Journal of Fluorescence, Vol. 5, No. 1, 1995

Video Fluorescence Microscopic Techniques to Monitor Local Lipid and Phospholipid Molecular Order and Organization in Cell Membranes During Hypoxic Injury

Xue Feng Wang,[1] K. Florine-Casteel,[2] John J. Lemasters,[1] and Brian Herman[1,3,4]

Received November 16, 1994; accepted November 16, 1994

Digitized video microscopy is rapidly finding uses in a number of fields of biological investigation because it allows quantitative assessment of physiological functions in intact cells under a variety of conditions. In this review paper, we focus on the rationale for the development and use of quantitative digitized video fluorescence microscopic techniques to monitor the molecular order and organization of lipids and phospholipids in the plasma membrane of single living cells. These include (1) fluorescence polarization imaging microscopy, used to measure plasma membrane lipid order, (2) fluorescence resonance energy transfer (FRET) imaging microscopy, used to detect and monitor phospholipid domain formation, and (3) fluorescence quenching imaging microscopy, used to spatially map fluid and rigid lipid domains. We review both the theoretical as well as practical use of these different techniques and their limits and potential for future developments, and provide as an illustrative example their application in studies of plasma membrane lipid order and topography during hypoxic injury in rat hepatocytes. Each of these methods provides complementary information; in the case of hypoxic injury, they all indicated that hypoxic injury leads to a spatially and temporally heterogeneous alteration in lipid order, topography, and fluidity of the plasma membrane. Hypoxic injury induces the formation of both fluid and rigid lipid domains; the formation of these domains is responsible for loss of the plasma membrane permeability barrier and the onset of irreversible injury (cell death). By defining the mechanisms which lead to alterations in lipid and phospholipid order and organization in the plasma membrane of hypoxic cells, potential sites of intervention to delay, prevent, or rescue cells from hypoxic injury have been identified. Finally, we briefly discuss fluorescence lifetime imaging microscopy (FLIM) and its potential application for studies monitoring local lipid and phospholipid molecular order and organization in cell membranes.

KEY WORDS: Video fluorescence microscopy; membrane; fluorescence polarization microscopy; fluorescence resonance energy transfer; fluorescence quenching imaging; fluorescence lifetime imaging microscopy; hypoxic injury; lipid.

INTRODUCTION

Digitized video microscopy (DVM) is a powerful technique with which scientists can examine a variety of cellular functions as they actually occur in single living cells [1–3]. The advantage that DVM provides over more classical experimental strategies which employ large cell populations or intact organs is that many of the events which occur within a cell or tissue population are *heterogeneous* in nature, and may occur in a rapid or sudden fashion (i.e., important individual events may be buried in the noise of the population). This is particularly important in studies of plasma membrane activity, as regional differences in membrane structure and function would be obscured in studies using large cell populations. In addition, the organization of the plasma membrane could be altered by the isolation procedures commonly employed to obtain purified plasma mem-

[1] Department of Cell Biology and Anatomy, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599–7090.
[2] Department of Pathology, Duke University Medical Center, Durham, North Carolina 27710.
[3] Cell Biology Program, Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599–7090.
[4] To whom correspondence should be addressed.

1053–0509/95/0300–0071$07.50/0

branes. The spectroscopic techniques of fluorescence polarization, fluorescence resonance energy transfer (FRET), and fluorescence quenching have provided important information about membrane structure and function [4–6], but have historically employed purified membrane fractions, artificial membranes, or large populations of cells and thus could potentially suffer from the concerns listed above. However, the combination of the light microscope with these spectroscopic techniques can overcome these limitations and provide important additional information regarding the dynamics of membrane structure. Recent advances in optical microscope technology and digital image processing techniques coupled with the availability of highly sensitive video cameras and newer, more specific fluorescent probes of membrane function have provided the opportunity to examine the structure and function of plasma membranes in single intact cells with very high temporal and spatial resolution.

In this paper, we discuss three quantitative video fluorescence microscopy techniques which have been adapted for use with fluorescence spectroscopic approaches to monitor the molecular organization in the plasma membrane of single living cells. These include (1) fluorescence polarization imaging microscopy, used to measure plasma membrane lipid order, (2) fluorescence resonance energy transfer (FRET) imaging microscopy, used to detect and monitor phospholipid domain formation, and (3) fluorescence quenching imaging microscopy, used to spatially map fluid and rigid lipid domains. We review these different technologies and their practical utilization, limits, and potential future developments. As an example of the application of these technologies to a biological question, recent findings regarding plasma membrane lipid order and topography during hypoxic injury in rat hepatocytes [7–9] are discussed. The development of these digitized video microscopic techniques has led to an increased understanding of the role of the plasma membrane in hypoxic injury and has pointed to specific sites for the development of interventional strategies designed to protect cells against hypoxic injury. Lastly, we discuss fluorescence lifetime imaging microscopy (FLIM) and how FLIM can be applied to studies of plasma membrane lipid/phospholipid cytoarchitecture and function.

FLUORESCENCE POLARIZATION IMAGING MICROSCOPY

One of the most widely used methods for measuring membrane lipid order has been fluorescence polarization spectroscopy [10,11]. Localized alterations in lipid order are thought to modulate the activity of plasma membrane proteins (e.g., receptors, transport enzymes) [12]. The ability to measure plasma membrane lipid order with high temporal and spatial resolution would be of great value in understanding the effects of lipid organization on plasma membrane protein activity [13,14]. Fluorescence polarization is based on the principle that fluorescent molecules absorb light most efficiently when their absorption transition moments are near parallel to the electric field vector of the excitation light [15]. For fluorophores embedded in a membrane, only those molecules whose absorption dipole moments are oriented exactly or near parallel to the plane of polarization of the excitation light will be excited. Light emitted from the excited fluorophores will also be polarized, but the amount of polarized emission parallel to the plane of excitation will decrease if there is rotational motion of the fluorophores during its excited-state lifetime. In a bilayer, rotation is related to the local acyl-chain order, with highly ordered lipid (gel phase) allowing limited angular rotation and disordered lipid (fluid phase) allowing greater rotational motion.

There are a large number of reports employing measurements of fluorescence polarization (anisotropy) in studies of plasma membrane lipid structure/function. Most of these studies employ measurements of cells or membranes in suspension and thus provide only averaged information regarding plasma membrane structure and function. The rationale for two-dimensional fluorescence polarization microscopic measurements arises because the physical state of the plasma membrane is spatially heterogeneous not only between cells, but also within an individual cell. For example, we have been monitoring alterations in plasma membrane lipid order and topography during hypoxic injury in rat hepatocytes. During hypoxic injury, plasma membrane blebs form which contain membrane that is structurally distinct from that found in the cell body region [8,9]. Even though there are a large number of reports employing fluorescence polarization for studies of membrane lipid order and topography, only a few reports use fluorescence polarization microscopy coupled with imaging technologies for the study of cellular membrane structure and physiology.

Fluorescence polarization microscopic measurements can be performed in two ways. The first is steady-state fluorescence polarization imaging: In this approach, a standard fluorescence microscope equipped with excitation and emission polarizers is used to generate two-dimensional maps of fluorescence anisotropy which can then be used to determine the rotational mobility of a fluorescent analog and detect anisotropic orientational

distributions [8,16,17]. The second approach is time-dependent fluorescence polarization imaging. In time-dependent fluorescence polarization imaging, the alterations in the rotational mobility of the fluorophore are monitored during the excited-state lifetime, thereby providing two-dimensional maps of the time-dependent emission anisotropy and rotational correlation time of the molecule of interest. Two-dimensional time-dependent emission anisotropy measurements can be made using fluorescence lifetime imaging microscopy (FLIM) (see below). The use of fluorescent probes to monitor molecular order and motions within liposome bilayers has been recently reviewed [11]. In this review, the advantages and/or limitations of fluorescent versus other methods of monitoring membrane order and dynamics were discussed. Fluorescence polarization using multiple probes to evaluate the fluidity of liposomes and other lipid assemblies has also been recently described [18]. This publication focused on (1) how to select the proper extrinsic probe, (2) how to optimize polarization measurements in terms of both theoretical and practical issues, (3) the determination of the location of the fluorescent probe in the sample being examined, (4) how to introduce the probe into the sample, (5) the ideal ratio of probe to the lipid, and (6) how to overcome artifacts introduced by light scattering.

