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# Analysis of the Fluorescence Intensity Profile in Contact Lenses using Laser Scanning Confocal Microscope

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## I. OBJECTIVES

The Laser Scanning Confocal Microscope was used in fluorescence mode to characterize the fluorescence intensity profiles in new (unworn) and worn contact lens samples. In particular, special attention is paid to following issues:

1) Is there any significant difference in the fluorescence intensity profile across the lens thickness between new wet lenses and new dry lenses?

2) Is the fluorescence intensity uniformly distributed across the lens thickness throughout the lens as a function of radial distance from the center?

3) Is the fluorescence intensity profile changed while the lenses are being worn? Is the fluorescent material depleted from the lenses while they are worn?

## II. LENS SAMPLES

1) 10 new (unworn) disposable contact lenses of lot number 991204:

* For characterization the fluorescence intensity across the lens thickness at several different locations both for wet and dry lenses.
* These lenses were placed on a PS mount such that the lens and the mount were as concentric as possible.
* To test the fluorescence intensity profile in dry lenses, a new wet lens was placed on a PS mount and left in a Petri dish loosely covered for 80 days.
* To test the fluorescence intensity profile across the lens thickness in different locations of the lens, one side of the PS mount with a lens was elevated by 2-10 microscope slide glasses. This makes it possible to focus on different parts of the lens with image symmetry, regardless of the relative location of the lens.

2) 5 worn disposable contact lenses: To compare with new (unworn) lenses

* Lens samples: 991204-131-OD, 991204-131-OS, 991204-132-OS, 991304-51-OD, 991304-51-OS

* When the worn lens was badly deformed/curved/curled such that it was impossible to focus at the lens center, a tiny piece of specimen was cut off from the relatively flat area.

* When the worn lens was still flexible without too much deformation, it was placed on a microscope slide glass and the imaging was done at relatively flat areas close to the lens center.

## III. INSTRUMENTAL SETUP

* Microscope: Leica TCS NT/SP Series Laser Scanning Confocal Microscope (LSCM)
* Excitation Laser Wavelength: 488 nm, Ar+ laser
* Fluorescence Detection Wavelength: 500 ~ 650 nm in Fluorescence Mode
* Objective: 10x dry, NA=0.3
* Detector Pinhole: 1.0 Airy disk
* PMT: 601
* Zoom: 1 or 2
* Accumulation: 1
* Scan Mode: xy
* Scan Speed: medium
* Scan Direction: mono
* Scan Format: 512x512

The experimental condition was the same as the study in the previous report. The laser excitation wavelength was 488 nm and the imaging was done in fluorescence mode with Leica laser scanning confocal microscope. Laser intensity was kept as low as possible while the photomultiplier was set high in linear response range, in order to minimize the unwanted bleaching during the experiment. The color shown in this report is chosen such that as long as there is no blue spot/area, there is no saturation in fluorescence intensity.

All imaging was done at the same microscopy setting with respect to the laser power to ensure the same excitation intensity to make the comparison easier.

## IV. RESULTS AND DISCUSSIONS

### *1. Characterization of New 991204 Wet Lenses*

#### 1.1. Effect of drying/exposure time: At the center of a new 991204 lens

A new 991204 lens was opened and the excess saline fluid was removed by holding the lens vertically on a paper tissue. The lens was placed on a PS mount, which was then mounted on the microscope stage as shown in **Figure 1(a)**. The lens and PS mount were arranged as concentric as possible. The center area (**Figure 1 (b)**) was imaged at 100 optical sections from top to bottom at 1 μm interval. It took roughly 5 min from the moment the lens was opened until the best area to scan, in both xy and z positions, was properly located. Hence the first run of the 100 section imaging was performed at 5 min after the lens was opened. Upon completion of the first run, the experiment was repeated with different number of section for 3 more times at 15 min, 19 min, and 23 min to monitor the change in the fluorescence intensity as a function of drying time while the lens is kept exposed to the air.

An example of the compiled images of the 100 sections is given below (**Figure 1(c)**). The reconstructed 3D images (**Figure 2**) show that the lens thickness is rapidly shrinking as the water is evaporated.

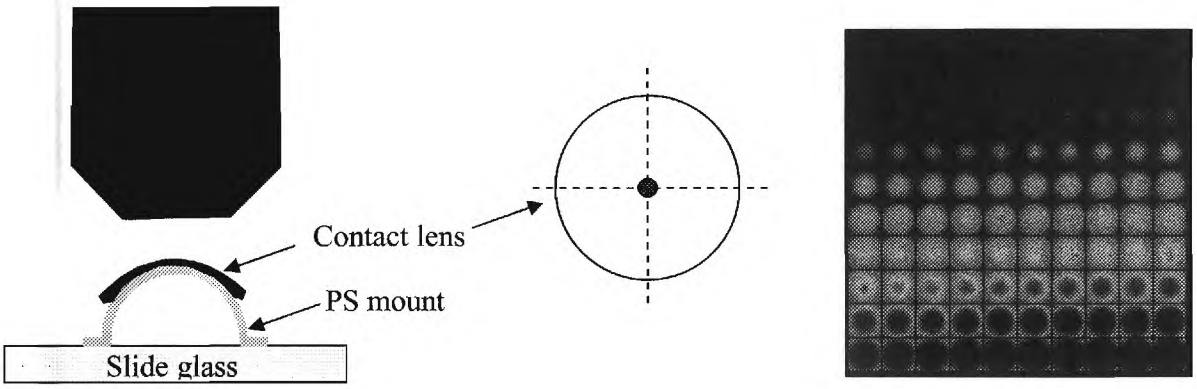


**Figure 1**. (a) Experimental setup for imaging of new (unworn) contact lenses. The lens was placed on a PS mount such that they can be as concentric as possible. (b) The red dot denotes the center location of the PS mount (and lens) where the imaging was made. (c) An example of a compiled image of the 100 xy section images at different lens thickness from the lens top to bottom at 1 μm sectioning interval.

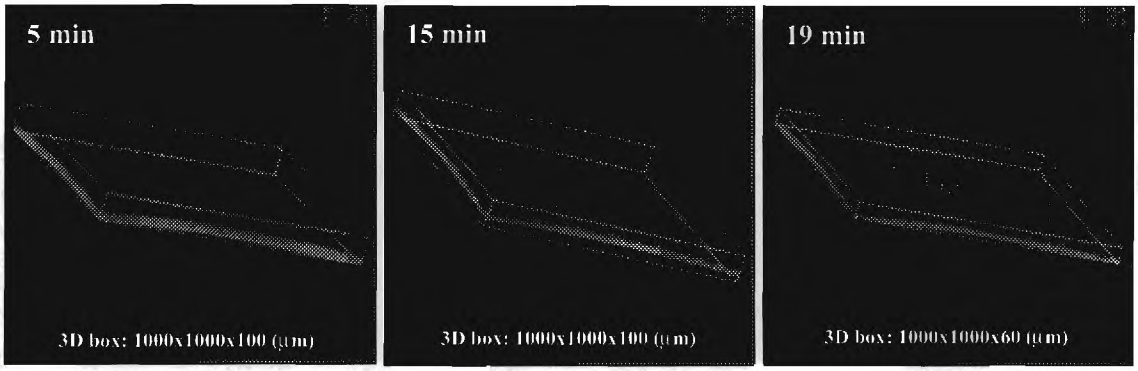


**Figure 2.** 3D images of a new (wet) lens 991204 at the lens center as a function of air exposure (drying) time. The lens becomes thinner as the drying progresses.

For quantitative analysis, a circle (circumference = 266 μm) is drawn at the center part of the image and the fluorescence intensity along this circle is taken from each section image.

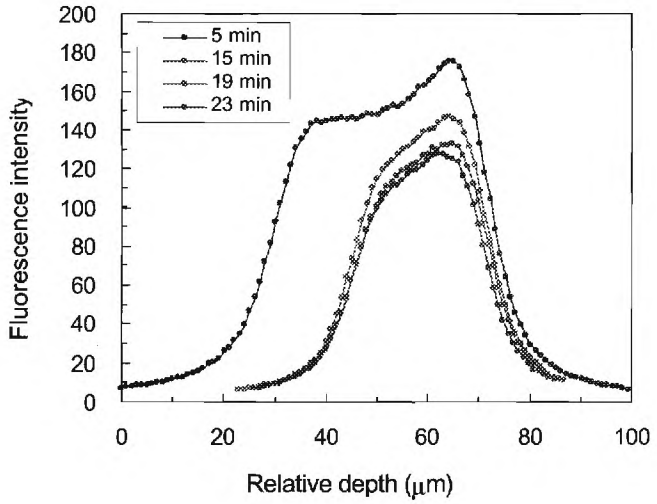


**Figure 3.** Fluorescence intensities of a new (wet) lens 991204 at the center of the lens as a function of air exposure (drying) time, which are taken from the images in **Figure 2.** Please see the text for detail explanation.

