# Assessment of Membrane Fluidity in Individual Yeast Cells by Laurdan Generalised Polarisation and Multi-Photon Scanning Fluorescence Microscopy

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In Fluorescence Spectroscopy, Imaging and Probes - New Tools in Chemical, Physical and Life Sciences (R Kraayenhof, AJWG Visser and HC Gerritsen, Eds.), Springer Series on Fluorescence: Methods and Applications, Vol. 2, Springer, Heidelberg, 2002, Chapter 14, pp 241-252. ISBN 3-540-42768-6.

Here we describe techniques that we developed for monitoring membrane fluidity of individual yeast cells during environmental adaptation and physiological changes. Multi-photon scanning fluorescence microscopy using laurdan as a membrane probe enables determination whether fluidity changes seen by spectroscopy reflect universal responses or changes only of sub-populations.

Yeast membranes are a primary site of environmental response and adaptation. Using fluorescence spectroscopy with DPH polarization and laurdan Generalized Polarization (GP), we previously found rapid "average" membrane fluidity modulation in yeast populations during growth and in response to nutrients or environmental stresses. To determine whether such responses reflect all cells we conducted the first multi-photon scanning fluorescence microscopy study of yeasts, measuring laurdan GP. We assessed membrane fluidity responses of individual yeasts related to growth phase, heat stress and ethanol stress.

Average fluidity decreased as cultures aged, however the decreased fluidity was due in some cases to an increasing proportion of uniformly low fluidity (high GP) cells, which were shown by vital dye to be dead. When yeasts were heat stressed, the mean laurdan GP increased in all cells, thus the entire population evidenced damage (viz. decreased membrane fluidity) to the same degree. On the other hand, with ethanol stress fluidity increased (GP decreased) on exposure of cells. All cells were affected although not to the same degree, and with variable recovery. The recovery assessed from GP microscopy was highly variable, and greater by that seen by spectroscopy.

## 1 Introduction

In order to provide background to these studies, we need to introduce three major concepts: the rationale for studying yeast membranes and their fluidity; the use of multi-photon scanning fluorescence microscopy; and the analysis of laurdan Generalized Polarization (GP).

In brief, using fluorescence spectroscopy we previously studied membrane fluidity modulation in baker's yeast, brewer's yeast, and in *Saccharomyces cerevisiae* S288c for which the genome sequence is known. We determined membrane fluidity by fluorimetry, measuring polarization of DPH (1,6-diphenyl-1,3,5-hexatriene) fluorescence. More recently, to reduce problems with cell density-dependent scattering of the polarized light, we utilized the environmentally sensitive spectra of laurdan (6-lauroyl-2-dimethylamino naphthalene). We found rapid membrane fluidity modulation in yeasts in response to environmental stresses (heat and ethanol), during growth in batch culture and as a physiological response to glucose availability.

### 1.1 Yeast membrane fluidity

The plasma membrane provides the semi-permeable barrier that allows all cells to exist. When yeasts encounter changes in the environment such as nutrient depletion, metabolite accumulation or temperature variation, the plasma membrane must adapt prior to internal structures. Thus the membrane is the primary site of response to environmental change.

Fluidity of membranes is a key factor in their function, affecting cell permeability and important activities such as nutrient transport and pH maintenance. Membrane function, which is largely dependent on membrane enzymes and transport proteins such as the yeast plasma membrane $H^+$-transporting ATPase, is also affected by changes in membrane fluidity [1].

Elucidation of molecular mechanisms of adaptation of yeasts to stress is highly relevant to commercial production of yeast products, with temperature and ethanol stresses of most economic significance. Furthermore, knowledge of yeast adaptation processes at the molecular level is applicable to studies of adaptive responses of higher organisms. Stressful environmental change may directly affect membrane fluidity, either transiently or permanently [2-4]. Cells that survive and adapt must therefore modulate their membrane fluidity to compensate.