Microscopic fluorescence polarization measurements on single cells present certain problems not associated with fluorometric measurements [13,14]. The major difficulties include the depolarizing effects of the microscope optics, the orientational dependence of the observed fluorescence polarization, and the lower fluorescence signals obtainable from individual cells. The depolarization effects of the microscope optics arise from the necessity of using objective lenses with high apertures for maximal collection of fluorescence, rather than a narrow slit of effectively zero aperture. A theoretical treatment of high-aperture observation and the corrections required for use with high-numerical-aperture microscope objectives has been previously described [13,19]. The orientational dependence of the observed fluorescence polarization is due to the fact that the surface of all cells is three dimensional, rather than a two-dimensional bilayer. Thus, surface curvature, in addition to the rotational mobility of the fluorophore during its excited-state lifetime, will determine the final observed depolarization. Corrections for the effects of surface curvature require knowledge of the probe excited-state orientation distribution in order to determine orientation-dependent fluorescence polarization [8]. Lastly, since microscopic measurements are performed on single living cells, it is imperative to keep the probe concentration as low as possible in order to minimize alterations in membrane function. Thus, the overall fluorescence signal from membranes covering single cells is relatively low compared to that obtained with suspensions of cells. This problem can be overcome by using highly sensitive image devices and additional image processing techniques. In summary, successful use of the microscope to undertake measurements of fluorescence polarization requires the consideration of a variety of factors and requires a balance between selection of probes which can accurately sense different membrane structure and function, yet provide the requisite sensitivity for accurate detection of a relatively small numbers of fluorophores.

In the future, several developments can be anticipated. The current selection of probes is limited. For example, [1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene, *p*-toluenesulfonate] (TMA-DPH), a widely used fluorophore for measurements of plasma membrane lipid order, absorbs maximally in the ultraviolet wavelength range (i.e., 360 nm), where cells are most susceptible to damage. In addition, NADP(H) and other cellular constituents absorb strongly in this wavelength range and therefore background nonspecific autofluorescence can diminish the sensitivity (signal to noise) of the measurement. In order to improve sensitivity, new types of probes for the study of plasma membrane lipid order and topography need to be developed [20,21]. In terms of the instrumentation to perform microscopic fluorescence polarization measurements, more accurate polarization measurements will require the ability to perform polarization detection over a wider range of angles and the use of more sensitive imaging devices. By combining total internal reflection fluorescence (TIRF) microscopy and polarization measurements, it has recently become possible to observe molecular motion, order, rotation, and orientation in greater detail with higher accuracy in a flat bilayer [14]. In addition, surface plasmon techniques [22] can be used to further enhance surface fluorescence polarization imaging. Recently, confocal fluorescence microscopes have become widely available for imaging cell structure and function in three dimensions. In principle, it is possible to modify a confocal microscope for confocal polarization measurements [23,24]. The advantage of such an approach is that better spatial resolution as well as three-dimensional interrelationships with regard to the rotational mobility of membrane components become amenable to study. Another improvement for polarization imaging measurements may evolve from "phase-lock" polarization detection techniques, in which the excitation and emission polarization are modulated and detected using

a polarization modulator [25]. This technique will significantly increase dynamic range and sensitivity for fluorescence polarization imaging. Thus, though the use of fluorescence polarization imaging to study molecular events and structure in living cellular membranes is only just beginning, it is likely that its application will rapidly expand.

FLUORESCENCE RESONANCE ENERGY TRANSFER (FRET) IMAGING MICROSCOPY

Fluorescence resonance energy transfer (FRET) imaging [26,27] is a microscopic technique for quantifying the distance between two molecules conjugated to different fluorophores. In principle, if one has a donor molecule whose fluorescence emission spectrum overlaps the absorbance spectrum of a fluorescent acceptor molecule and is spatially close enough to the acceptor molecule, the donor will transfer its excitation energy to the acceptor nonradiatively via a dipole–dipole interaction. This energy transfer manifests itself as both quenching of donor fluorescence (in the presence of acceptor) and an increase in the emission of acceptor fluorescence (sensitized emission). Because FRET decreases in proportion to the inverse sixth power of the distance between the two probes, this phenomenon is effective at measuring separation of the donor and acceptor labeled molecules when they are within 10–100 ° of each other [28]. Recently, two reviews on FRET theory, relevant literature, practical suggestions, application in the study of membrane organization, and new developments in FRET techniques have been published [27,29]. In these papers, extensive bibliographies are provided as well as a table containing R_0 values for a variety of different donor–acceptor pairs. In principle, the measurement of FRET through a microscope can provide the same information that is available from macroscopic solution measurements of FRET; however, FRET microscopy has the additional advantage that the spatial distribution of the FRET can be measured throughout the image rather than providing average information over the entire object of interest. Because energy transfer occurs over distances of 10–100 °, a FRET signal corresponding to a particular field (or pixels) within a microscope image provides additional information beyond the microscopic limit of resolution down to the molecular scale. The ability to measure interactions and distances of molecules provides the principal and unique benefits of FRET for microscopic imaging [26,27,30]. FRET imaging is particularly useful in examining temporal and spatial changes in the distribution of fluorescent molecules in membranes of the living cell [4,29,31].

In practice, there are two ways to perform FRET microscopy imaging, through the use of steady-state or time-resolved methods. In steady-state sensitized ratio FRET imaging, FRET imaging is performed using a standard fluorescence microscope equipped with excitation/emission filters and sensitive video cameras which are currently used for the popular technique of ratio imaging. In FRET imaging, one typically measures the emission at two wavelengths, that of the donor and acceptor. Since FRET results in an increase in acceptor emission (sensitized emission) and a decrease in donor emission, the ratio of the intensity of the acceptor-to-donor fluorescence emission when excited at donor excitation can be taken as a convenient experimental measure of FRET [26]. The value of this ratio depends on the average distance between donor–acceptor pairs. Since this method is simple and easy to perform, it has been used in a variety of different FRET imaging applications [9,32]. The drawback of using steady-state FRET imaging is that it is difficult to perform rigorous quantitative measurements, since there are several sources of distortion which need to be corrected for (e.g., polarization effects, overlapping of the donor and acceptor emission spectra). Time-resolved FRET imaging allows the quantitative measurement of donor–acceptor separation distances and is based on measuring the lifetime of the donor in the presence and absence of the acceptor. FRET imaging can be performed using FLIM.

Successful undertaking of FRET imaging requires that several points be considered. First, the concentration of the donor and acceptor fluorophore and the ratio of donor to acceptor need to be tightly controlled. In order to achieve high *S/N*, high fluorescence signals are preferred. However, too high a concentration of dye can cause self-quenching and disordered membrane function [33]. Second, photobleaching needs to be prevented. Almost all fluorescent molecules are sensitive to photobleaching. Photobleaching can alter the donor–acceptor ratio and therefore the value of FRET. To prevent photobleaching, highly sensitive image devices and lower levels of excitation intensity are preferred. Third, ideally, the donor emission spectrum should substantially overlap the absorption spectra of the acceptor. Fourth, there should be relatively little direct excitation of the acceptor at the excitation maxima of the donor. Fifth, the emission of both the donor and acceptor should occur in a wavelength range in which the detector has maximum sensitivity. Sixth, there should be little if any overlap of the donor absorption and emission spectra, thus mini-

mizing donor–donor self-transfer. Seventh, the emission of the donor should ideally result form several overlapping transitions and thus exhibit low polarization. This will minimize uncertainties associated with the K^2 factor. Lastly, when antibodies are used for FRET imaging, it is necessary to determine whether the antibody reagent itself may be affecting the FRET measurements. For example, because antibody labeling may affect the original molecule's structure and therefore distance information, the correct selection of labeling procedure and antibody needs to be seriously evaluated.