Several observations can be made from the **Figure 3.** First, the fluorescence intensity is neither symmetrical nor uniform across the lens thickness, which is probably because of the two reasons: 1) the fluorescent dye absorption was not uniform across the lens thickness when the lens was originally made, and 2) the fluorescent dye has higher quantum yield in wet area. The water content is obviously lower at the top part of the lens due to the evaporation during the imaging process. Second, the fluorescence intensity starts to increase at deeper z location (meaning at lower height and close to the lens bottom) at later experiment times, clearly showing

that the lens becomes thinner as the drying progresses. It is also observed that the fluorescence intensity decreases only a little at the bottom of the lens where the lens is in contact with the PS mount, due to the limited water evaporation. Third, the fluorescence intensity more or less reaches the steady value after about 20 minute exposure, while the peak intensity becomes lower as the lens gets dried further. However, it will take a lot longer time than 20 min to completely dry the lens throughout the whole lens thickness.

**1.2. Effect of drying/exposure time: At off-center of a new (unworn) 991204 lens**

A new 991204 lens was opened and the excess saline fluid was removed as mentioned earlier. The lens was placed on a PS mount in such a way that the lens and PS mount were as concentric as possible. This was then mounted on the microscope stage as shown in **Figure 4(a)** with 3 slide glasses (each 1 mm thick) stacked under one side of the PS mount, so that the imaging can be made for the area (**Figure 4(b)**) that is away from the contact lens center, yet maintaining symmetry in xy direction. The highest area at this configuration was imaged at 100 optical sections from top to bottom at 1 μm interval. The first run of the 100 section imaging was performed at 4 min after the lens was opened. Upon the completion of the first run, the experiment was repeated 3 more times at 8 min, 12 min, and 22 min to monitor the change in the fluorescence intensity as a function of drying time while the lens is kept exposed to the air. An example of the compiled images of the 100 sections is given below (**Figure 4(c)**).

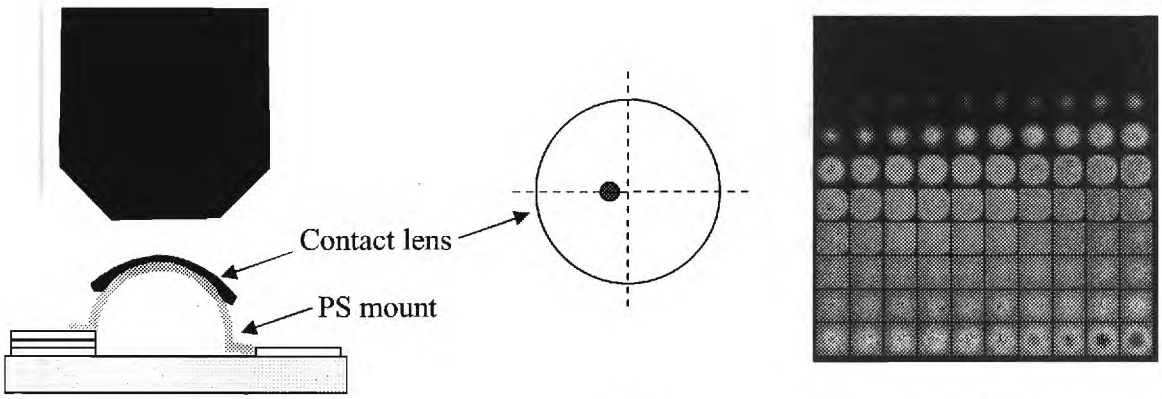


**Figure 4**. (a) Experimental setup for imaging of new (unworn) contact lenses with stacked slide glasses (each 1 mm thick) under one side of the PS mount to change the imaging location. (b) The red dot denotes the location of the lens where the imaging is made. (c) An example of a compiled image of the 100 xy section images at different lens thickness from the lens top to bottom at 1 μm sectioning interval.

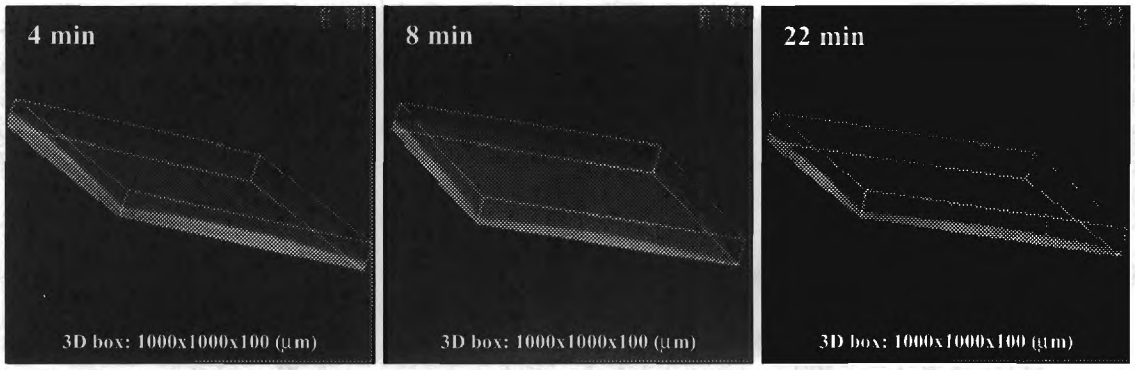


**Figure 5**. 3D images of a new (wet) lens 991204 as a function of air exposure (drying) time. The location of the visible top of the lens is lowered, clearly showing that the lens becomes thinner as the drying progresses.

For quantitative analysis, a circle (circumference = 266 μm) is drawn at the center part of the image and the fluorescence intensity along this circle is taken from each 100 section image. In general, the same argument can be made as described in the section 1.2 above, regarding to the thickness shrinkage upon drying. However, since the lens thickness at this location is bigger than the case in the previous section 1.2 (see Figure 3), the drying seems far less progressed at the time scale of 4-22 min, again indicating that the lens has to be left exposed to the air for a long time to ensure complete drying (see the section 2 below).

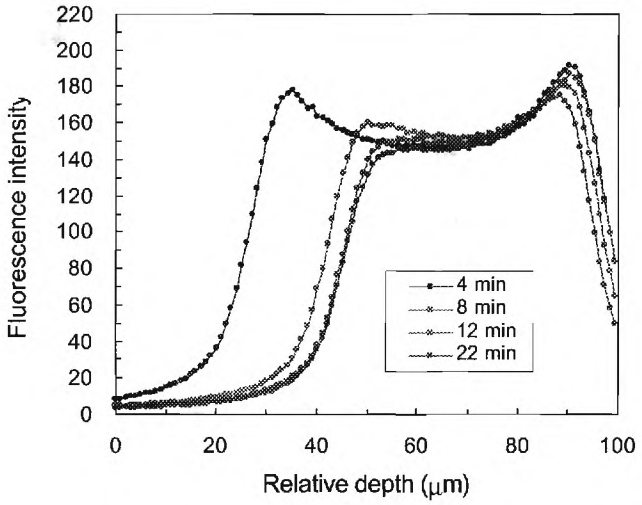


**Figure 6**. Fluorescence intensities of a new (wet) lens 991204 as a function of air exposure (drying) time, which are taken from the images in **Figure 2**. Please see the text for detail explanation.

## 1.3. Fluorescence intensity distribution at different locations of the lens: A new 991204 lens

A new 991204 lens was drained and placed on a PS mount in such a way that the lens and PS mount were as concentric as possible. This was then mounted on the microscope stage with the same concept to change the imaging location with 3, 6, 9 slide glasses (each 1 mm thick) stacked under one side of the PS mount (**Figure 7(a)**). For each different number of Hence the imaging was made for the locations shown in **Figure 7(b),** which are away from the center of the contact lens, while the imaging symmetry in xy direction was maintained. The location in **Figure 7(b)** is relative, because no other attention was made except the number of the slide glass. The highest area at these configurations was imaged at 100-160 optical sections from top to bottom at 1 μm interval. Imaging was done at 5 min from the moment the lens was opened with a new lens for each location.

