performing Förster resonance energy transfer (FRET) measurements. Unlike fluorescence intensity which has been traditionally used in FRET experiments, fluorescence lifetimes do not depend on fluorophore concentration, detection efficiency, illumination intensity and uniformity. Therefore, lifetime FRET overcomes the artefacts arising from intensity measurements and provides improved precision in investigating the protein unfolding procedure. Herein, we developed a novel technique for performing lifetime FRET using a maximum likelihood estimator (MLE) adapted from single molecule studies. We demonstrate the feasibility of our detection technique by monitoring the unfolding procedure of the membrane protein Bacteriorhodopsin (bR) labelled with the FRET pair Alexa Fluor 488 (donor) and Alexa Fluor 647 (acceptor). A home-built laser scanning confocal microscope and two avalanche photodiode detectors (APDs) are used for detection with high sensitivity and the fluorescence decays are collected using time correlated single photon counting (TCSPC). When determining a fluorescence lifetime with less than 2000 photons, the conventionally used least squares approach is not appropriate. Therefore, a MLE previously developed in our lab defined by multinomial statistics is used to accurately extract molecular fluorescence lifetimes from as little as 10 photons. These are then used to calculate FRET efficiencies and hence the conformational state of bR.

2999-Pos
Exploiting the Rise Time of Acceptor Fluorescence by FRET-FLIM in Living Cells
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Previously (Borst et al. (2008) Biophys. J. 95) a time-resolved fluorescence methodology has been described for quantitative determination of Förster resonance energy transfer (FRET) between donor-acceptor pairs in biological macromolecules by analyzing the time resolved rise of acceptor fluorescence upon donor excitation. The main advantage is that only those molecules are monitored involved in the energy-transfer process. This contrasts with the more conventional method that measures time-resolved fluorescence of donor molecules and thereby probing a mixture of FRET-active and FRET-inactive populations. We have extended the determination of rise times of acceptor fluorescence to measure FRET in living cells with fluorescence lifetime imaging microscopy (FLIM). Parameters describing the rise of acceptor fluorescence and the decay of donor fluorescence can be determined via simultaneous global analysis of multiple FLIM images thereby increasing the accuracy of the recovered parameters. In the present study, plant protoplasts were transfected with a visible-fluorescent-protein fusion composed of a 6-amino-acid peptide flanked by enhanced GFP (eGFP) and mCherry for illustration of the new data analysis method. It is demonstrated that the distances estimated with the present method are substantially smaller (and more realistic) than those estimated from average donor fluorescence lifetimes. The latter over-estimation is due to a fraction of non-transferring donor molecules, which makes the average fluorescence lifetime of the donor longer. In addition, combining fit results of fluorescence kinetics at different detection wavelengths allows correcting for the contribution of molecules that are not able to transfer their excitation energy. The high fraction of non-transferring eGFP in the construct is explained by taking the presence of non-matured mCherry into account. Software to reproduce the presented results is provided in an open-source and freely available package called "TIMP" for "The R project for Statistical Computing".

3000-Pos
Macrophages Create a Lysosomal Synapse to Digest Aggregated Lipoproteins
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Atherosclerosis is the underlying cause of the majority of heart attacks and strokes. Lipoprotein accumulation and degradation by monocyte-derived macrophages is a central event in the pathogenesis of atherosclerosis. Data show that the vast majority of low density lipoprotein (LDL) in atherosclerotic plaques is aggregated and avidly bound to the subendothelial matrix. We employ various microscopy and biochemical techniques in both cell culture and animal models to investigate the mechanism of uptake of aggregated LDL (agLDL) by macrophages. We show that macrophages create an extracellular, acidic compartment where the cells contact the agLDL, and lysosomal contents are delivered to these compartments, thereby forming an extracellular hydrolytic compartment - a lysosomal synapse. Active acidification of these special-

ized compartments and aggregate catabolism were observed by fluorescence ratiometric time-lapse imaging of an *in vitro* cell culture model. Biochemical assays, employing radiolabeled agLDL demonstrated an increase in free cholesterol in aggregates contained in the lysosomal synapse. This cholesterol can be delivered to the cell, initiating the process of macrophage cholesterol loading and ultimately causing progression of the atherosclerotic plaque.

Although *in vitro* systems provide insight into macrophage uptake of lipoproteins, ultimately we must strive to understand the function and behavior of macrophages in intact animals. Relatively little is known of the fate of monocytes once they migrate into atherosclerotic lesions. To this end, we employ intravital multiphoton microscopy in mouse models of atherosclerosis to allow observation of macrophages within the artery wall.

Our studies elucidate the mechanism of a novel pathway for catabolism of agLDL by macrophages. Better understanding of the mechanisms by which macrophages interact with lipoproteins in the subendothelium may lead to new approaches to inhibit lipid accumulation in macrophages and thus, may be of therapeutic value in preventing atherosclerosis.