In the future, the following developments may play an important role in the successful application of FRET imaging techniques. As previously mentioned, donor and acceptor fluorophore labeling for quantitative FRET imaging is a critical problem. New labeling methods need to be developed. Recently, green fluorescent protein (GFP) has been introduced as a marker for gene expression [34]. GFP is a unique protein which can also be used for FRET measurements. By inserting the GFP cDNA into the donor molecule cDNA using molecular biology techniques, direct labeling of the donor molecule can be achieved. It also has been reported that a rhodamine-like fluorescent protein also exists. Thus, it may be possible to obtain directly labeled donor and acceptor molecules where the label is actually part of the molecule itself. Of course, it will be imperative to prove that the insertion of GFP does not affect the activity of the labeled molecules in any way. The continuing development of FLIM will become more important in the increasing research applications of FRET microscopy. FLIM will provide more quantitative FRET imaging measurements; however, currently existing FLIM instrumentation is too expensive to be adopted for a wide variety of applications. It is likely that within a short time, commercially available FLIM will be available at a reasonable price, which will serve to enlarge the number of FRET imaging applications. Other types of FRET imaging instruments will be developed for different applications. Confocal FRET imaging is one logical extension of this instrument. Recently, near-field optics [35,36] have been developed, and by combining near-field optics with FRET, observation of membrane–substrate interactions with high resolution will be possible.

FLUORESCENCE QUENCHING IMAGING MICROSCOPY

There are several good review papers on the use of fluorescence quenching for probing membrane structure [37,38]. Generally, fluorescence quenching experiments are relatively easy to perform, because they do not require sophisticated instrumentation. Fluorescence quenching techniques have become a popular technique for the study of protein structure and obtaining membrane topographical information. The concept of fluorescence quenching is based on the Stern–Volmer equation. As is the case with fluorescence polarization and FRET measurements, fluorescence quenching measurements can be made using steady-state or time-resolved methods. Fluorescence quenching can occur via different mechanisms (e.g., collisional vs. concentration dependent) and occur over different time scales. Depending on which fluorescence quenching process is operant, different time scales can exist and thus dictate the optimal measurement approach (i.e., steady state vs. time resolved). In studies examining membrane topography, brominated or nitroxide-labeled phospholipids have become popular probes because of their depth of penetration into the lipid bilayer. In lipid bilayers, these quenching lipids align with their alkyl chains perpendicular to the bilayer plane. The quencher group can be positioned at different places along with the chain. In the membrane, the quenching process occurs in a relatively slow lateral motion, and the quenching by the quencher lipids is considered to be largely a steady-state phenomenon [37–39]. Certain spin labels (e.g., TEMPO) have also found utility in studies of membrane structure (see below). The quenching of biomacromolecular fluorescence by solute quenchers has also become a widely used and powerful technique to obtain topographical structural information about proteins, nucleic acids, and membrane systems [37–39]. These quenching techniques are based upon the accessibility of intrinsic (i.e., the amino acid tryptophan) or extrinsic fluorescence probes which are attached to a biomacromolecule or to small quenchers (e.g., iodide, acrylamide, oxygen). Conformational changes in the biomacromolecule can then be monitored in terms of changes in the accessibility of the quencher to the intrinsic or attached extrinsic fluorophore.

As was the case for fluorescence polarization and FRET measurements, extension of solution fluorescence quenching principles to the microscope and the two-dimensional state is a relatively easy procedure. It has recently become possible to carry out two-dimensional fluorescence quenching imaging of a single cell membrane by simply modifying existing video fluorescence microscopy techniques [9]. Such two-dimensional fluorescence quenching can provide spatial information regarding the topographical structure and dynamics of the

cell membrane. To the best of our knowledge, even though there exists a large literature reporting on the use of fluorescence quenchers, our recent publication is the first to describe such an application of fluorescence quenching imaging [9].

In practice, the most important aspect of performing fluorescence quenching measurements is in the selection of the proper fluorescent probes and quenchers. The size, charge, and polarity of the quencher need be to considered. In addition, environmental factors such as pH, temperature, pressure, and viscosity need to be carefully controlled. Moreover, quenching measurements are affected by photobleaching. Therefore, highly sensitive image devices for low-light detection are needed to prevent photobleaching. The future of fluorescence quenching techniques will likely rest on the development of more specific and efficient quenchers that display high selectivity for specific lipid phases or distinct phospholipid/lipid molecules.

MULTIPLE MICROSCOPIC TECHNIQUES FOR THE MEASUREMENT OF PLASMA MEMBRANE LIPID STRUCTURE IN HEPATOCYTES DURING HYPOXIC INJURY

We have applied fluorescence polarization, FRET, and TEMPO quenching imaging to the study of plasma membrane lipid structure in hepatocytes during injury due to chemical hypoxia. We describe our research aims, experimental procedures, and results as an example of application of these microscopic techniques.

Hypoxic Injury in Hepatocytes

There is increasing evidence that changes in the physical state of the plasma membrane are a major factor in the evolution of irreversible hypoxic injury. A number of recent studies from our laboratory in which the progression of cell injury was examined in single rat hepatocytes indicated that hypoxic injury in rat hepatocytes is characterized by the formation of plasma membrane blebs [7,40]. These blebs initially form from the tips of microvilli and then fuse and coalesce, resulting in the appearance of a few large blebs. The onset of cell death was found to be a rapid event associated with the rupture of one of these large blebs. As such dramatic alterations in plasma membrane structure occur during hypoxic injury, we hypothesized that alterations in the physical state of the plasma membrane may underlie the injury process. To test this hypothesis, we wished to define changes in plasma membrane lipid structure that occur during the evolution of hypoxic injury in hepatocytes by using video fluorescence microscopy. In order to identify and monitor lipid and phospholipid molecular order, several different fluorescence imaging approaches were developed, which are described in detail below. The results of these studies, when combined with data obtained from both our laboratory as well as those of other investigators, has led to the findings that activation of a pH-dependent phospholipase A_2 leads to alterations in the physical state of the plasma membrane resulting in destabilization of the plasma membrane and eventually in the breakdown of the plasma membrane permeability barrier and onset of irreversible injury (cell death) [41–44].

Multiparameter Digitized Video Microscopy (MDVM) System

The various digitized video fluorescence microscope techniques described in this paper are based on the design of a multiparameter digitized video microscope (MDVM) (Fig. 1) [45,46]. The MDVM system is comprised of a Zeiss IM-35 inverted microscope equipped with epi-illumination and phase contrast optics. The excitation light source is either a xenon or mercury arc lamp. Fluorescence images are collected with a microchannel plate (MCP) image intensifier (Genllsys, Dage-MTI, Inc., Michigan City, IN) coupled by a relay lens to a CCD camera (Model CCD-72, Dage-MTI) operating at video frame rates. Images are acquired and digitized at 8 bits per pixel resolution by an imaging board set (Datacube Inc., Danvers, MA) running in a SUN 3/160 UNIX computer. Computer-driven filter wheels allow for selection of both the intensity and wavelength of excitation. The high sensitivity of the image intensifier camera, together with the frame-averaging capability of the computer, allow work at very low levels of excitation energy and fluorophore concentration, thus preventing photobleaching or photodamage that might lead to disruption of normal cell activity. The image intensifier camera can also be used to obtain bright-field images, allowing the acquisition of phase and fluorescent images from same cells. Image acquisition and analysis software were developed in our laboratory. A temperature-controlled perfusion chamber is used to maintain physiologic pH, CO_2 levels, and temperature during the experiment [40].