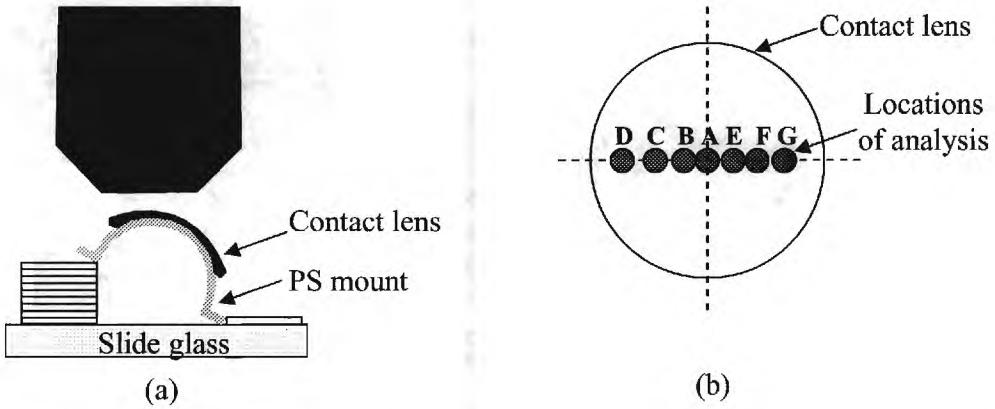


**Figure 7**. (a) Experimental setup for imaging of new contact lenses with stacked slide glasses (each 1 mm thick) under one side of the PS mount to change the imaging location. (b) The red dots indicate the relative locations of a new 991204 wet lens where the analyses were performed for the images and fluorescence intensities shown in **Figure 8** and **Figure 9**. The radial distance from the lens center is increasing in the order A<B<E<F<C<G<D.

The fluorescence intensity profiles across the lens thickness were taken for each of the xy section image in **Figure 8**. Then they are plotted in **Figure 9** such that each profile has symmetrical lens thickness in x axis (lens thickness) to make the comparison easier. From **Figure 9**, several observations can be made. First, as the radial distance from the lens center increases, the lens thickness increases. Second, the fluorescence intensity distribution is not uniform across the lens thickness, which is increasingly more evident as the area of the interest is further away from the lens center and hence the lens thickness increases. Third, as the area of interest is moved from the center (A→B→C→D or A→E→F→G), the fluorescence intensity at the middle of the lens, $I_m$, becomes lower. Fourth, the fluorescence intensity profile is not quite the same at different locations of the same lens thickness. At locations E and F, where the lens thickness was between the values at the location B and C, the middle point intensity, $I_m$ is far smaller than those at B and C. In other words, since the lens thickness is increasing in the order

of A<B<E<F<C<G<D, and it takes longer time to dye through the middle section of the lens thickness when the lens is thicker, it is expected to observe the $I_m$ in the order, $I_m(A)>I_m(B)>I_m(E)>I_m(F)>I_m(C)>I_m(G)>I_m(D)$, while the analysis shows that $I_m(A)\sim I_m(B)>I_m(C)>I_m(E)>I_m(F)>I_m(D)>I_m(D)$. It is expected to see more signigicant drying effect in the thinner parts of the lenses, i.e., in $I_m(A)$ and $I_m(B)$.

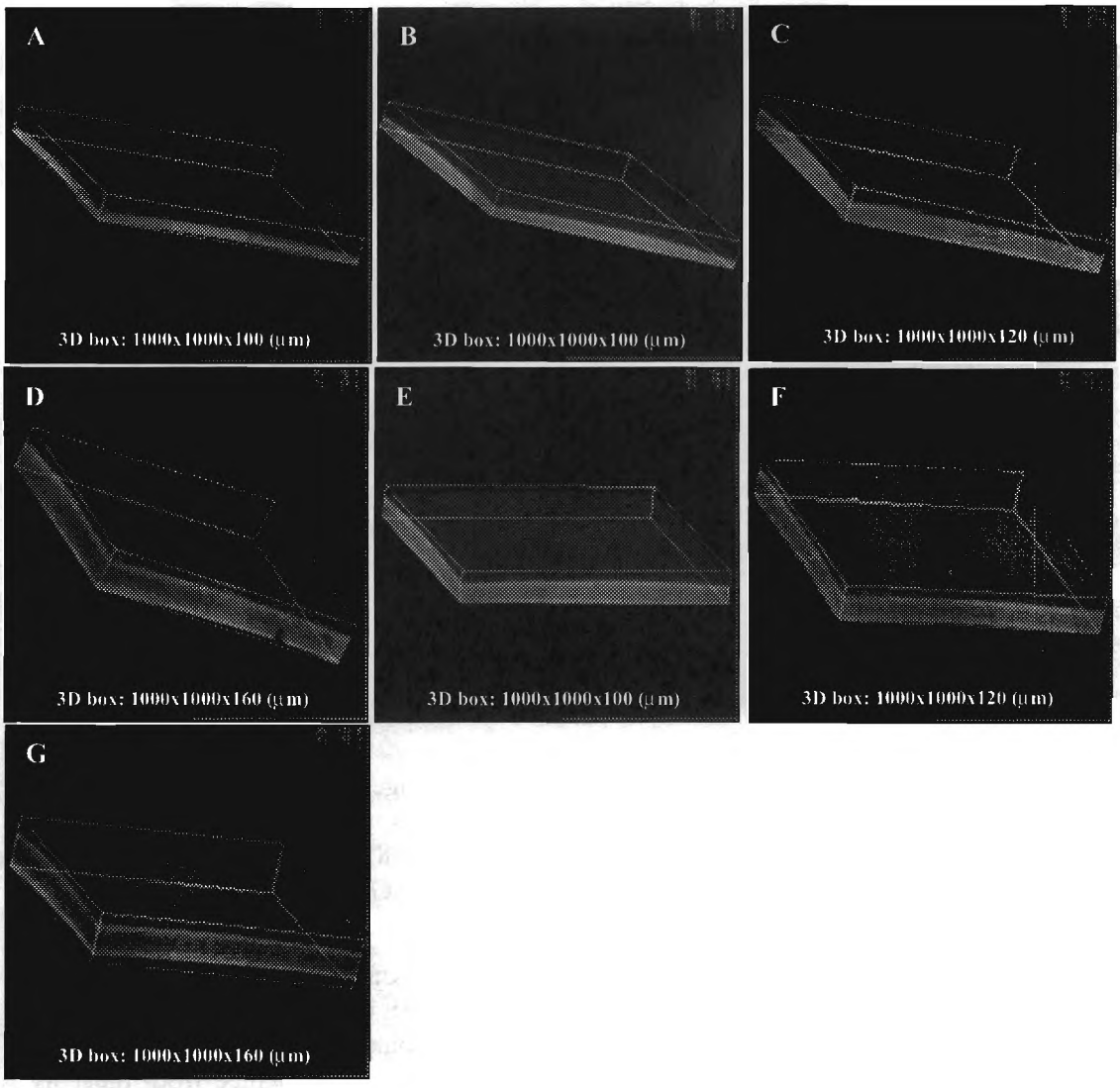


**Figure 8**. 3D images of a new (wet) lens 991204 at different locations of the lens, as indicated in **Figure 7(b)**. As the radial distance from the lens center increases, the lens thickness increases. The fluorescence intensity distribution is not uniform through the lens thickness, which is increasingly more evident as the lens thickness increases.

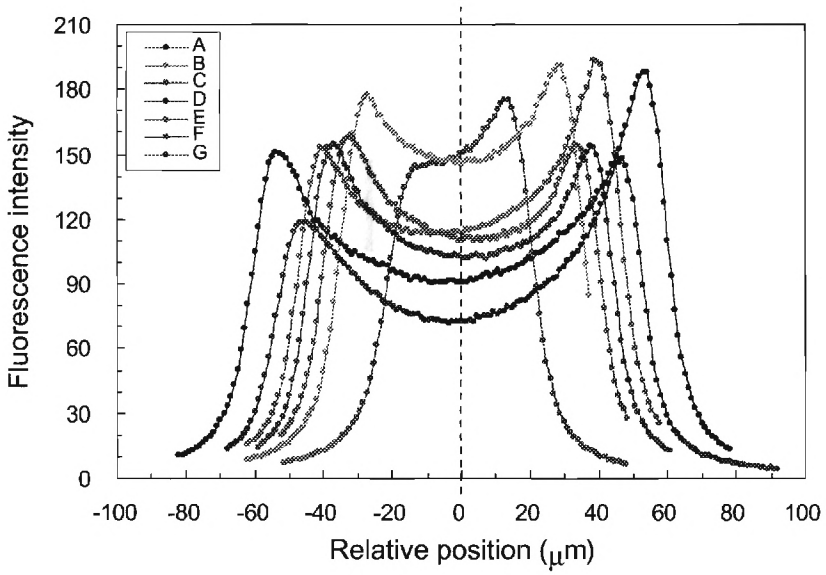


**Figure 9**. Fluorescence intensities of a new (wet) lens 991204 at various locations of the lens. This lens has non-uniform distribution of the fluorescence intensity across the lens thickness, indicating less than 100% absorption of the dye throughout the lens. As the area of interest is farther away from the lens center, the lens thickness becomes larger and the fluorescence intensity profile becomes more nonuniform in general, with higher intensity near lens surfaces.

