shape shows that the C=O and C-D mode frequency deviations have a negative correlation factor: solvent-solute structures having positive deviations of the C=O frequency have their C-D frequencies decreased. The waiting time dependence of the 2D-IR signal indicates that the inhomogeneous distribution evolves to a more homogenous distribution during the first 600 fs. This is seen from the result in Figure 2 that shows the spectrum becoming upright as the correlation becomes lost. It is well known that for uncorrelated distributions the node separating the positive and negative parts of the 2D spectrum is parallel to the $\omega_τ$ axis and that correlation tilts this node depending on the sign of the correlation. The $\Delta_{\text{CD/CO}}$ was estimated from relative values of the cross and diagonal peak intensities to be $\sim-0.85 \pm 0.20$ cm⁻¹ which is in the range of the DFT computed value.

The experimentally determined results (anharmonicity and anti-correlated frequencies) matched well with the theoretical predictions. This observation of C-D/C=O coupling makes more promising the goal of utilizing these types of interactions in tracking structural changes of proteins.

**Acknowledgment:** This work was done with K. Kumar and R. M. Hochstrasser and supported by NIH Grant P41RR001348.
References


Research Article

**Single Molecule Real-Time Imaging of Biologically Related Processes**

Core Researcher: Alexey Sharonov

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**Introduction:** Single molecule detection methods, related to detection of fluorescence from single fluorophores, are an important tool for investigation of numerous biologically important processes. Scanning methods of single molecule imaging (such as confocal microscopy) have limited time resolution in terms of detection of time-triggered events and molecular spatial motion. Wide field illumination and direct optical imaging allow recording of simultaneous events over the whole field of view and can follow the molecular motion along the sample plane (1). Both epi-fluorescence and total internal reflection (TIR) microscopy are useful for fluorescence single molecule imaging. We would like to report in this research article a summary of our investigations in real-time imaging of single proteins and peptides and their interaction with artificial and living cell membranes.

Localization of excitation is extremely important for single molecule observation. In TIR geometry the excitation is through the evanescent field localized within a few hundred nanometers of the surface and observation of single fluorescent molecules is possible even in highly fluorescent environments (2, 3).

![Diffusion in lipids and cell membranes](image)

**Figure 1:** Figure 1: Single protein (ACBP) tracking in POPC supported bilayer. Molecules diffusing on supported bilayer show fast ($D \approx 2.9 \mu m^2/s$, red) and slow ($D \approx 0.07 \mu m^2/s$, blue) components. Both undergo Brownian type of diffusion.

Diffusion in lipids and cell membranes: We have applied this technique to the surface diffusion of lipids in supported lipid bilayers. The bilayer was formed on the glass surface and consists of a double layer of amphiphilic lipids, analogous to a cell membrane. Fluorescently labeled acyl-CoA-binding protein (ACBP) serves as a probe for bilayer properties; it binds easily to the bilayer and diffuses in the membrane plane with the...
lips. If the bilayer is in a liquid phase, where the lipids are free to move in a layer, one would expect Brownian motion with the mean square displacement being linearly proportional to time. The diffusion coefficient derived from this dependence characterizes the bilayer fluidity. We tracked single ACBP molecules attached to lipids and statistically determined diffusion coefficients. We found that the diffusion in the supported bilayer has two components. The fast one, undergone by ~80% of the molecules is $D \sim 2.9 \, \mu m^2/s$ which is typical for unrestricted motion of lipid in the supported bilayer. The slow component, $D \sim 0.08 \, \mu m^2/s$, represents defects in the lipid packing (see Fig.1) that create traps for diffusing molecules.

We also studied the surface diffusion in a living cell membrane. At a very early endocytosis step fluorescently labeled protein transferrin binds to an appropriate receptor on the plasma membrane surface. Tracking of single transferrin/receptor complexes within the membrane plane gives information on the diffusion of single membrane transferrin receptors in a plasma membrane. Quantitative analysis of recorded data was performed with software algorithms for deconvolution of images developed at RLBL. Spot coordinates are fitted to diffraction limited fluorescent spots with two 2D Gaussian profiles and tracked. With single particle tracking microscopy hundreds of fluorescent spots could be followed simultaneously. Due to multiple complex interactions of membrane proteins with other proteins diffusion in the plasma membrane is not purely Brownian. About 54% of the molecules were anchored at certain positions around which they are moving slowly ($D \sim 0.01 \, \mu m^2/s$). 33% of the 46% not anchored molecules had Brownian type of motion much slower ($D \sim 0.25 \, \mu m^2/s$) than in the artificial lipid bilayer.