Fluorescence Polarization Imaging

Digitized fluorescence polarization microscopy (DFPM) was used to monitor lipid order during hypoxic

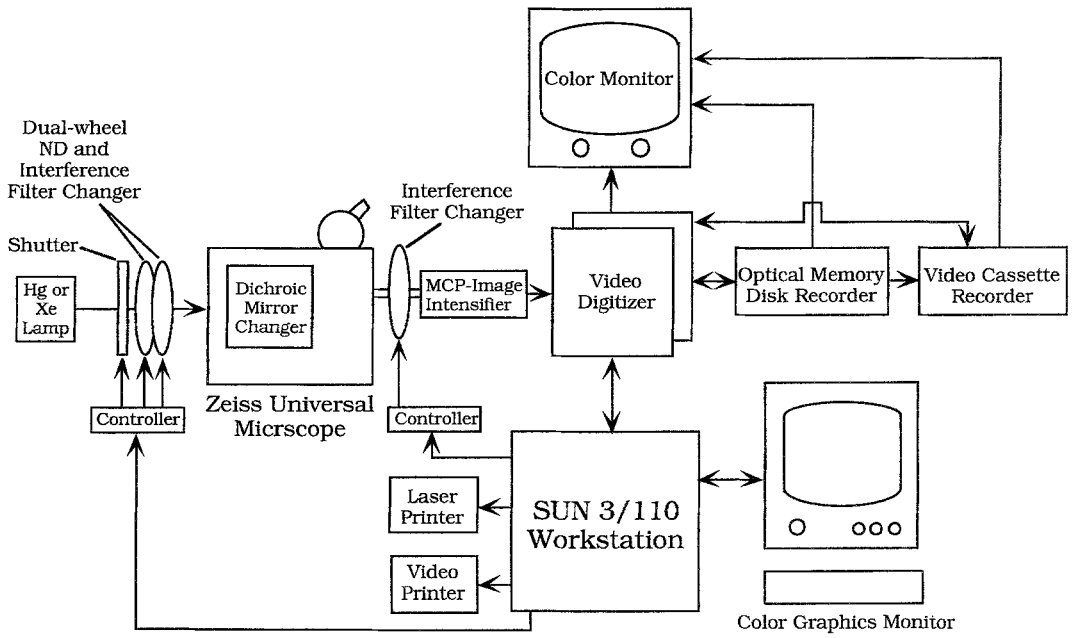

Fig. 1. Schematic diagram of multiparameter digitized video microscopy (MDVM) system.

and anoxic injury [8,16]. DFPM allows plasma membrane lipid order to be spatially and temporally quantitated. However, as previously discussed, there are two issues associated with quantitative measurements of fluorescence polarization through the optics of the microscope which need to be addressed. First, the high numerical aperture of the objective lens leads to partial depolarization of collected fluorescence. This as well as other polarization-dependent light transmission properties of other microscope optical components must be corrected for. The second issue concerns the effect of cell curvature on the observed polarization ratios. Since membrane-bound fluorophores are oriented predominantly parallel to the phospholipid fatty acyl chains, their orientation relative to the plane of polarization of exciting light will change along a curved membrane surface. Thus, corrections for the effects of membrane curvature on the observed polarization measurements must also be made. By a modification of the method of Axelrod [19], expressions relating the observed polarization ratio to lipid order, position on the cell surface, and lens numerical aperture were developed. Models for the angular freedom of movement of TMA-DPH in both fluid and gel-phase states were developed, tested using liposomes composed of fluid or gel-phase lipids at room temperature, and corrected for the effects of cell curvature and objective numerical aperture. The polarization ratio values at any position on the cell perimeter as a function of lipid order were then calculated [16].

The information on lipid order that is obtainable from a single cell by this method is dependent on three factors: cell geometry, the properties of the membrane probe, and the particular combination of excitation/emission polarizer orientations used in image acquisition. In studies examining hepatocyte hypoxic injury, the hepatocyte and its blebs were modeled as the simple case of a spherical membrane surface (a plasma membrane bleb) labeled with a rod-shaped probe of known orientation in the membrane (TMA-DPH). Using this approach, it was possible to describe the plasma membrane lipid order profile around the bleb perimeter with a set of four fluorescence images. A different cell geometry, or a probe with different absorption/emission dipole orientations or diffusion properties, would require the appropriate modifications to the equations used in order to determine the functional dependence at the polarization ratio on lipid order and membrane surface location. Image acquisition at other polarizer orientations might also be necessary.

A set of polarization measurements consisted of four images, each an average of 64 frames (2-sec illumination time), obtained as follows. A film polarizer was placed in the excitation light path in series with a 365-nm bandpass filter, a neutral density filter (to minimize probe photobleaching), and a heat filter. Fluorescence was observed through a 395-nm dichroic mirror in series with a 420-nm longpass filter and another film polarizer. A pair of images was obtained with the emission polarizer oriented first parallel and then perpendicular to the

polarization direction of the exciting light. The excitation polarizer was then rotated 90° and a second pair of images was obtained, again with the emission polarizer oriented parallel and perpendicular to the exciting light polarization direction. For each set of measurements the camera/intensifier gain was set for maximal image intensity without pixel saturation. Figure 2 is an example of a DFPM measurement in a rat hepatocyte following 30 min of chemical hypoxia. Cultured hepatocytes on glass coverslips were labeled with TMA-DPH (5 m*M*) in a probe-containing buffer. After a 1 min of labeling at 37°C, the TMA-DPH-containing solution was removed and fluorescence polarization measurements were made. Cell viability during the measurements was monitored using the fluorescent probe propidium iodide, which stains the nuclei of nonviable cells. Fluorescence polarization ratios ($F_{\parallel}/F_{\perp}$) were computed on a pixel-by-pixel basis from digitized image pairs after background subtraction and image alignment. For display as pseudocolor ratio images, polarization ratios were converted to gray levels (0–255), using a multiplication factor of 100, and were then assigned color values ranging from violet (low ratio) to white (high ratio). From the ratio images the corresponding cone angles were calculated. The two blebs on the left and right sides of the cell are nearly identical and the $F_{\parallel}/F_{\perp}$ ratios correspond to cone angles of 10–20° and a lipid order parameter of S = 0.91–0.98 [$S = (1/2) \cos q_{max} (1 + \cos q_{max})$]. For comparison, using lipid vesicles roughly the size of rat hepatocytes, we found cone angles for TMA-DPH of 32 ± 4° (S = 0.78 ± 0.05) and 17 ± 2° (S = 0.935 ± 0.015) in fluid and gel-phase lipid vesicles, respectively [16]. The data indicated that the fluorophore resided in a rigid membrane environment after hypoxic injury in rat hepatocytes.

Fluorescence Resonance Energy Transfer (FRET) Imaging

Sensitized fluorescence was monitored to indicate the extent of FRET and hence the distance between two fluorophore-conjugated lipid analogs (donor and acceptor) during hypoxic injury [9]. Since FRET results in an increase in acceptor emission (sensitized fluorescence) and a decrease in donor fluorescence, using direct excitation of the donor, the ratio of acceptor to donor fluorescence (I_A/I_D) (which is measured at the peak of the acceptor and donor emission intensity, respectively) was used as an experimental measure of FRET [47]. The value of this ratio depends on the average distance between the donor–acceptor pairs, and thus on the state of aggregation of lipids in the membrane. The main advantage of this approach for the study of single cells is its speed, simplicity, and experimental convenience. Its main drawback is that FRET efficiencies cannot be calculated from these data. Uncertainties in determining FRET efficiency in plasma membranes are due to (1) the complex geometry of the cell surface, (2) the nonrandom distribution of lipids in the plasma membrane, (3) the variation of the donor and acceptor relative to one another when in the plasma membrane, and (4) the fact that each donor in the cell membrane sees a unique surrounding distribution of possible acceptors. FRET efficiencies could in theory be calculated from the I_A/I_D ratio, but only through the use of a rather complicated, cumbersome calibration procedure [48]. For these reasons only relative changes in average proximity of the donor and acceptor which can be monitored without relating these changes to absolute distance were monitored.

The lipid analogs *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-hexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (NBD-PE) and 2-(12-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)dodecanoyl-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (NBD-PC) were used as donors, and *N*-(lissamine rhodamine B sulfonyl)-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (Rhd-PE) was used as acceptor [9]. Selection of optimal donor–acceptor ratio is important to ensure optimal transfer efficiency [47]. Before measurements were performed on hepatocytes, donor–acceptor phospholipid concentrations were varied to determine the maximum amount of energy transfer observed for a given donor–acceptor pair. From these measurements, a donor concentration of 2 m*M* and a donor–acceptor ratio (NBD-PE:Rhd-PE) of 1:2.5 (acceptor concentration of 5 m*M*) were found to provide maximum energy transfer signal without photodynamic damage to the hepatocyte plasma membrane. Hepatocytes were incubated with the donor–acceptor phospholipids for 2 min at 37°C and washed twice in buffer, and FRET imaging measurements were carried out.