## *2. Characterization of New 991204 Dry Lenses*

A new 991204 lens was opened and the excess saline fluid was removed by holding the lens vertically on a paper tissue as described above. The lens was placed on a PS mount, which was left in a Petri dish for **80 hrs** to ensure complete drying. It was found that the lens was dried maintaining its apparent original shape without any noticeable extra curvature, bent, or roughness. However, the lens and PS mount were appreciably off-centered each other as indicated in **Figure 10**.

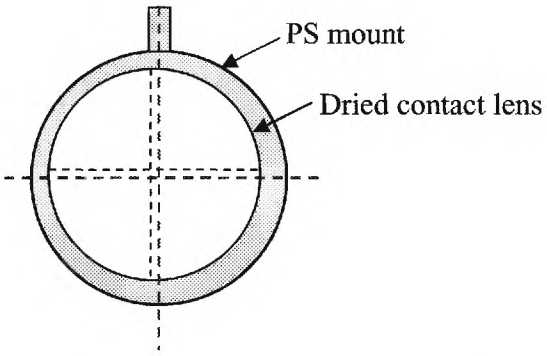


**Figure 10**. Relative placement of the dry contact lens on a PS mount, indicating they are not perfectly concentric to one another. This sample was used for the characterization of the dry lens, reported in **Figure 12** through **Figure 19**.

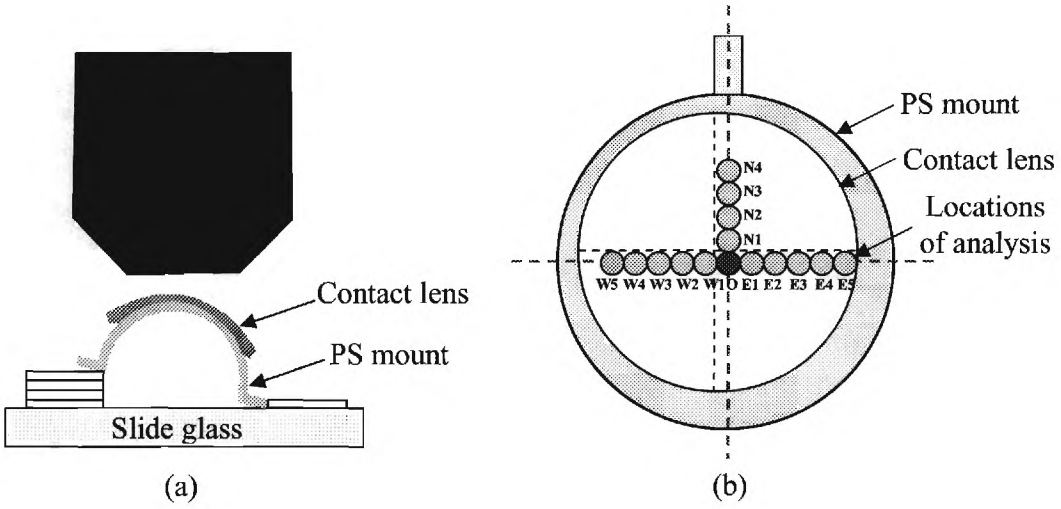


**Figure 11**. (a) Experimental setup for imaging of new contact lenses with stacked slide glasses (each 1 mm thick) under one side of the PS mount to change the imaging location, where the number of the slide glass varies 0-10. (b) The red dot indicates the location of the analysis

without any slide glass, and the pink dots with 2-10 slide glasses. The results are shown in **Figure 12** through **Figure 19**.

The PS mount with a dry lens was placed on a microscope stage with 0, 2, 4, 6, 8, and 10 slide glasses (each 1 mm thick) (**Figure 11(a)**). The center area was imaged The highest area (center) of the specimen was chosen for imaging at each configuration (**Figure 11(b)**), at 66-160 optical sections from top to bottom at 1 μm interval. The locations indicated in Figure 11(b) are only relative, without quantitative meaning in absolute scale. The only controlled variable was the number of the slide glasses (each 1 mm thick) used.

## 2.1. Fluorescence intensity distribution across the *dry* lens thickness: At the center of a *new (unworn) dry* 991204 lens

3D images in **Figure 12** and the fluorescence intensity profile in **Figure 13** was plotted in **Figure 13** such that the position zero represents the middle section of the lens thickness for easier comparison. **Figure 13** actually includes 3 sets of data to show the reproducibility. When compared with the new (wet) lenses, it is found that 1) the dried lens has much smaller thickness, 2) the overall fluorescence intensity in a dry lens is lower for given lens thickness, and 3) the fluorescence intensity distribution in a dry lens is much more homogeneous across the lens thickness (see **Figure 2** and **Figure 3** for comparison). This can be explained with the same argument made with the wet lens profile. First, even though the fluorescent dye absorption was not originally uniform throughout the lens thickness when the lens was made, it became more uniform in a reduced thickness. Second, the drying is complete and hence the water content is homogenous, which offers an environment where the fluorescence quantum yield is uniform throughout the lens thickness even to the bottom of the lens.

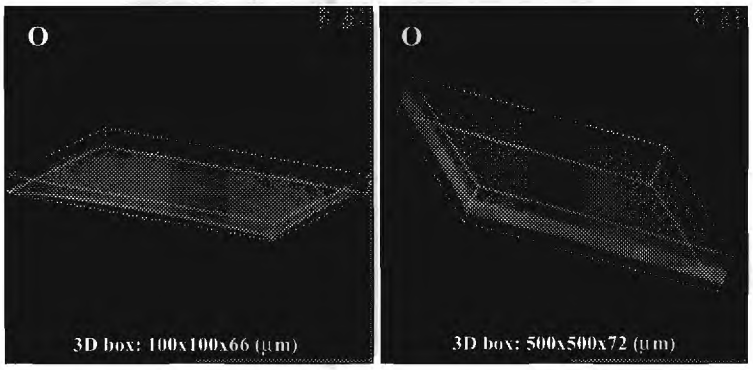


**Figure 12.** 3D images of a new lens 991204 ***dried*** on a PS mount for 80 hrs at the center of the lens, taken at the location O in **Figure 11(b)**. The right image was taken at higher zoom factor to better observe the variation across the lens thickness.

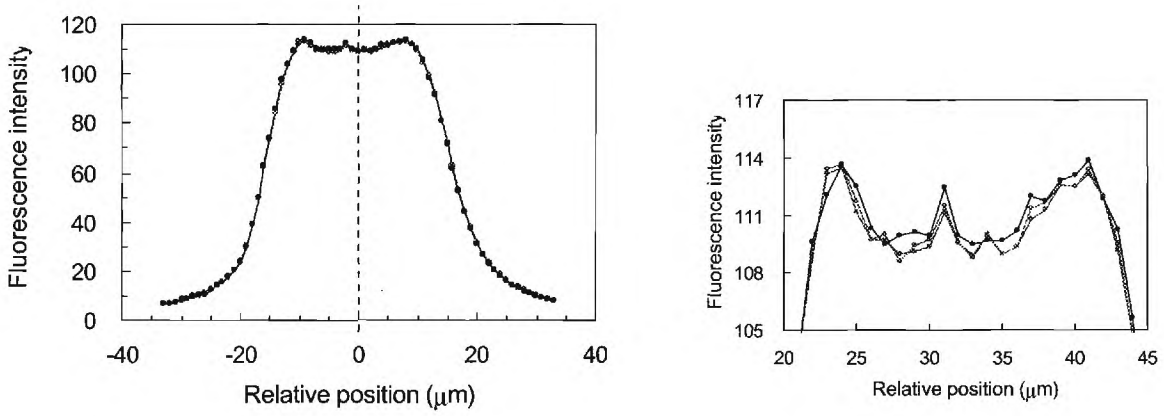


**Figure 13**. Fluorescence intensity profiles across the lens thickness of a new lens 991204 ***dried*** on a PS mount for **80 hrs** at the vicinity of the lens center (location O), which are taken from the images used in **Figure 12**. The smaller graph at right is an enlarged portion of the high intensity area, showing excellent reproducibility of the 3 set of data.