In heat stress the plasma membrane is considered a primary site of heat damage, although the mechanism is controversial. The "fluidization" hypothesis maintains that heat increases fluidity to unstable levels, resulting in breakdown of structure and function [5]. On the other hand, we believe that rather than fluidization, heat damage to membrane proteins impacts on membrane function. The latter theory is supported by data from a number of studies [6-8]. It is thought that heat responses are triggered by damaged cytosolic proteins, although membrane proteins may be more vulnerable since heat damage may involve oxygen-derived free radicals, which partition into membranes [9]. In support of the former theory we found yeast membrane fluidity relates inversely to heat tolerance [1, 10-12]. However, more strikingly supporting the latter theory, we found that during heat stress membrane fluidity progressively and irreversibly decreased [1].

When considering ethanol stress, survival and adaptation to ethanol (from fermentation of sugars) are important criteria in beer and wine production. Like heat, ethanol causes accumulation of damaged proteins and induces similar responses [13], although it definitely increases membrane fluidity due to its solvent action. In preliminary experiments, we found that yeasts exposed to high concentrations of ethanol showed an immediate increase in fluidity. In the case of cells early in the growth phase the fluidity increase was followed by a marked, sustained decrease that may reflect denaturation of membrane proteins [2, 3, 14].

However, while these studies provided useful information on rapid membrane responses of yeasts, a disadvantage is that average responses of whole populations were measured. Furthermore, information of subcellular location of responses was not available. Recently, the technique of multi-photon scanning fluorescence microscopy has become available. This technique has a number of advantages over confocal microscopy, as described below. Multi-photon fluorescence microscopy enables immediate and direct visualization of events at the individual cell and sub-cellular levels. Therefore, we conducted the first study of yeasts by multi-photon scanning fluorescence microscopy.

### 1.2 Multi-photon scanning fluorescence microscopy

The technique of multi-photon scanning fluorescence microscopy has been applied to study of laurdan GP in model membranes and mammalian cells [15, 16]. Multi-photon scanning fluorescence microscopy involves using laser beams and optics to focus the incident beam on a small point, rather than illuminating the whole specimen. In addition, using the intense radiation allows the possibility of multiple photons simultaneously exciting a fluorophore. Consequently, for two-photon excitation the wavelength of the incident light may be approximately doubled, reducing the energy of the incident radiation from the high energy UV region to the lower energy red region of the visible spectrum. This technique has a number of advantages over conventional

confocal fluorescence microcopy [15]. Photobleaching is dramatically reduced by point illumination of specimen (fluorescence excitation is localized to the focus region), and the use of lower energy red to near-infrared irradiation (700 nm – 1050 nm). This also allows for study of UV-excitable dyes without the expense of UV-laser systems. In addition, the penetration of longer wavelength radiation into biofilms of microorganisms or animal or plant tissues is much greater, allowing analysis of events at much greater depth.

### 1.3 Determination of membrane fluidity using laurdan Generalized Polarization

Laurdan [17] in membranes is known to be sensitive to the polarity of the environment, exhibiting a 50 nm red shift in emission spectrum over the gel to liquid-crystalline phase transition [15, 18]. The spectroscopic property Generalized Polarization (GP), derived from fluorescence intensities at critical wavelengths, can be considered as an index of membrane fluidity [19]. The GP is calculated in an analogous way to the Polarization parameter, exchanging values at critical wavelengths for the polarization orientations. Thus the GP is calculated from relative fluorescence intensities at wavelengths at the red and blue edges of the spectrum, representing gel (approx. 440 nm) and liquid crystalline (approx 490 nm) phases of bilayer systems. The GP is calculated as follows [19]:

$$GP = \frac{I_{gel} - I_{lc}}{I_{gel} + I_{lc}} \qquad (1)$$

The GP may theoretically range from –1 to +1. This GP parameter is inversely related to membrane fluidity; high GP values are found in gel phase, and low in liquid-crystalline phase [15]. Laurdan has been used to analyze membrane structure and organization in model phospholipid systems [19-21] and mammalian cell membranes [22-25] by cuvette fluorimetry and two-photon microscopy [15, 16]. As noted above, we reported the first use of laurdan to detect rapid membrane fluidity modulation in microorganisms by fluorimetry – specifically in yeast populations during growth and under stress. [2, 3, 26]. In addition we pioneered the use of multi-photon scanning fluorescence microscopy of microorganisms [26, 27].