Fluorescence images were collected from labeled cells with two filter arrangements: (a) excitation (460 nm) and emission (520 nm) filters for donor, and (b) excitation filter for donor (460 nm) and emission filter for acceptor (620 nm). By ratioing the images obtained with filter combination (b) with respect to filter combination (a), regional changes in the relative concentration of donor and acceptor were imaged very sensitively. An example of FRET images obtained with the NBD-PE/Rhd-PE lipid probe pair at different stages of hypoxic injury is shown in Fig. 3. A high amount of FRET (large ratio: red) indicates a closer packing of PE lipid mole-

cules, whereas a lower ratio (blue) signifies less FRET and a looser packing of PE lipid molecules.

A normoxic hepatocyte demonstrates a relatively loose packing of PE lipid molecules as indicated by a low FRET image (Fig. 3A). As previously discussed, in hepatocytes, hypoxic injury is associated with plasma membrane blebbing. This blebbing occurs in three stages; stage I is characterized by the development of small surface blebs, stage II is characterized by the fusion and coalescence of stage I blebs, and stage III is characterized by rupture of one of the stage II cell surface blebs. During stage I blebbing, cells display a mixture of areas of high and low FRET in the cell body, while blebbed regions display a low degree of FRET (i.e., looser packing of lipid) (Fig. 3B). In late stage II blebbing, FRET increased both in nonblebbed and blebbed regions (Fig. 3C); at this point in the injury process, blebs show a high degree of FRET, potentially indicative of changes in lipid topography that occur just prior to bleb rupture. A FRET image of a hepatocyte in stage III of blebbing is shown in Fig. 3D. One of the surface blebs has ruptured, resulting in the onset of cell death; a high degree of FRET (closer packing of lipid) was found in the plasma membrane. Quantitation of the FRET imaging data in both nonblebbed and blebbed regions of hepatocytes during hypoxic injury indicated that the nonblebbed regions (cell body) were the first to demonstrate an increase in FRET, which continued to increase up until just prior to loss of cell viability. Conversely, blebbed regions of the plasma membrane showed a more delayed and smaller increase in FRET (about 50%) relative to that seen in the nonblebbed regions. Similar results were obtained using NBD-PC/Rhd-PC as a donor–acceptor pair. Interestingly, when NBD-PC and Rhd-PE were used as a donor–acceptor pair, no increase in FRET was seen (compared to normoxic cells) during hypoxic injury, indicating that plasma membrane phospholipids of the same type increase their packing density as hypoxic injury progresses.

Fluorescence Quenching Imaging

TEMPO (2,2,6,6-tetramethylpiperidinyl-1-oxy) was employed as a quencher of plasma membrane TMA-DPH fluorescence to measure plasma membrane lipid structural changes during hypoxic injury [9]. TEMPO is a water-soluble lipophilic spin label that has been shown to selectively partition into fluid regions of membranes and cause contact quenching of membrane probe fluorescence [49]. We have found it to be a very efficient fluorescence quencher for several membrane-localized fluorophores, a notable exception being the long-chain indocarbocyanine dyes. The use of TEMPO and TMA-DPH allowed the development of a difference ratio imaging method to measure the degree of TEMPO quenching imaging; the spatial organization and quantitative amount of TEMPO quenching in membrane reflects the order of the phospholipids of the plasma membrane. For example, fluid regions of the plasma membrane would have a higher amount of quenching compared to rigid gel-phase regions of the plasma membrane.

TEMPO quenching measurements were performed using MDVM. A 365-nm bandpass filter and a neutral density filter were used in the excitation light path. The fluorescence image was observed through a 395-nm dichroic mirror in series with a 420-nm longpass filter. Fluorescent-probe-containing buffer was prepared by adding an aliquot from a 2 m*M* stock solution of TMA-DPH in dimethylformamide to buffer (to a final probe concentration of 5 m*M*), followed by brief vortex mixing. Cultured hepatocytes at various stages of hypoxic injury were mounted on the microscope stage. Once a cell was located, TMA-DPH-containing buffer was added for 2 min, the cells were washed twice, and then TEMPO quenching measurements were made. An image of the cell was obtained before TEMPO addition (the 'before' image, I_B); then 25 m*M* TEMPO was added and after a 2-min equilibration period (determined empirically in other studies) another image of the cell was obtained (the 'after' image, I_A). Following acquisition of the before and after images, a difference ratio image $[(I_B - I_A/I_A] \times C$, was calculated on a pixel-by-pixel basis, where C is a constant used to bring the values of the ratio image into the full range of gray-scale values. The resultant difference image is thus related to the degree of quenching; the difference image was pseudocolored and displayed on the computer monitor. A high ratio represents greater TEMPO accessibility, more quenching, and a more fluid lipid environment, whereas a low ratio represents less TEMPO accessibility and a more rigid lipid environment based on the phase partition behavior of TEMPO.

TEMPO quenching imaging of a TMA-DPH-labeled hepatocyte at different stages of hypoxic injury is shown in Fig. 4. A high amount of quenching (large ratio: red) indicates a more fluid membrane, whereas a lower ratio (blue) signifies less quenching and a more rigid (gel) membrane [39]. A normoxic hepatocyte has a relatively fluid membrane as indicated by a high ratio after TEMPO addition, as shown in Fig. 4A. Within 15–20 min after initiation of hypoxia, cells are in stage I of blebbing and small blebs have formed. Initially, stage I blebs are more fluid than the normoxic plasma membrane. Subsequently, these cells display a mixture of areas of high and low quenching in both the cell body and blebs. However, on average, the cell body displays less

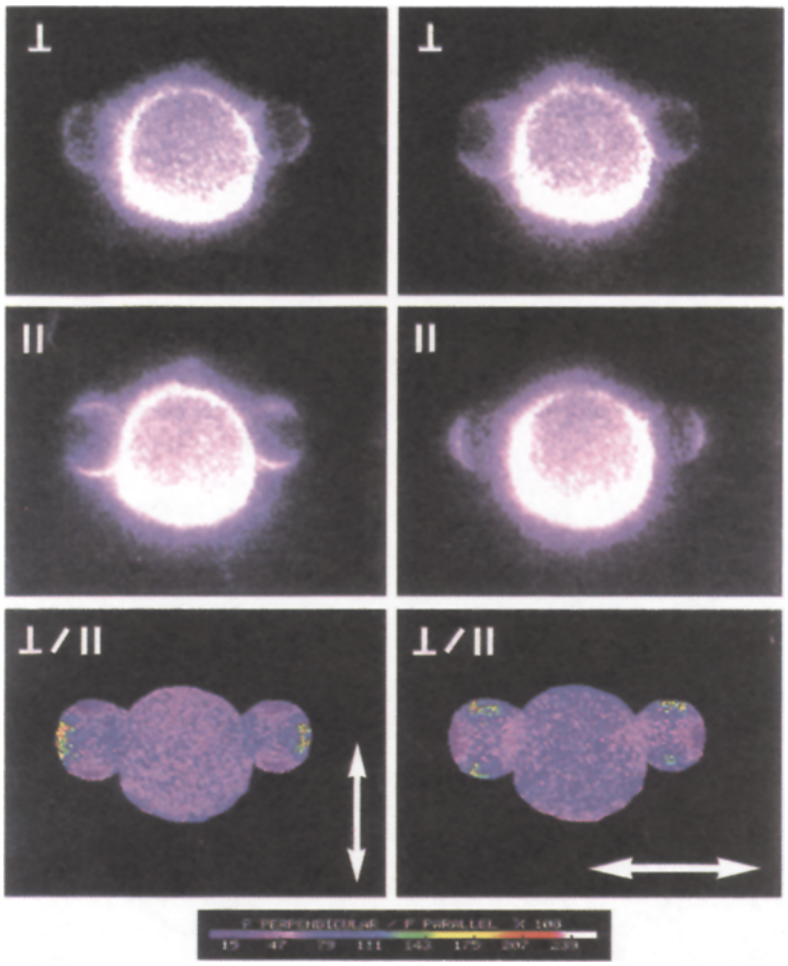

Fig. 2. Digitized microscopic fluorescence polarization measurement of a hepatocyte labeled with TMA-DPH after 30 min of hypoxic injury. Top and middle: fluorescence images, after background subtraction, obtained with the emission polarizer oriented perpendicular and parallel (top and middle) to the excitation light polarization direction for each of two excitation polarizer orientations (indicated by the arrows). Bottom: pseudocolor polarization ratio images. Cell diameter is 25 μm.