## 2.2. Fluorescence intensity distribution across the *dry* lens thickness at locations N1-N4: A *new (unworn) dry* 991204 lens

A series of analysis is made on the new dry 991204 lens at locations N1 through N4 indicated in **Figure 11(b)**, the 3D images are collected in **Figure 14**. The fluorescence intensity profiles across the lens thickness plotted in **Figure 15** such that the relative position zero represents the middle point of the lens thickness, to make the comparison easier.

3D images in **Figure 14** and the fluorescence intensity profile in **Figure 15** also show similar trend that 1) the dried lens has much smaller thickness, 2) the fluorescence intensity is lower, and 3) the fluorescence intensity distribution is much more homogeneous across the lens thickness, than the wet lens (see **Figure 2** and **Figure 3** for comparison). This can be explained with the same argument made with the wet lens profile. First, even though the fluorescent dye absorption was not uniform throughout the lens thickness when the original wet lens was made, it became more uniform in a reduced thickness, and second, the drying is complete and hence the water content is homogenous, which offers an environment where the fluorescence quantum yield is uniform throughout the lens thickness even to the bottom of the lens.

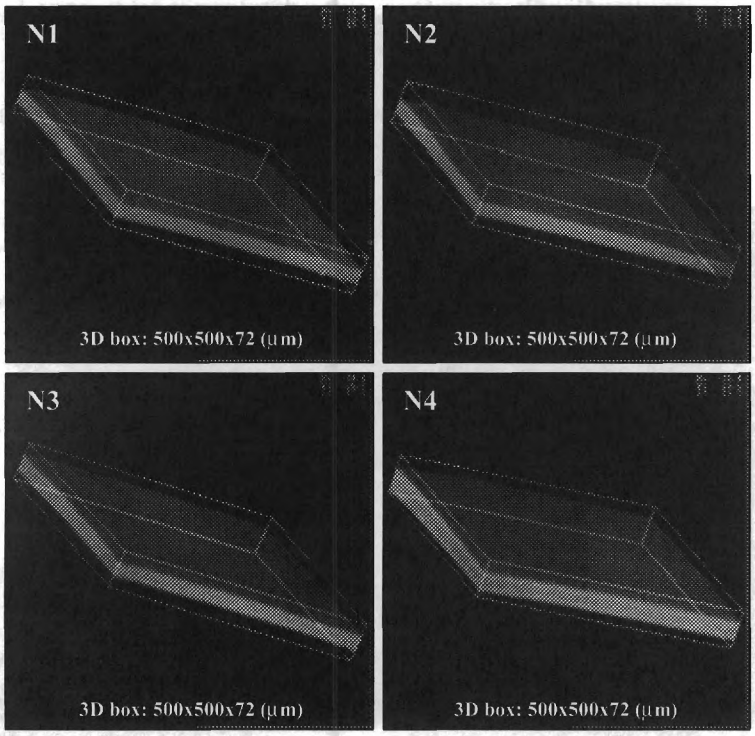


**Figure 14**. 3D images of a new lens 991204 ***dried*** on a PS mount for 80 hrs at the lens locations N1 through N4 in **Figure 11(b)**.

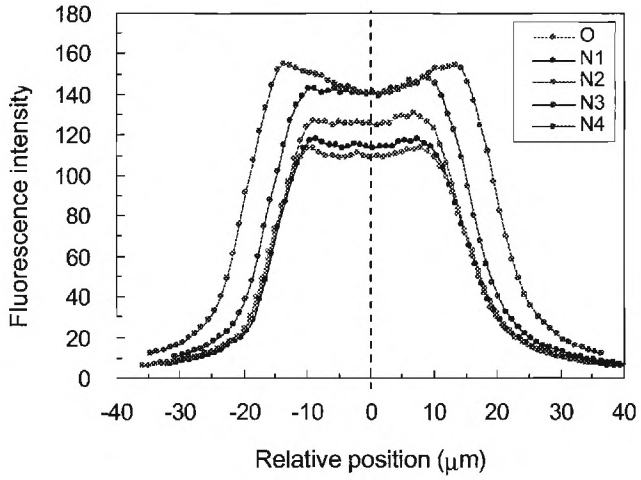


**Figure 15**. Fluorescence intensities of a new lens 991204, ***dried*** for 80 hrs at room temperature, at various locations of the lens. This lens originally has distinct non-uniform distribution of the fluorescence intensity across the lens thickness (see **Figure 9**), however, the distribution became more uniform upon drying.

## 2.3. Fluorescence intensity distribution across the dry lens thickness at locations W1-W5: A *new (unworn) dry* 991204 lens

A series of analysis is made on the new dry 991204 lens at locations W1 through W5 indicated in **Figure 11(b)**, the 3D images are collected in **Figure 16**.

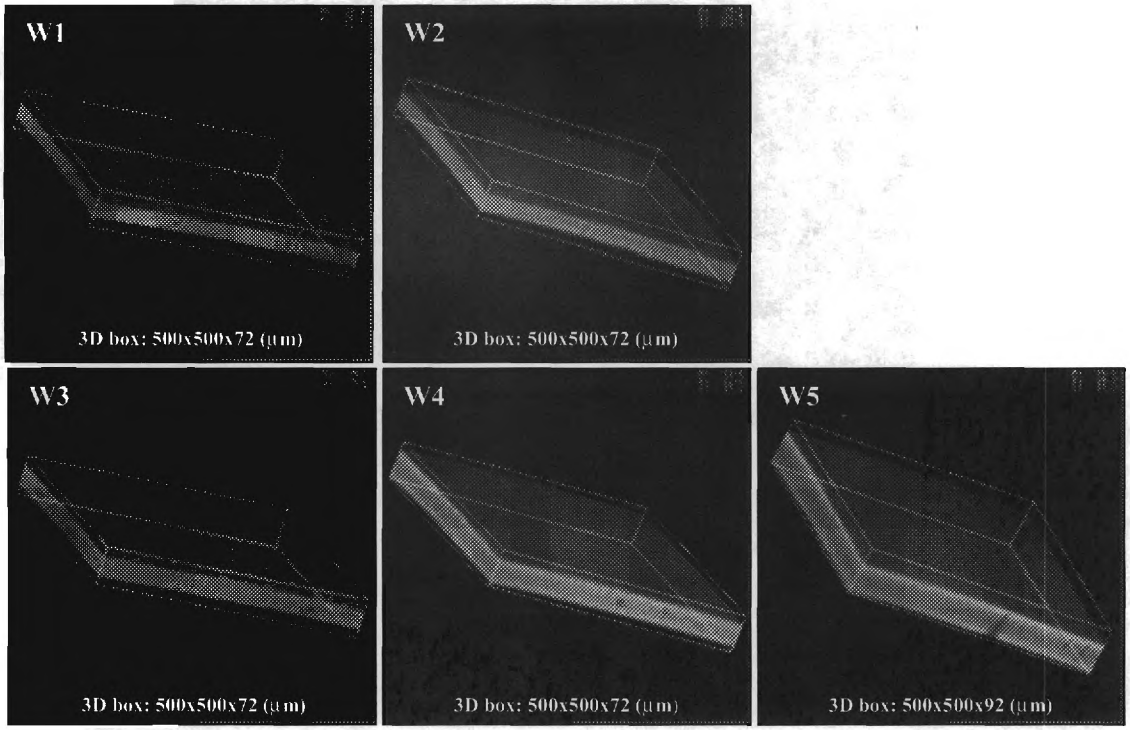


**Figure 16**. 3D images of a new lens 991204 ***dried*** on a PS mount for 80 hrs, taken at the lens locations W1 through W5 in **Figure 11(b)**.

The fluorescence intensity profiles across the lens thickness were plotted in **Figure 17** in the same way as above to make the comparison easier. As shown in Figure 9 and discussed in section 1.3 in pages 7-8, the $I_m$ again does not follow the simple trend which is expected from the lens thickness variation and the longer diffusion distance as a function of the lens thickness.

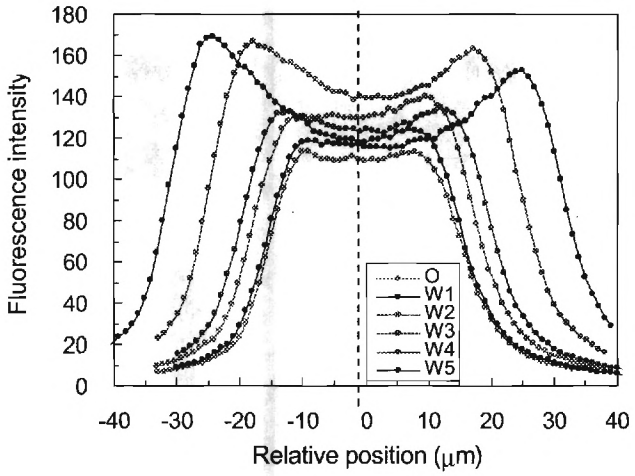


**Figure 17**. Fluorescence intensities of a new lens 991204, ***dried*** for 80 hrs at room temperature, at various locations of the lens W1 through W5.