## 2 Materials and Methods

These studies included three *Saccharomyces cerevisiae* strains: a wild type baker's yeast [28], a wild type brewer's yeast [27], and strain S288c for which the genome sequence is known [29]. Cells from a slope were inoculated into YNBG broth containing 1% glucose and 0.67% Yeast Nitrogen Base (Difco), and incubated overnight on an orbital shaker (30 ± 1°C, 180 rpm). Growth media was filter sterilized as autoclaving resulted in increased background fluorescence. Culture grown overnight (starter culture) was inoculated in fresh YNBG broth at 0.1 $OD_{600nm}$ and continued on the shaker (experimental culture). This provided a starter culture population in relatively similar growth phase for all experimentation. It is important to briefly define the phases of aerobic growth in diauxic yeast cultures. We previously defined these stages [30] as initial lag (adaptation), respiro-fermentative growth on glucose (about 12 h under our conditions), diauxic lag phase (adaptation), respiratory growth on the accumulated ethanol (up to 80 or more h, note this would not occur under anaerobic conditions), stationary phase (maintenance phase – nutrients exhausted), followed eventually by death phase (duration of stationary phase depends upon cell conditions). Experimental cultures were analyzed at various stages of growth, as indicated below.

The fluorescent membrane probe laurdan was obtained from Molecular Probes, Eugene, OR, (USA). Cells at appropriate culture phases were labeled by mixing with laurdan stock in ethanol (1 μL per mL cells at $OD_{600nm}$ = 0.4, final laurdan concentration 5μM) and incubating for 1 h in the

dark, under the same conditions as the main culture. Where required, cells were diluted in filtered culture supernatant. The negligible addition of ethanol was previously shown not to affect the yeast cells.

Two-photon fluorescence scanning microscopy and GP analysis was performed as described previously [15] with excitation at 770 nm (equivalent to 1-photon excitation at 385 nm), and emission analyzed using two 46-nm bandpass filters centred at 446 and 499 nm. The two filters were exchanged after each full scan. To compensate for photobleaching, three scans in the sequence red-blue-red were taken, and the red scans averaged. Laurdan-labeled cells were analyzed against unlabelled cells to subtract background autofluorescence.

As GP image was derived from 3 independent images, immobilization of cells was important, as movement would result in non-overlapping images. In our experiments we devised and partially optimized two methods for fixing live yeast cells, to give comparable results. In the first method, pelleted cells were mixed with low temperature melting agar (to avoid unintended heat shock of cells) and set to consistent depth under cover slips on microscope slides. The cover slips were then sealed to prevent specimens drying out during microscopy. This method provided good immobilization of cells, although it precluded addition of reagents during analysis. The second method involved attaching cells to poly-D-lysine coated cover slips in 8-chambered format (Fisher Scientific). This allowed up to 500 μL of culture supernatant or other solutions to be added to the fixed cells, and replacement if required with fresh solutions. However the immobilization of cells by the latter technique was variable and worked well for some, but not all yeast strains. The adhesion of cells was growth phase dependent, reduced over time on the cover slip, and was affected by ethanol addition. In some experiments, while cells remained adherent to the cover slip, they tended to wobble, thus leading to poor image quality. However, when conditions were optimal the cover slip/well method provided useful data. Further studies are progressing to optimize live cell fixation, utilizing techniques such as a poly-lysine based flow cell biofilm preparation method [31], a mucin-based biofilm preparation method [32], and a biotin-streptavidin method [33]. Since this study was conducted, the microscopes in the laboratory of E.G. have been modified to enable simultaneous 2-channel measurements. R.P.L. has also obtained a 2-channel system.

## 3 Results and Discussion

Using the fluorescent probe laurdan, we viewed optical sections of cells to assess membrane fluidity, represented as GP. We assessed membrane fluidity of the three yeast strains as a function of growth phase in batch culture, heat stress and ethanol stress. Laurdan-labeled cells were analyzed against unlabeled cells to subtract background autofluorescence. Fig. 1 displays GP images of three yeast strains at various stages of the growth curve. The 6 h images represent respiro-fermentative growth on glucose, 24 h respiratory growth on ethanol, 3–4 d late respiratory growth to stationary phase, and 8 d stationary phase. At 6 h and less at 24 h, one can see the typical morphology of growing yeasts, which devide by budding – a larger parent cell with a smaller bud growing from it. One can also see the large cell vacuole in some cell sections, viewed as a large black area (i.e. no fluorescence above background detected). The microscopy study confirmed our previous fluorimetry studies, in that average fluidity decreased (GP increased) as cultures progressed.