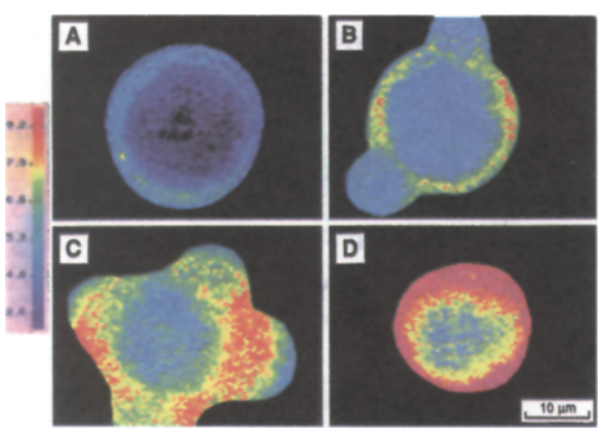

Fig. 3. Temporal and spatial changes in the FRET images (from I_A/I_D ratio) of hepatocytes at different stages of hypoxic injury. Following hypoxic injury the hepatocytes were labeled with NBD-PE/Rhd-PE lipid probe pair (D:A, 1:2.5) and images recorded with a 60×, 1.3 numerical-aperture glycerol immersion objective. A large amount of FRET (large ratio: red) indicates a tighter lipid domains, whereas a smaller ratio (blue) signifies less FRET and a more fluid membrane.

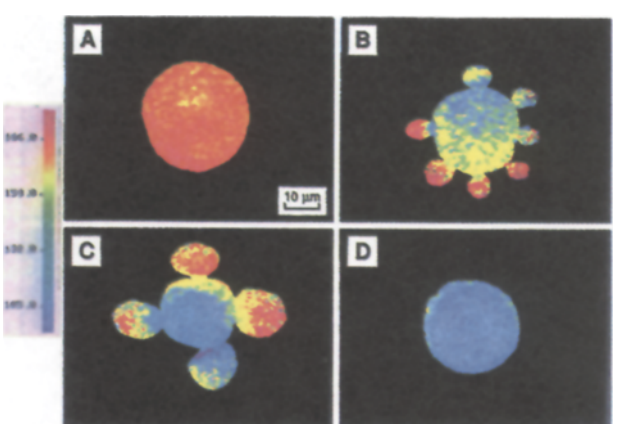

Fig. 4. TEMPO quenching images of TMA-DPH-labeled hepatocytes at different stages of hypoxic injury. TEMPO quenching images were recorded with a 40×, 1.3 numerical-aperture glycerol immersion objective. A high amount of quenching (large ratio: red) indicates a more fluid phase membrane, whereas a lower ratio (blue) signifies less quenching and a more rigid (gel phase) membrane.

quenching than the blebbed regions of the cell, indicating that the plasma membrane of the cell body is in a more rigid state than that of the blebs (Fig. 4B). These data indicate that the plasma membrane of hypoxic cells undergoes a transition from a fluid to a more rigid (gel) state as the injury evolves and that there exists spatial heterogeneity with respect to the extent and amount of gel-state lipid that forms. Over the next 20–40 min, cells enter stage II of blebbing. Blebs remain fluid for a longer time than the rest of the plasma membrane (Fig. 4C); in fact, little change in order of the lipids in the blebs occurs throughout the injury process. Figure 4D shows a cell in stage III of blebbing. This cell is dead; all of the blebs had ruptured and the nucleus was positive for propidium iodide staining (data not shown). The entire plasma membrane seems to be in a gel state, as evidenced by a low degree of quenching over the entire cell surface. To determine whether these results could be artifactual to the use of TMA-DPH, similar experiments using a variety of plasma-membrane-localized fluorophores (NBD-PC, NBD-PE, and Rhodamine PE) were performed. In all cases examined in stage I of hypoxic injury, the blebbed regions of the plasma membrane were more fluid than the rest of the cell surface (K. Florine-Casteel and B. Herman, unpublished data). Thus, the data obtained with TEMPO quenching imaging suggest that there is a transition from a fluid to a more rigid state in the plasma membrane as hypoxic injury progresses. Importantly, the use of an imaging approach to measure TEMPO quenching allowed us to discern that the transition in lipid order from that in a normoxic plasma membrane to that of a hypoxic membrane occurs in a spatially heterogeneous fashion, with blebbed portions of the membrane remaining in a fluid state while the membrane surrounding the cell body (nonblebbed region) becomes much more rigid.

FLUORESCENCE LIFETIME IMAGING MICROSCOPY (FLIM)

The previously described digitized video fluorescence microscopy measurements were performed under steady-state conditions. However, steady-state fluorescence microscopy is limited in its ability to quantitate and study rapid dynamic events (i.e., on the order of nano- to milliseconds) in plasma membranes. Measurements of time-dependent fluorescence emission anisotropy and time-resolved spectra of fluorescent plasma membrane protein or lipid probes can provide quantitative dynamic information about plasma membrane lipid fluidity, topography, and interactions among membrane components. Use of these techniques requires the ability to measure the fluorescent lifetime of the probe of interest in a spatially defined manner at high temporal resolution in intact single living cells. Recent technological advances in laser light sources, high-speed and highly sensitive image detection devices, and image processing techniques have allowed the development of fluorescence lifetime imaging microscopy (FLIM) [51–55]. FLIM is an extremely important advance, as it allows for the first time the combination of the sensitivity of fluorescence lifetime to environmental parameters to be monitored in a spatial manner in single living cells. As such, FLIM provides a sensitive technique capable of obtaining data regarding the dynamics and heterogeneous nature of plasma membrane components which cannot be obtained by using the methods mentioned above, which are based on time averaging of the fluorescent signal.

FLIM uses a very fast and highly sensitive MCP gated image intensifier [50,54]. The configuration of the system is shown in Fig. 5. A picosecond pulsed light source consisting of a mode-locked YAG laser and a dye laser with a third harmonic generator and cavity dumper provides picosecond pulses tunable from the UV to the IR at rates from single shot to 76 MHz. The pulsed excitation light is guided to a fluorescence microscope using a series of mirrors, and the beam is adjusted to illuminate the entire field of view. The fluorescence microscope has two output ports, one for the gated image intensifier and the other for single-photon counting (SPC) detection. The SPC detection system is used for sensitive measurement of fluorescent lifetimes with high temporal resolution. Time-resolved fluorescence microscopic images are obtained by using a high-speed gated MCP image intensifier (minimum gatewidth 3.0 ns). The principal features of the gated image intensifier are very small image distortion, wide spectral response, compact size, and high sensitivity, gain, and spatial resolution. The input gate signal for the gated image intensifier is delayed relative to the excitation laser pulse, using a digital delay/pulse generator. The gated image intensifier is operated at a maximum frequency of 10 kHz, and therefore the laser pulse frequency is fixed at 10 kHz at the cavity dumper. The output image of the intensifier on the phosphor screen is focused at unity magnification onto a slow-scan cooled CCD camera. For weak fluorescence emission, target integration in the CCD is effective at enhancing the signal-to-noise ratio (*S/N*) of the detected image. In a given experimental situation, the available fluorescence light level determines the integration time required to obtain acceptable images with adequate signal-to-noise ratio. A method to calculate