**Spatial resolution:** The spatial resolution of traditional optical microscopy is limited by diffraction. However the position of a single fluorescent molecule can be determined with high accuracy at nanometer precision. If the surface density of molecules is low enough and molecules are well enough separated, it is possible to determine accurately the position of each molecule in an entire image. High-resolution imaging is based on collecting of many images of non-overlapping molecules and then combining these images to achieve the high resolution image of localized molecules (5-8). In our approach, we used fluorescent molecules freely diffusing in solution and binding to our object of interest at low enough rates to have well separated molecules in every time frame [see also the Research Article by E. Mei]. We demonstrated a high-resolution image of two 100 nm POPC large unilamellar vesicles (LUV, Figure 2) by overlapping 853 coordinates of single NileRed molecules, collected in 4095 sequential frames. A similar framing approach was used to locate TMR molecules diffusing near a patterned surface (4).
Optical cross sectioning: The evanescent wave in TIRFM doesn’t penetrate deeply enough from the glass/media interface to allow other than surface regions to be accessed. We have developed a new excitation scheme for imaging optical cross sections in a grazing angle excitation configuration that allows visualization and recording of single molecules far from the interface.

By means of this grazing angle cross sectioning we have visualized molecular motion on a cell membrane, internalization events and diffusive and directed motion in the cytoplasm. For example the primary steps of receptor-mediated endocytosis of transferrin were examined in monkey kidney fibroblast cells (COS7). The binding of transferrin leads to the formation of receptor-transferrin clusters at clathrin-coated pits, which are internalized to form vesicles in the cytoplasm. Transferrin receptors localized on the entire cell surface were thereby visualized in 3D space (see Figure 3).

In summary real-time imaging methods with single-molecule sensitivity that have been developed at RLBL were successfully applied to protein and peptide interactions with artificial and cell membranes, to trafficking in living cells and to conformational dynamics of single proteins.

Acknowledgment: This work was done in collaboration with K. Burgess and F. Schroeder (Texas A&M) and supported by NIH Grant P41RR001348.

References

Current Technological Development and Research at the RLBL

The main subjects under investigation at RLBL are shown below. If your research may be interfaced with any of these approaches we urge you to contact us. A full description of each of these topics is also available at our Web site http://rlbl.chem.upenn.edu.

- **Two-dimensional infrared spectroscopy (2D-IR) and infrared analogues of NMR**: Heterodyned photon echo spectroscopy and spectrally resolved three pulse IR photon echoes are employed to investigate the amide I, amide II and amide A region and other transitions of peptides and small proteins. Single or dual frequency experiments are feasible to obtain quantitative information on the anharmonic potential surfaces, the vibrational mode coupling, the frequency distributions and vibrational frequency correlation functions with the highest time resolution. Efforts are under way to couple the 2D-IR technique with temperature jump and pH jump methods.
Methodologies to investigate protein folding and macromolecular conformational dynamics: Detection and characterization of intermediate states in conformational dynamics and unfolding is another developing technology at RLBL. Laser-based temperature-jump instruments and a fast mixing device are available for these investigations in combination with transient absorption techniques in the visible and infrared spectral regions for bulk samples.

Investigations of single molecular assemblies using either confocal microscopy or total internal reflection fluorescence microscopy: It is now possible to examine the properties of single molecules using fluorescence and phosphorescence in association with confocal microscopy. Studies using resonance fluorescence energy transfer, dual wavelength detection, polarization resolution, two photon absorption are feasible for single molecule investigations of biomolecules and imaging techniques of single cells. The RLBL also has coupled single molecule detection methods with mature time correlated photon counting technology for fluorescence lifetime imaging with polarization resolution. Excitation wavelengths available cover the whole visible range from 400 – 850 nm and include continuous wave and pulsed picosecond and femtosecond lasers. Additionally, an objective-type total internal reflection microscope (TIRFM) that images the fluorescence of single molecules excited by the evanescent generated by total internal reflection by means of a fast CCD camera has recently been set up and coupled to various laser sources. Typical penetration depth of the evanescent field is ~150 nm, ideally suited for example to study single molecules located in cell membranes.

Energy transfer and fluorescence monitoring of biological dynamics: Monitoring fluorescence lifetimes and anisotropies reveals details of structural dynamics of peptides and proteins. Techniques have been developed at the RLBL to monitor these properties of fluorescing species on the femtosecond to nanosecond timescale. Vibrational energy dynamics, electron transfer reactions, and UV cross-linking are open for investigation.

A Selection of Recent Publications


2D IR spectra of the $^{13}$C=O isotopomers of alanine...


Application for Use of the RLBL

Title:

Investigators (PI first)  Degree  Department / Institution / Address

1.

2.

3.

NIH Support Sources  NIH Start/End Date  Other Support Sources
Grant Number(s)   (MM/DD/YY-MM/DD/YY)   Agency and Grant Number(s)

1.

2.

Abstract: Describe briefly (200-250 words) the scientific goals and methods.

Logistics: Equipment to be supplied by applicant, needed from RLBL, and anticipated time.

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