## 2.4. Fluorescence intensity distribution across the dry lens thickness at locations E1-E5: A *new (unworn) dry* 991204 lens

A series of analysis is made on the new dry 991204 lens at locations E1 through E5 indicated in **Figure 11(b)**, the 3D images are collected in **Figure 18**. The fluorescence intensity profiles across the lens thickness are plotted in **Figure 19** in the same way as above to make the comparison easier.

The overall observation is the same as discussed in sections 2.2 and 2.3.

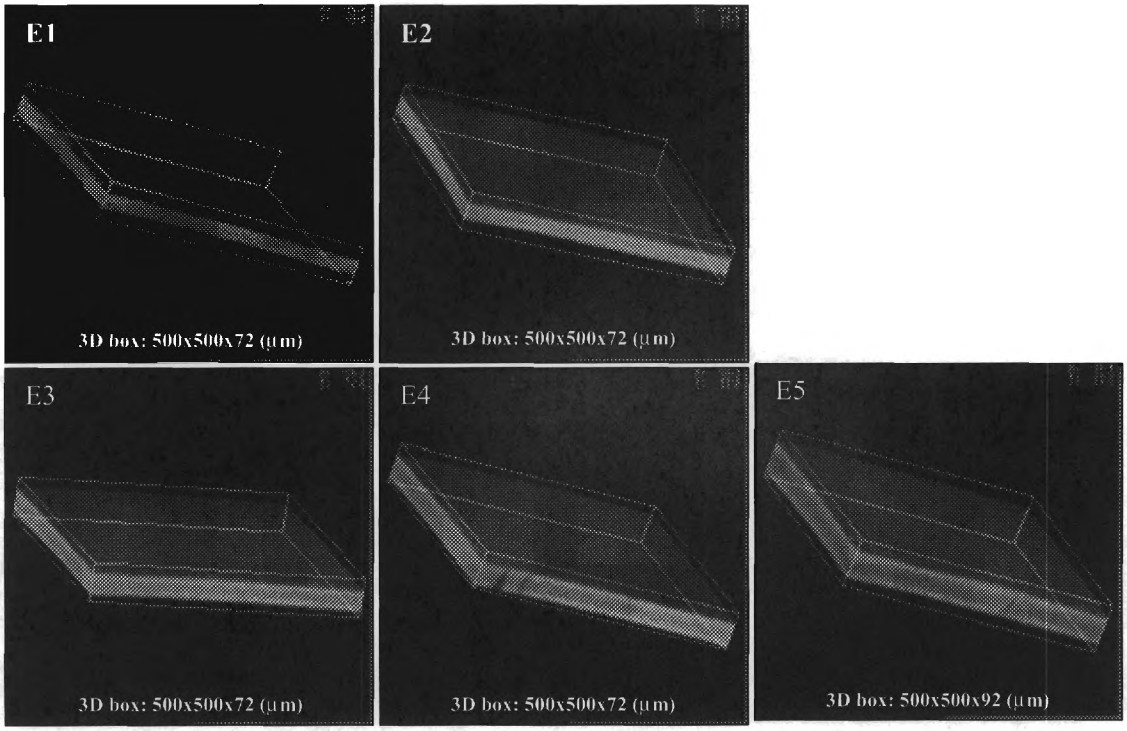


**Figure 18**. 3D images of a new lens 991204 ***dried*** on a PS mount for 80 hrs, taken at the lens locations E1 through E5 in **Figure 11(b)**.

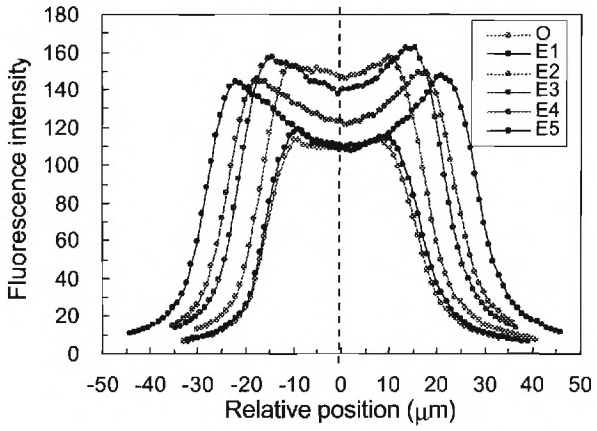


**Figure 19**. Fluorescence intensities of a new lens 991204, ***dried*** for 80 hrs at room temperature, at various locations of the lens E1 through E5.

## *3. Characterization of Worn 991204 Lenses*

### 3.1. 991204-131-OD-Worn

This lens was folded in squashed taco shape, already almost dried, and pretty rigid. It was not possible to find any flat area to image on without breaking the sample. A small piece of specimen was taken by a sharp razor blade around a lens edge area.

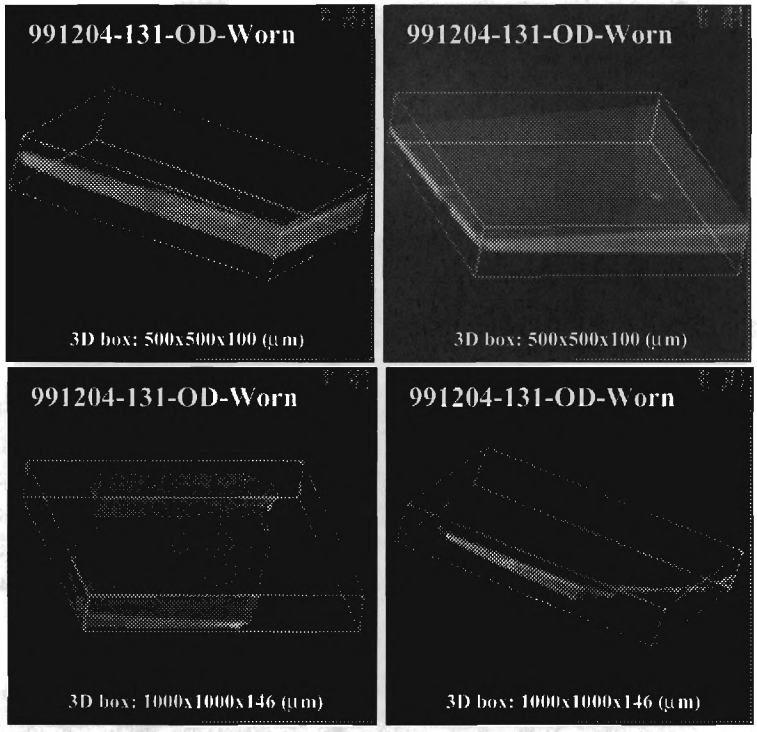


**Figure 20**. 3D images of a tiny specimen cut from a worn 991204-131-OD lens, at various viewing angles.

**Figure 20** shows that this specimen was cut in a direction such that it reveals the lens thickness variation very clearly.

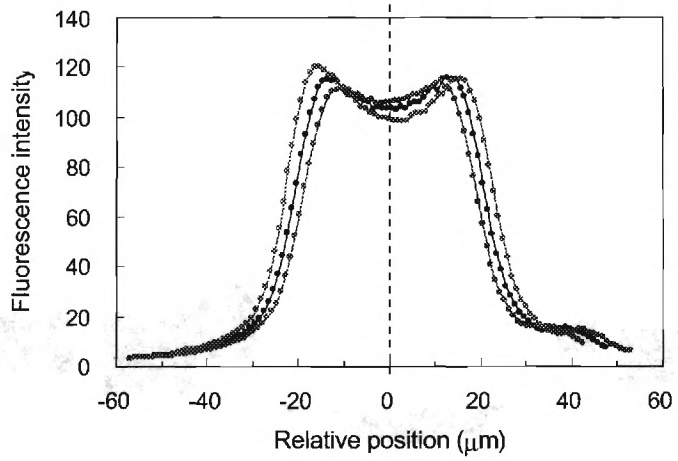


**Figure 21**. Fluorescence intensities of a worn 991204-131-OD lens.

## 3.2. 991204-131-OS-Worn

This lens was in deformed/irregular taco shape with high rigidity and brittleness. Care was taken to take a tiny specimen from a reasonably flat area using a sharp razor blade, but the lens was broken into several pieces. It was unknown where the specimen was located with respect to its original lens configuration, however, the 3D images below show that they are probably from a thick part of the lens. The fluorescence intensity was averaged from 4 sets of data at each location. Since this specimen has much larger lens thickness, the fluorescence intensity profile is much more non-uniform across the lens thickness.