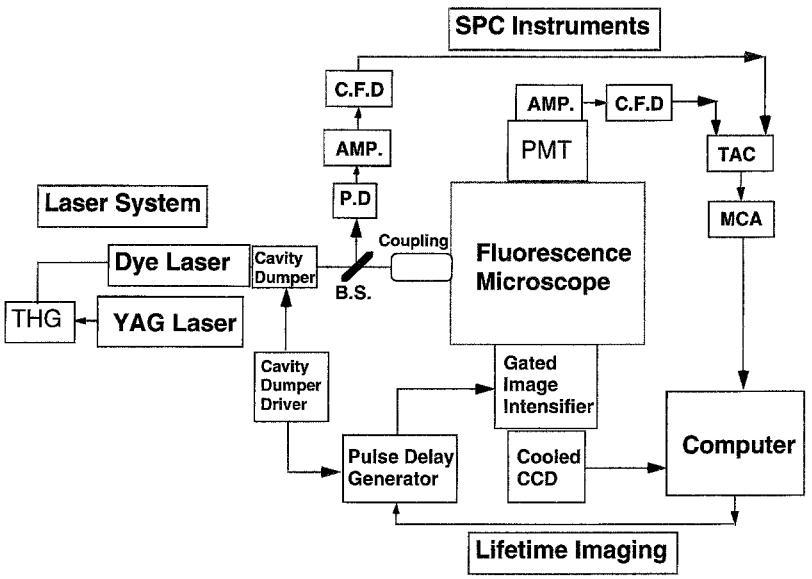

Fig. 5. Schematic diagram of fluorescence lifetime imaging microscope (FLIM). In the SPC (single-photon counting) mode, a photomultiplier (PMT, C2773, Hamamatsu Photonics K.K.), a photodiode (P.D), amplifiers (AMP, HP-8447F), constant-fraction discriminators (C.F.D, TC454, Oxford Instruments Inc.), a time-to-amplitude converter (TAC, TC864, Oxford Instruments Inc.), and a multichannel analyzer (MCA, Oxford Instruments Inc.) are used to measure fluorescence lifetimes from a single spot. The gated image intensifier and cooled CCD are used to image fluorescence lifetimes as described in the text.

lifetime images from FLIM images for single-exponential decay of fluorescence which results in extremely short calculation times has been developed [51,56]. Fluorescence lifetimes and preexponential factors can be calculated directly without fitting a large number of data points as required by conventional least-squares methods.

Recently, FLIM has been applied to study the extent of membrane fusion of individual endosomes in single cells [57]. By using fluorescence resonance energy transfer techniques and FLIM, the extent of fusion and the number of fused and unfused endosomes were clearly visualized. Currently, we are applying FLIM to monitor the time-dependent emission anisotropy of fluorescently labeled fatty acids and phospholipid probes in hepatocytes during hypoxic injury. We hope these studies will allow a more accurate description of the changes in membrane organization that accompany hypoxic injury. For example, do phospholipids in different portions of the membrane display distinct rotational mobilities? Are particular rotational mobilities associated with sites of loss of the plasma membrane permeability barrier? Does the segregation of plasma membrane phospholipids underlie the mechanism of the formation of lipid domains with varying degrees of gel and fluid phases? What characterizes a plasma membrane that is irreversibly injured by hypoxia from one that is reversibly injured? These questions and others can be addressed by employing the powerful combination of time-resolved (lifetime) measurements with the ability to visualize the spatial location of these changes in individual cells as these processes unfold. In the future, if proper probes can be developed and the cost of instrumentation can be decreased, FLIM should find wide versatility as a research tool for studies on membrane local lipid and phospholipid organization. FLIM will become a powerful approach to provide more quantitative information and insight about membrane structure and dynamics.

CONCLUSIONS

In this paper, we have reviewed several different digitized video fluorescence microscopic techniques for the study of molecular order in cell plasma membranes. Using these techniques, it is possible to measure detailed changes in plasma membrane structure and organization. Fluorescence polarization imaging provides two-dimensional lipid/phospholipid membrane order and viscosity mapping. FRET imaging can be used to monitor lipid/phospholipid domain formation, interaction, and topography. Fluorescence quenching imaging can be used to examine the spatial heterogeneity in membrane fluidity. These different approaches can be used to investigate membrane structure and function and provide comple-

mentary information. The use of these different technologies was illustrated in studies addressing alterations in plasma membrane lipid architecture that accompany hypoxic injury in rat hepatocytes. The data indicate that shortly after induction of hypoxia, major alterations in plasma membrane lipid and phospholipid order and topography occur, which initially leads to a more fluid plasma membrane. Subsequently, as injury progresses, there occurs a spatially and temporally heterogeneous change in both the organization and the degree of fluidity/rigidity of the membrane of hypoxic cells. These findings have identified the plasma membrane as a site of hypoxic injury. Digitized video fluorescence microscopy can be used to extract important spatial and temporal information from cell membranes and the hope is that the methods described in this paper will be adopted and modified by other groups for different applications. Application of these techniques, as well as others yet to be developed (e.g., near-field microscopy), will undoubtedly provide a more detailed view of cellular microarchitecture and function.

ACKNOWLEDGMENTS

This work was supported by grants AGO7218 and DK30874 from the National Institute of Health, the Gustavus and Louise Pfeiffer Research Foundation, and grant J-1433 from the Office of Naval Research. The work on the FLIM project is supported by grants from the NSF, American Cancer Society, North Carolina Biotechnology Center, and Whitaker Foundation and a UNC Research Council award. We gratefully acknowledge support from Hamamatsu Photonic Systems, Olympus Co., and ISS Co. for our FLIM research.

REFERENCES

1. D. L. Taylor and Y.-L. Wang (Eds.) (1989) *Fluorescence Microscopy of Living Cells in Culture A&B (Methods in Cell Biology, Vol. 30)*, Academic Press, San Diego, California.
2. D. L. Taylor, M. Nederlof, F. Lanni, and A. S. Waggoner (1992) The new version of light microscopy, *Am. Sci.* **80**, 322–335.
3. B. Herman and J. J. Lemasters (1993) *Optical Microscopy: Emerging Methods and Applications*, Academic Press, San Diego, California.
4. C. D. Stubbs and B. W. Williams (1992) Fluorescence in membranes, in J. R. Lakowicz (Ed.), *Topics in Fluorescence Spectroscopy*, Plenum Press, New York, pp. 231–272.
5. T. G. Dewey (1991) Fluorescence energy transfer in membrane biochemistry, in T. G. Dewey (Ed.), *Biophysical and Biochemical Aspects of Fluorescent Spectroscopy*, Plenum Press, New York, pp. 197–230.
6. W. T. Mason (1993) *Fluorescent and Luminescent Probes for Biological Activity*, Academic Press, San Diego, California.
7. J. J. Lemasters, J. DiGuiseppi, A. L. Nieminen, and B. Herman (1987) Blebbing, free Ca^{++} and mitochondrial membrane potential preceding cell death in hepatocytes; *Nature* **325**, 78–81.
8. K. Florine-Casteel, J. J. Lemasters, and B. Herman (1991) Lipid order in hepatocyte plasma membrane blebs during ATP depletion measured by digitized video fluorescence polarization microscopy, *FASEB J.* **5**, 2078–2084.
9. X. F. Wang, J. J. Lemasters, and B. Herman (1993) Plasma membrane architecture during hypoxic injury in rat hepatocytes measured by fluorescence quenching and resonance energy transfer imaging, *Bioimaging* **1**, 30–39.
10. B. R. Lentz (1988) Membrane "fluidity" from fluorescence anisotropy measurements, in L. M. Loew (Ed.), *Spectroscopic Membrane Probes*, CRC Press, Boca Raton, Florida, Vol. I, pp. 13–44.
11. B. R. Lentz (1993) Use of fluorescent probes to monitor molecular order and motions within liposome bilayers, *Chem. Phys. Lipids* **64**(1–3), 99–116.
12. R. C. Alvia, C. C. Curtain, and L. M. Gordon (1988) *Lipid Domain and the Relationship to Membrane Function*, Alan R. Liss, New York.
13. D. Axelrod (1989) Fluorescence polarization microscopy, in D. L. Taylor and Y.-L. Wang (Eds.), *Methods in Cell Biology, Vol. 30*, Academic Press, San Diego, California.
14. D. Axelrod (1989) Total internal reflection fluorescence microscopy, in D. L. Taylor and Y.-L. Wang (Eds)., *Methods in Cell Biology, Vol. 30*, Academic Press, San Diego, California.
15. C. Weber (1986) Solution spectroscopy and image spectroscopy in D. L. Taylor *et al.* (Eds.), *Applications of Fluorescence in the Biomedical Sciences*, Alan R. Liss, New York, pp. 601–615.
16. K. Florine-Casteel, J. J. Lemasters, and B. Herman (1990) Phospholipid order in gel- and fluid-phase cell size liposomes measured by digitized video fluorescence polarization microscopy, *Biophys. J.* **57**, 1199–1215.
17. J. A. Dix and A. S. Verkman (1990) Mapping of fluorescence anisotropy in living cells by ratio imaging: Application to cytoplasmic viscosity, *Biophys. J.* **57**, 231–240.
18. V. Borenstain and Y. Barenholz (1993) Characterization of liposomes and other lipid assemblies by multiprobe fluorescence polarization, *Chem. Phys. Lipids* **64**(1–3), 117–127.
19. D. Axelrod (1979) Carbocyanine dye orientation in red cell membrane studied by microscopic fluorescence polarization, *Biophys. J.* **26**, 557–574.
20. R. P. Haugland (1992) *Handbook of Fluorescent Probes and Research Chemicals*, Molecular Probes, Junction City, Oregon.
21. J. W. Kok and D. Hoekstra (1993) Fluorescent lipid analogues: Applications in cell and membrane biology, in W. T. Mason (Ed.), *Fluorescent and Luminescent Probes for Biological Activity*, Academic Press, San Diego, California.
22. M. Malmqvist (1993) Biospecific interaction analysis using biosensor technology, *Nature* **361**, 186–187.
23. J. B. Pawley (1990) *Handbook of Biological Confocal Microscopy*, Plenum Press, New York.
24. E. W. Hansen, R. Allen, and M. F. Riley (1985) Laser scanning phase modulation microscope, *J. Microsc.* **140**, 371–381.
25. D. A. Beach, K. S. Wells, and C. Bustamante (1987) Differential polarization microscope using an image dissector camera and phase-lock detection, *Rev. Sci. Instrum.* **58**, 1987–1995.
26. B. Herman (1989) Resonance energy transfer microscopy, in D. L. Taylor and Y.-L. Wang (Eds.), *Methods in Cell Biology*, Vol. 30, Academic Press, San Diego, California, pp. 219–243.
27. R. M. Clegg (1995) Fluorescence resonance energy transfer (FRET), in X. F. Wang and B. Herman (Eds.), *Fluorescence Image Spectroscopy and Microscopy*, Wiley, New York.
28. L. Stryer (1978) Fluorescence energy transfer as a spectroscopic rule, *Annu Rev. Biochem.* **47**, 819–846.
29. P. Wu and L. Brand (1994) Review: Resonance energy transfer: Methods and applications, *Anal. Biochem.* **218**, 1–13.