3001-Pos
Broad-Beam Fluctuation Spectroscopy for Non-Flow Cytometry and Clinical Diagnostics
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Fluorescence fluctuation spectroscopy (FFS) and related techniques such as FCS, PCH and FIDA have been valuable tools for biophysical research, but have not found widespread clinical application. Many clinical blood diagnostics measure the concentration of particular cell types in the blood. These tests are typically performed using fluorescent antibodies and flow cytometry. We present a novel scanning FFS system, which we term Broad-beam Scanning Fluctuation Spectroscopy (BSFS), with application to cytometry. BSFS uses a much larger (~1 nL) observation volume than conventional FCS, so that the fluctuations measured result from cells, rather than individual molecules. This technique is well suited to the measurement of cell concentration, as the correlation analysis also yields a measurement of the sample volume, allowing an absolute concentration to be determined. BSFS is a viable alternative for a variety of cell-based clinical diagnostics, while lacking the optical and fluidic complexity of a flow system.

3002-Pos
Mobility Analysis in Living Yeast using 4Pi CFM
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Continuous fluorescence microphotolysis (CFM) is a powerful tool to analyze molecular mobilities and association reactions in single living cells but has mostly been restricted to diffraction limited focal volumes. Recently, we introduced the theoretical and experimental framework to combine CFM with super-resolution microscopy by utilizing a 4Pi point spread function in mobility analysis.

Here we show that this methodology can be readily applied to living yeast cells under physiological buffer conditions using water-immersion lenses. Yeast cells are relatively small with a typical diameter of 5 μm and the intracellular individual compartmentalization with a large vacuole and the nucleus results in small volume elements with unhindered diffusion. Therefore diffusion measurements with an engineered point spread function should be beneficial to recover the diffusion coefficient as this approach is less susceptible to improper positioning of the laser.

Using 4Pi CFM we were able to clearly recover the diffusion coefficient of GFP in the cytoplasm and the nucleus of living yeast cells. Additionally, the mobility of GFP-tagged proteins involved in nucleo-cytoplasmatic transport was analyzed. While the diffusion coefficient of a GFP-tagged cargo was determined to be in a range expected for a molecule of this respective size, we found evidence that the diffusion coefficient of a GFP-tagged transport receptor was reduced compared to the expected value for purely free diffusion. This might indicate that the molecule is subject to a certain degree of unspecific binding in the cytoplasm of yeast.

3003-Pos
Scanning Laser Image Correlation (SLIC) Measurements in Zebra Fish Larvae
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Scanning Laser Image Correlation (SLIC) is a technique to measure the flow of small particles and to observe local flow patterns over an area. SLIC can be applied to situations including blood cells flowing through blood vessels or tracer particles flowing through microfluidic channels. The main advantage of SLIC over other flow measurement techniques is that SLIC can be scaled to measure flow in areas ranging from microns to centimeters wide. To accomplish this, an image is first acquired through laser scanning and analyzed with number and brightness analysis (N&B) to identify regions of flow. This is used as a guide to manually (or potentially automatically) select a pattern within the image, such as a line along the center of a channel, that is then scanned repeatedly with the laser beam. Since the entire image is not scanned in each measurement SLIC measurements can be obtained quickly and efficiently. The results of these scanned patterns are analyzed with the recently developed pair correlation technique to extract the rate of flow and to identify characteristic flow patterns such as turbulence, particles that adhere to the channel walls, and variable velocity along the length of the channel.

In the work presented here we demonstrate the effectiveness of SLIC by measuring blood flow in a zebra fish model. With SLIC, we are able to obtain blood flow measurements equivalent to those obtained with other techniques. We are also able to map the rate of flow and to observe variability in flow rate over time. This indicates that SLIC has potential to measure blood flow in other animals as well and may hold potential as the basis of a medical device.

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3004-Pos
Fluorescent Lifetime Imaging of Lignin in the Plant Cell Wall

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Lignin, a highly complex but integral part of plant cell wall , is indigestible and therefore a concern in biomass deconstruction for cost effective biofuel production. A way to address this issue is to manipulate the plant to generate a cell wall that is amenable to breakdown. However, little is know about the actual assembly of lignin during plant cell wall biosynthesis. Fluorescent Lifetime Imaging Microscopy (FLIM) utilizes the lifetime of the auto-fluorophore signal generated, rather than its intensity , to create an image. An interesting characteristic of lignin is that it is highly auto-fluorescent in the UV excitation region due to its phenolic ring composition. Thus, it may be possible to infer structural and organizational information of lignin polymerization using this imaging technique. In this study FLIM is used to resolve differences in lignification in the plant cell wall during development.