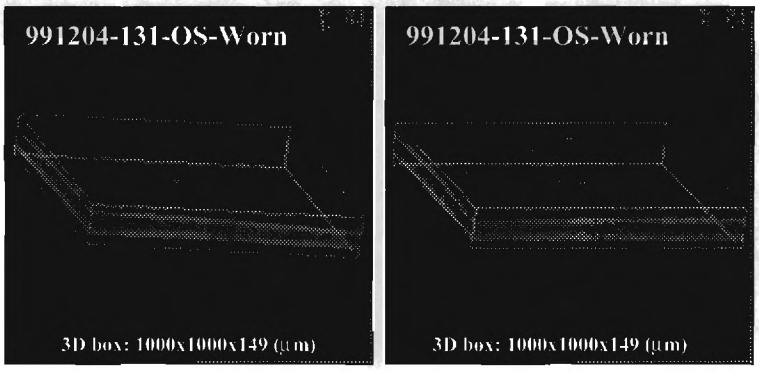


**Figure 22**. 3D images of a worn 991204-131-OS taken from a tiny specimen cut from the worn lens.

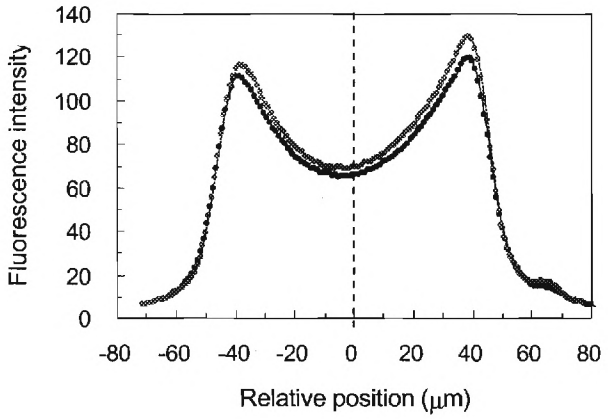


**Figure 23**. Fluorescence intensities of a worn 991204-131-OS lens, at two locations of the lens.

## 3.2. 991204-132-OS-Worn

This lens was bent near the center and reasonably flexible. It was placed on a PS mount and imaged at a location with minimum visible curvature, which is not an area very close to the center of the lens. By the time the experiment was over, the lens was changed into quite rigid state.

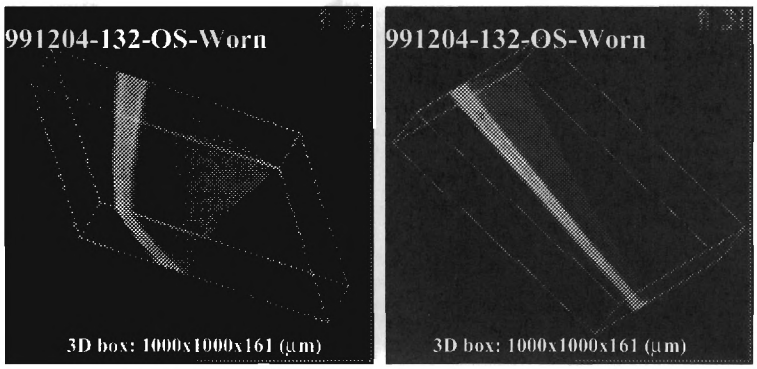


**Figure 24**. 3D images of a worn 991204-132-OS taken from a tiny specimen cut from the worn lens, represented in various viewing angles.

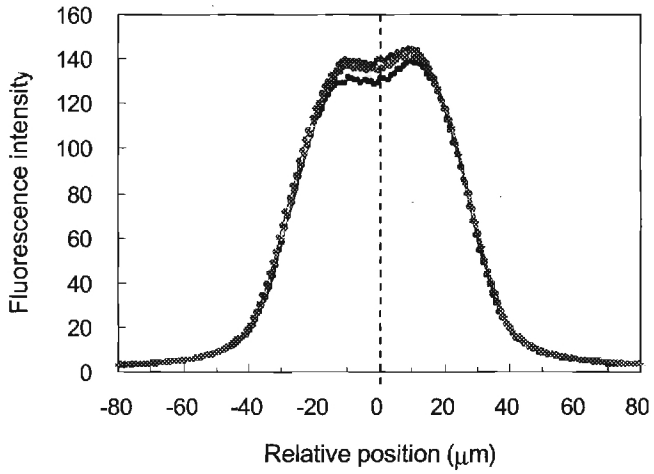


**Figure 25**. Fluorescence intensities of a worn 991204-132-OS lens.

## *4. Characterization of Worn 991304 Lenses*

### 4.1. 991304-51-OD-Worn

This lens was bent near the center and reasonably flexible. It was placed on a PS mount and imaged at a location with minimum visible curvature, which is not an area very close to the center of the lens. By the experiment was over, the lens was changed into quite rigid state.

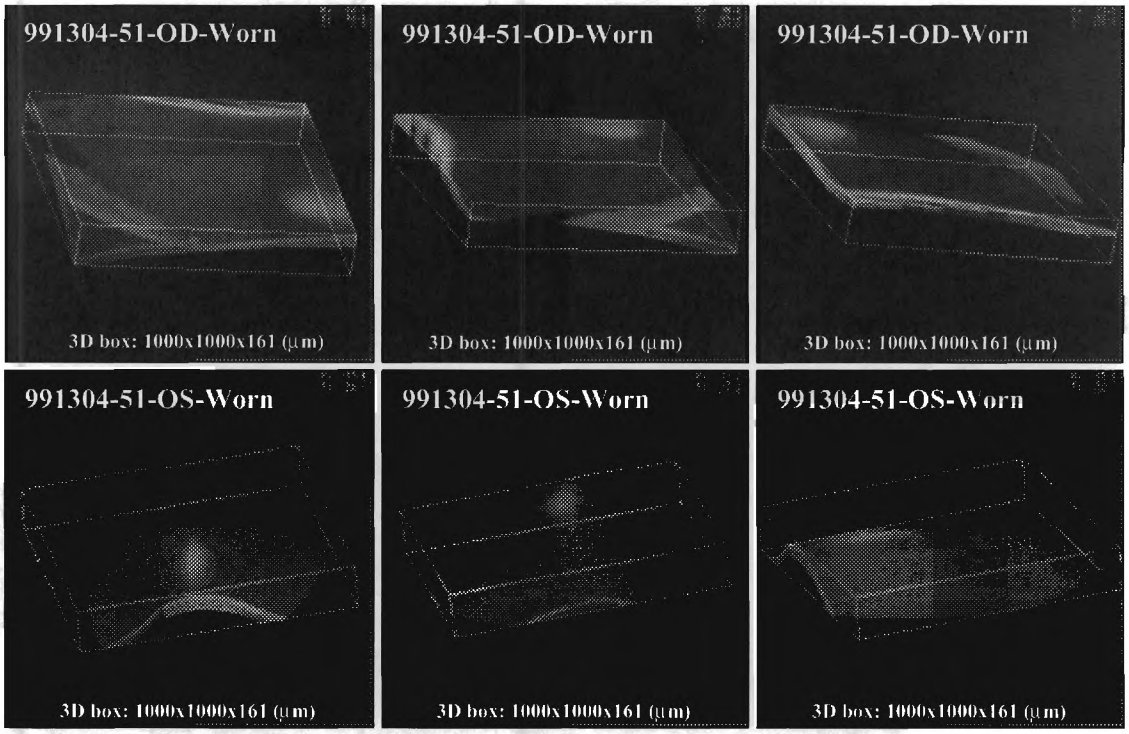


**Figure 26**. 3D images of a worn 991304-51-OD taken from a tiny specimen cut from the worn lens in various viewing angles.

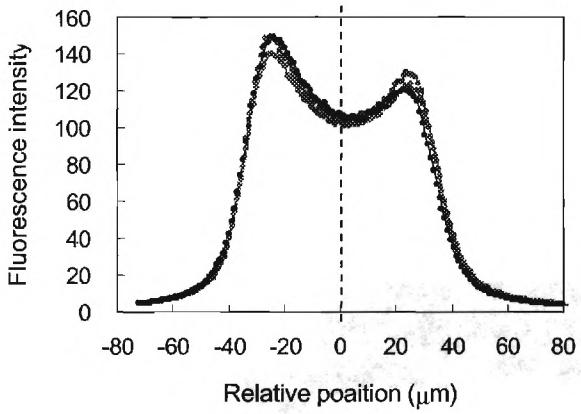


**Figure 27**. Fluorescence intensities of a worn 991304-51-OD lens, at various locations of the lens.

## 4.2. 991304-51-OS-Worn

This lens was in pretty dry and brittle shape. A tiny piece was taken from the lens.