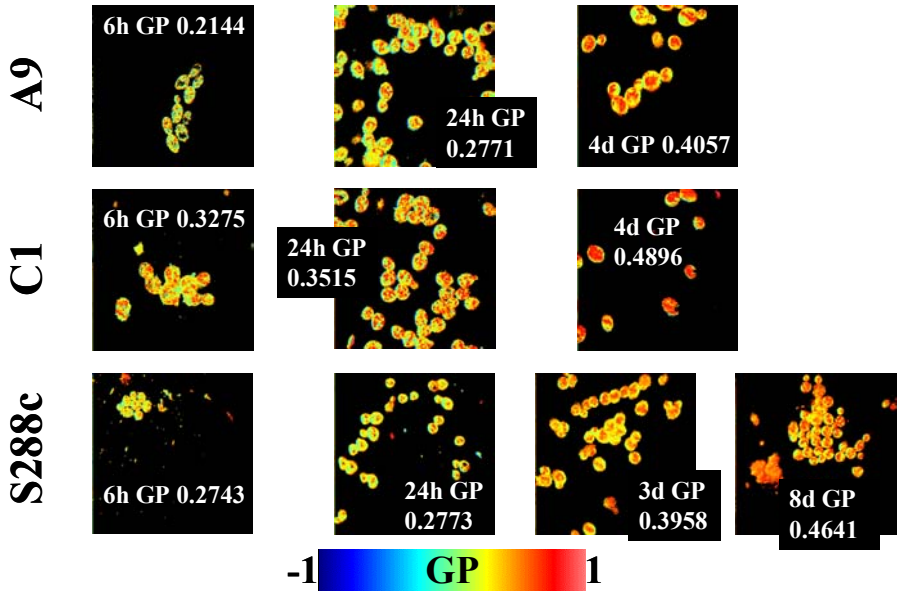


**Fig. 1.** Generalized Polarization images of three yeast strains related to culture time. The scanned intensity data were obtained using a Zeiss 40X 1.25 NA oil emulsion objective and corrected for photobleaching and unlabelled cell autofluorescence. The 256 x 256 pixel scans represent 23 x 23 μm, the depth scanned was 1 μm.

Fig. 2 compares the GP values for the yeast strain A9, obtained by microscopy and spectroscopy. It can be seen that the standard deviations of the former are much greater, as would be expected since a limited number of cells are analyzed per scan, where values are averaged from thousands of cells in the cuvette. For technical reasons including time of cell preparation for scanning, the data from microscopy are not as sensitive to change around the time of exhaustion of glucose, however the trend of both curves is of increased GP as cultures age. It should be noted that the absolute values of GP vary between different instruments, due e.g. to differing optics, differing wavelength-associated transmission characteristics of monochromators and/or differing wavelength-dependent efficiencies of photomultiplier tubes in detectors.

Unfortunately, in organisms as small as yeast, the resolution was not adequate to precisely define subcellular membranes. However, there seems to be clustering of like GP values (particularly high GP values). While we were unable to assign this clustering to particular subcellular structures, it may reflect membrane lipid rafts (clustering of particular species of membrane lipids into defined domains. Further studies will attempt to establish whether the clustering of GP values represents lipid rafts, and whether membrane proteins are specifically associated with these.

Notwithstanding the lack of ability to delineate subcellular membranes, valuable information was gained on individual cell responses in a population. Examination of microscope GP images revealed in some (but not all) cases the decreased average fluidity may be due to an increasing proportion of uniformly low fluidity (high GP) cells with the remaining cells unchanged (with variable fluidity across the cell). This is not obvious in Fig. 1, but can be seen well in Fig. 4, comparing the control cells at 6 and 24 h. Although double labeling with a vital dye (methylene blue) caused quenching of fluorescence, it indicated that cells with uniform low fluidity were dead. Thus cells seemed to be entirely healthy or dead, with “frozen” membranes, with no intermediate form detectable.