3005-Pos
Using High Resolution Photoemission Electron Microscopy to Quantify the Absorption Properties of Human Eumealnin and Pheomelanin

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A novel approach to photoemission electron microscopy is used to enable the first direct measurement of the absorption coefficient from intact melanosomes. Two cases are examined in detail: bovine melanosomes from choroid and iris tissue, and human iris melanosomes isolated from different colored irides. The difference in absorption between newborn and adult bovine uveal melanosomes is in good agreement with that predicted from the relative amounts of the monomeric precursors present in the constituent melanin as determined by chemical degradation analyses. The results demonstrate that for melanosomes containing eumelanins, there is a direct relation between the absorption coefficient and the relative 5,6-dihydroxyindole: 5,6-dihydroxyindole-2-carboxylic acid (DHI:DHICA) content, with an increased UV absorption coefficient associated with increasing DHICA content. The human iridal melanosomes from different colored irides contain both eumelanin and pheomelanin; the ratio of which varies with iris color. Taking pigment composition for these melanosomes into account, the absorption coefficient of natural eumelanin is determined to be a factor of six greater than that of natural pheomelanin. This contrasts synthetic models for eumelanin and pheomelanin, which exhibit comparable absorption coefficients at this wavelength. This difference between natural and synthetic systems underscores the care that must be exercised in using such polymeric systems as models for photophysical and photochemical properties of melanosomes. The determined absorption coefficients for the iridal melanosomes further suggest that the correlation between epidemioleigical data and the eumelanin:pheomelanin ratio reflects an increased exposure of tissues to UV light rather than a increased reactivity of pheomelanin.

3006-Pos
Correlative TEM/FISH Imaging of Microbial Communities

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Biofilms are the predominant lifestyle for most microbes. Mixed microbial communities are adapted to their specific environment allowing them to live under sometimes extreme conditions and to perform complicated metabolic pathways. They often show high species interdependencies suggesting that such communities are highly organized. However, little is known about detailed interaction between the microbes. This is due to difficulties in both studying them at sufficient resolution to visualize the individual community members in their biofilm context and at the same time identifying each community member.

To overcome this problem we have developed a technique that combines TEM sample preparation and imaging with 16S rRNA Fluorescence In-Situ Hybridization (FISH). This technique can be applied both to thin resin sections and to cryo TEM, allowing the identification of the microbes for both TEM techniques.

We applied the technique first to already well characterized Acid Mine Drainage biofilms, and we currently transfer the same technique to the termite hindgut microbial community. Our goal here is to clarify the mechanism how the up to 200 reported species in the hindgut (Warnecke et al. (2007), Nature 450) manage to efficiently degrade lignocellulose. TEM shows the attraction of the bacteria towards the wood particles and interesting extracellular features such as vesicles, which seem to be involved in the digestion process. While cells with different morphologies are usually densely packed, even single bacteria prove the ability to digest the material by themselves as they manage to create tunnel-like structures through the wood. Since the termite hindgut community is regarded one of the most effective lignocellulose degrading systems, it is of great interest for the research on second generation biofuels.

Fluorescence Spectroscopy

3007-Pos
Exploiting the Environmental Sensitivity of Fluorescent Proteins Allows Unambiguous Discrimination of Genetically Identical Labels

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The application of transcriptional fluorescent fusion proteins has revolutionized the field of single molecule biophysics. Recent efforts to expand beyond GFP by way of mutagenesis have generated nearly comprehensive libraries of fluorescent proteins exemplified by a wide variety of spectral excitation/emission profiles, photostabilities, and quantum efficiencies. A different, but similarly applicable approach to expand the usefulness of these fluorescent constructs can be achieved by combining spectral imaging with multivariate analysis to quantitatively separate each of the emitting species present in a sample. A recent demonstration of this imaging methodology, under the extreme condition of two genetically identical fusion proteins (YFP) conjugated to two different membrane receptors (TLR4 and the BK channel), reveals that the slight perturbation of the local environment of the fluorescent reporter is sufficient for spectral separation, and quantitatively interpretable images. In this talk we will highlight several recent discoveries enabled by multivariate analysis of environmentally specific perturbations of fluorescence in both prokaryotic and eukaryotic systems, and demonstrate the implications of these findings on the commonly used analytical tools, fluorescence correlation spectroscopy (FCS) and fluorescence resonant energy transfer (FRET). Finally, the potential for mapping local chemical environments based on multivariate analysis of spectral images will be discussed.

3008-Pos
Accurate Color Tuning of Firefly Chromophore by Modulation of Local Polarization Electrostatic Fields

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In molecular biology, fluorescent proteins have become a unique marking tool for gene expression, environmental pollutants, and monitoring the dynamics of AIDS virus and single-molecule motors [1-3]. Particularly, the mutagenesis