30. P. S. Uster (1993). *In situ* resonance energy transfer microscopy: Monitoring membrane fusion in living cells, in N. Duzgunes (Ed.), *Membrane Fusion Techniques Part B*, Academic Press, San Diego, California, Vol. 221, pp. 239–246.
31. D. E. Wolf, A. P. Winiski, A. E. Ting, and R. E. Pagano (1992) Determination of the transbilayer distribution of fluorescent lipid analogues by nonradiative fluorescence resonance energy transfer, *Biochemistry* **31**, 2865–2873.
32. S. R. Adams, A. T. Harootunian, and R. Y. Tsien (1991) Fluorescence ratio imaging of cyclic AMP in single cells, *Nature* **394**(21), 694.
33. J. E. Sunderland and J. Storch (1993) Effect of phospholipid headgroup composition on the transfer of fluorescent long-chain free fatty acids between membranes, *Biochim. Biophys. Acta* **1168**, 307–314.
34. M. Chalfie, Y. Tu, and D. C. Prasher (1994) Green fluorescent proteins as a marker for gene expression, *Science* **263**, 802–805.
35. E. Betzig and R. Chichester (1993) *Science* **262**, 1422.
36. R. Kopelman and W. Tan (1993) Near-field optics: Imaging single molecules, *Science* **262**, 1382–1384.
37. M. R. Eftink (1991) in J. R. Lakowicz (Ed.), *Topics in Fluorescence Spectroscopy II*, Plenum Press, New York.
38. M. R. Eftink (1991) in T. G. Dewey (Ed.), *Biophysical and Biochemical Aspects of Fluorescent Spectroscopy*, Plenum Press, New York.
39. E. London (1982) Investigation of membrane structure using fluorescence quenching by spin-labels, *Mol. Cell. Biochem.* **45**, 181–188.
40. B. Herman, A. L. Nieminen, G. J. Gores, and J. J. Lemasters (1988) Irreversible injury in anoxic hepatocytes precipitated by an abrupt increase in plasma membrane permeability, *FASEB J.* **2**, 146–151.
41. D. C. Harrison, J. J. Lemasters, and B. Herman (1991) A pH-dependent phospholipase A_2 contributes to loss of plasma membrane integrity during chemical hypoxia in rat hepatocytes, *Biochem. Biophys. Res. Commun.* **174**, 654–659.
42. X. F. Wang, J. Gordon, J. J. Lemasters, and B. Herman (1994) Phospholipid A_2 activity and its contribution to plasma membrane lipid lateral diffusibility during chemical hypoxia in rat hepatocytes, *Biophys. J.*, in preparation.
43. G. J. Gores, A. L. Nieminen, B. E. Wray, B. Herman, and J. J. Lemasters (1989) Intracellular pH during 'chemical hypoxia' in cultured rat hepatocytes: Protection by intracellular-acidosis against the onset of cell death, *J. Clin. Invest.* **83**, 386–396.
44. R. W. Gross (1992) Myocardial phospholipases A_2 and their membrane substrates, *Trends Cardiovasc. Med.* **2**, 115–121.
45. J. DiGuiseppi, R. Inman, A. Ishihara, K. Jacobson, and B. Herman (1985) Application of digitized fluorescence microscopy to problems in cell biology, *BioTechniques* **3**, 395.
46. T. T. Tsay, R. Inman, B. Wray, B. Herman, and K. Jacobson (1990) Characterization of low-light-level cameras for digitized video microscopy, *J. Microsc.* **160**, 141–159.
47. B. Herman and S. M. Fernandez (1982) Fluorescent pyrene derivative of concanavalin A: Preparation and spectroscopic characterization, *Biochemistry* **21**, 3271–3275.
48. M. Ludwig, N. F. Hensel, and R. J. Hartzman (1992) Calibration of a resonance energy transfer imaging system, *Biophys. J.* **61**, 845–857.
49. H. M. McConnell, K. L. Wright, and B. G. McFarland (1972) The fraction of the lipid in a biological membrane that is in a fluid state, *Biochem. Biophys. Res. Commun.* **47**, 273–281.
50. X. F. Wang, A. Periasamy, D. M. Coleman, and B. Herman (1992) Fluorescence lifetime imaging microscopy: Instrumentation and applications, *Crit. Rev. Anal. Chem.* **23**, 369–395.
51. X. F. Wang, A. Periasamy, J. Gordon, and B. Herman (1994) Fluorescence lifetime imaging microscopy (FLIM) and its applications, in *Time-Resolved Laser Spectroscopy in Biochemistry*, SPIE Proceedings 2137, in press.
52. J. R. Lakowicz and K. W. Berndt (1991) Lifetime-selective fluorescence imaging using an RF phase-sensitive camera, *Rev. Sci. Instrum.* **62**, 1727–1734.
53. J. R. Lakowicz, H. Szmacinski, K. Nowaczyk, and M. L. Johnson (1992) Fluorescence lifetime imaging of calcium using Quin-2, *Cell Calcium* **13**(3), 131–147.
54. A. Periasamy, X. F. Wang, P. Wodnicki, G. Gordon, and B. Herman (1995) High-speed fluorescence microscopy: Lifetime imaging in the biomedical science, *J. Micros. Soc. Am.* in press.
55. T. W. Gadella, T. M. Jovin, and R. M. Clegg (1994) Fluorescence lifetime imaging microscopy (FLIM): Spatial resolution of microstructures on the nanosecond time scale, *Biophys. Chem.* **48**, 221–239.
56. X. F. Wang, T. Uchida, D. M. Coleman, and S. Minami (1991) A two-dimensional fluorescence lifetime imaging system using a gated image intensifier, *Appl. Spectrosc.* **45**, 360–366.
57. T. Oida, Y. Sato, and A. Kusumi (1993) Fluorescence lifetime imaging microscopy (flimscopy): Methodology development and application to studies of endosome fusion in single cells, *Biophys. J.* **64**, 676–685.