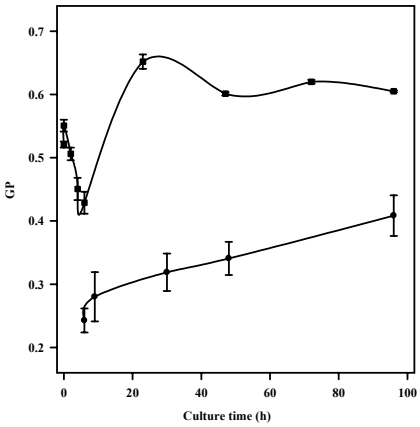


**Fig. 2.** Mean GP of cells from yeast strain A9 related to culture time. A by microscopy, E by spectroscopy (using an ISS PC2 spectrofluorimeter). Error bars represent standard deviation. Respiro-fermentative growth on glucose to 12h, respiratory growth on ethanol to 72 h.

Numerical analysis of histograms of GP images (Fig 3) showed that there were only two classes of cells: live cells with mean low GP, and dead cells with mean high GP. The histogram in Fig 3 shows a peak centred at GP 0.3176 representing a total of 57% of the data, with a second peak centred at GP 0.6392 representing 43% of the values. This correlates well with the fractions obtained by simply counting low average GP (live) cells and high average GP (dead) cells, giving 56 % and 44 %, respectively.

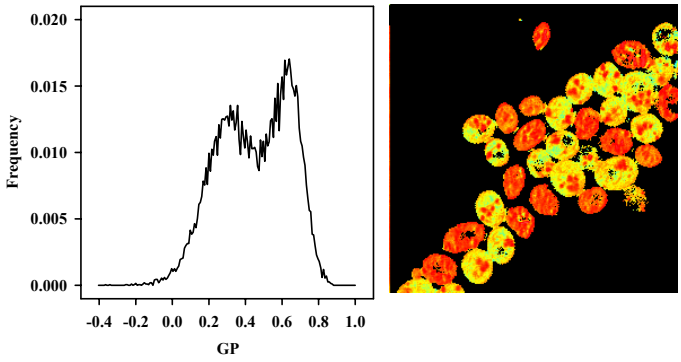


**Fig. 3.** GP frequency histogram and image of A9 cells in stationary phase at 4 d.

It should be noted that this phenomenon might not occur, for example it is not indicated in 6–24 h images of Fig. 1. Nevertheless, cuvette-based studies must be interpreted with caution and viability closely monitored, as increased GP may be due to death of a proportion of cells rather than an "average" cell response. It should be possible for investigators to determine a "correction factor" to determine the effect of skewing GP by the proportion of non-viable cells. However, this would need to be done for each instrument, due to the individual instrument factors discussed above.

In relation to heat stress, our previous DPH polarization data indicated that membrane fluidity decreased progressively in cells exposed to heat, and that the decrease was greater in respiro-fermentative cells. When cells were heat stressed (52 °C, 5 min) and assessed by GP microscopy (Fig. 4), the mean GP of respiro-fermentative cells increased from 0.199 ± 0.040 to 0.360 ± 0.028. The increase was seen consistently across all cells, rather than a sub-population change, thus the

entire population was damaged to the same degree. Respiratory cells had higher mean GP (0.379 ± 0.029) and were more resistant to heat stress, as the 5 min heat exposure did not markedly increase the mean GP of the latter cells (0.392 ± 0.012). In this case the microscopy data confirmed the previous fluorimetry data.

When ethanol stress was studied, our previous DPH polarization and laurdan GP data indicated that fluidity was increased within seconds by exposure of respiro-fermentative and respiratory cells to ethanol, followed by only slight recovery. By GP microscopy (Fig. 5) however, the results were inconsistent, but indicated potentially greater recovery of membrane fluidity, and generalized effect across all cells.

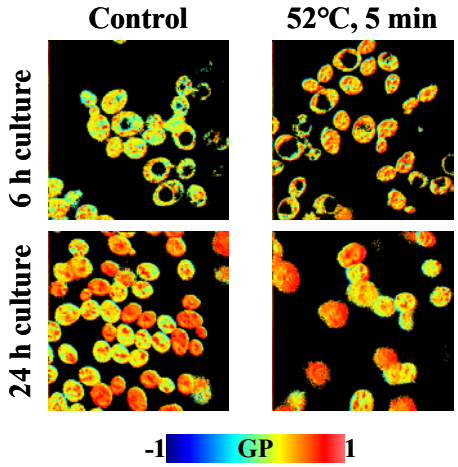


**Fig. 4.** Generalized Polarization images of yeast strain A9 growing on glucose (respiro-fermentative, 6h) and ethanol (respiratory, 24h) before and after heat stress (52 °C, 5 min). The data were obtained using a Zeiss 63X 1.25 NA oil immersion objective. The 256 x 256 pixel scans represent 36.2 x 36.2 μm.

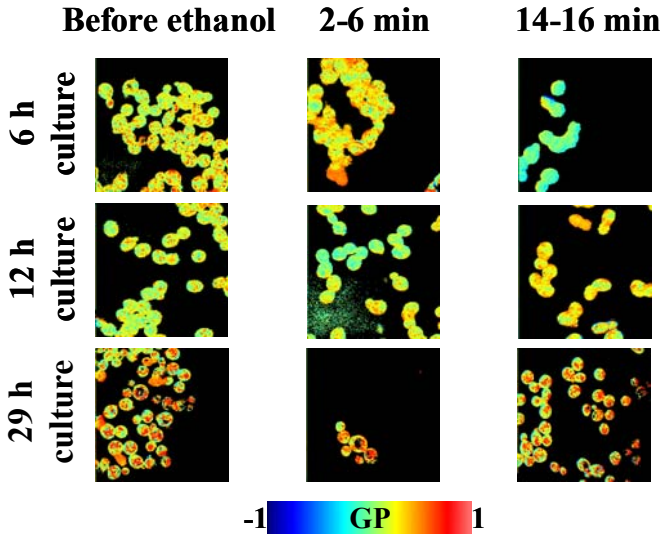


**Fig. 5.** Generalized Polarization images of yeast strain C1 during and at the end of growth on glucose (respiro-fermentative, 6 h, 12 h), and growth on ethanol (respiratory, 29 h) before and after addition of ethanol. The scanned intensity data were obtained using a Zeiss 40X 1.25 NA oil immersion objective as in Fig.1. The 256 x 256 pixel scans represent 23 x 23 μm.

The inconsistency may reflect sub-optimal immobilization of cells in presence of ethanol, and further studies aim to improve immobilization of cells in the cover slip/well system. Application of simultaneous 2-channel recording should also improve this situation.

## 4 Conclusions

In conclusion, we developed techniques for monitoring membrane fluidity in individual yeast cells during environmental adaptation and physiological change. The microscopy studies allow us to determine whether changes reflect ubiquitous responses, or changes only in sub-populations of cells. The GP images show evidence of subpopulation changes in relation to age of culture and to some extent for ethanol damage, although heat damage is more universal.

Multi-photon scanning fluorescence microscopy adds a further dimension to our analyses, providing information on responses of individual cells within a population. The observation in some situations that a variable proportion of dead cells skews mean GP values demonstrates that cuvette-based studies must be interpreted with caution. Increased GP may be due to death of a proportion of cells rather than an "average" cell response, necessitating simultaneous assessment of cell viability during experiments.

The ability to monitor responses of individual live cells is of critical importance in establishing adaptation of populations to minor or major environmental change. For example a measured average response may reflect high activity of a subpopulation of cells at a certain stage of their growth cycle. In the case of major stresses (e.g. heat, ethanol, osmotic, pH) it is not uncommon for a small proportion (0.001 to 10 %) of cells to survive. Whether this is simply a stochastic process, or related to the precise nature of the surviving cells, may be elucidated by following individual cell responses. Here we concentrated on membrane fluidity responses, although the technique could be used to assess a range of responses for which fluorescent probes are available.

**Acknowledgements.** The Laboratory for Fluorescence Dynamics is a National Research Resource, supported by the National Institutes of Health and the University of Illinois. E.G. acknowledges support from grant PHS P41 RR03155. R.P.L. acknowledges support from the Australian Research Council grants C29600212, IP97050 and R00107932.

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