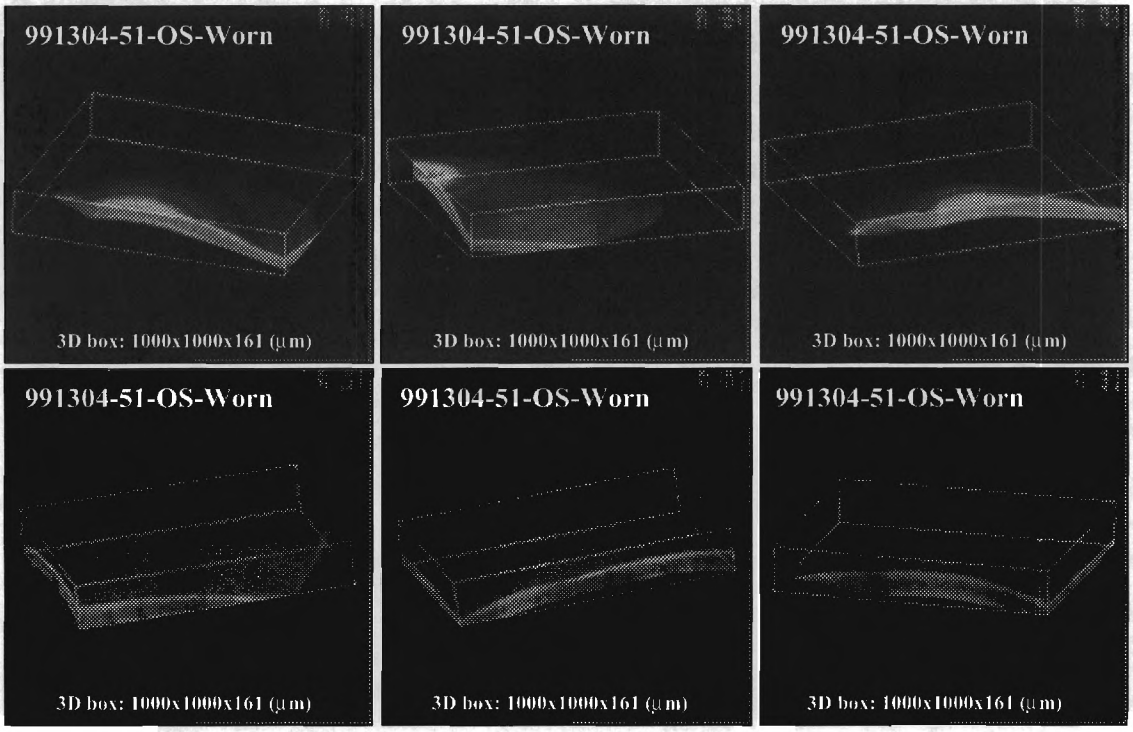


**Figure 28**. 3D images of a worn 991304-51-OS taken from a tiny specimen cut from the worn lens, at different locations and in various viewing angles.

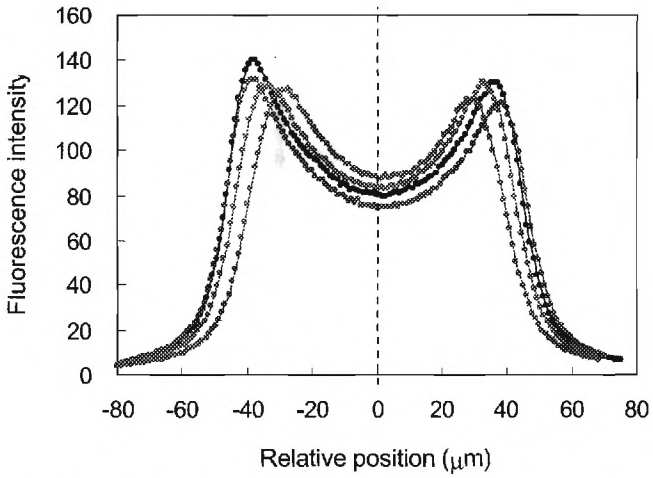


**Figure 29**. Fluorescence intensities of a worn 991304-51-OS lens, at various locations of the lens.

In **Figure 30**, the typical fluorescence intensity profile of each worn lens sample is compiled. In comparison with the new dry sample, since the worn samples still contain varying amount of water depending on the location of the lens and the lens thicknesses are hence in between the new wet lens and new dry lens, the fluorescence intensity profiles are in between those of new wet lenses and those of new dry lenses, in terms of the $I_m$ and intensity uniformity.

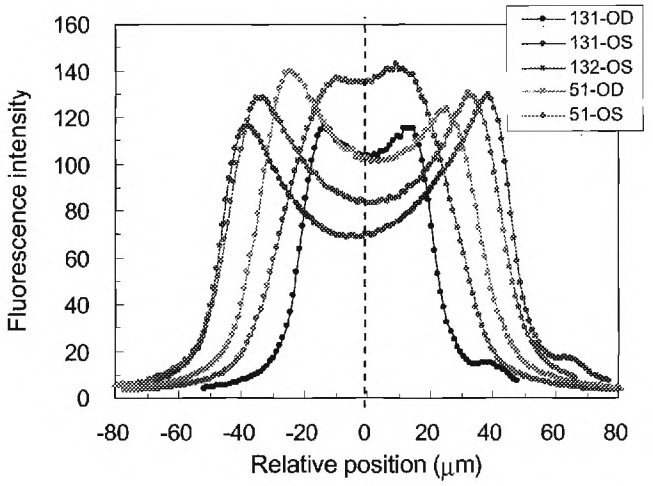


**Figure 30**. Typical fluorescence intensities of the worn lenses. The trend is the same in that as the lens thickness gets larger, the fluorescence intensity become more bimodal, with higher intensity near lens surfaces.

## *5. Comparison of Worn 991204 Lenses with a New Dry 991204 Lens*

The 3D images of the worn lenses above show that xy section imaging was done vertically across the lens thickness for only two worn lenses, 991204-131-OD and 991204-131-OS, as it was done so for all new lens samples. Hence, in **Figure 31**, the typical fluorescence intensity profiles of these two worn lens samples are compared with those of some new dry lens profiles. Again, the profiles of E2 and 991204-131-OD at similar lens thickness show that the intensity at the middle part of the lens thickness is lower for the worn lens. However, this may be due to the reduced water content in the worn lens. The overall trend of increasing/falling fluorescence intensity, i.e., the slope of the intensity at both ends in all the intensity profiles, near the lens surfaces seem not much different in worn lenses, when compared with the new wet or dry lenses. Also, it is not possible to make any definite judgment on whether the worn lenses lost detectable amount of fluorescent material very close to the lens surfaces during the usage, because the z resolution is only about 0.5 micron.

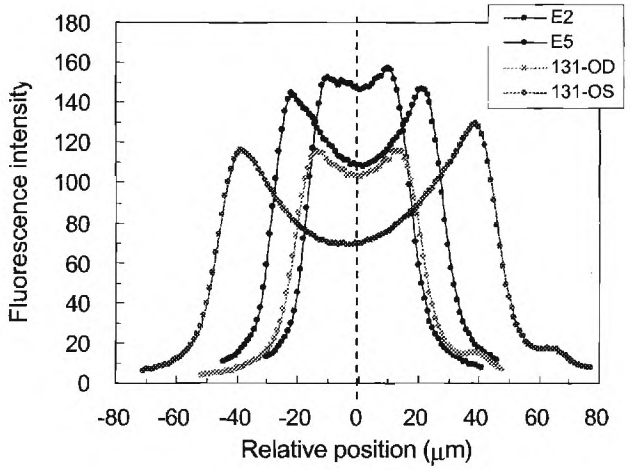


**Figure 31**. Fluorescence intensities of two worn lenses, 991204-131-OD and 991204-131-OS and a new dry 991204 lens at two different lens thicknesses.

# IV. SUMMARY

The fluorescence intensity profile across the lens thickness was studied using Leica laser scanning confocal microscope in fluorescence mode for new (wet) 991024 lenses, a dry 991024 lens and 5 worn lens samples at various lens locations. Lenses were mounted on a PS mount both for new wet samples and a new dry sample. For worn lenses, when the lens was in badly deformed dry state, a tiny specimen was taken for microscopic study, while when the lens is still rather "flexible", then the whole lens was mounted on a microscopy slide glass and efforts were made to image the area with minimum deformation relatively close to the lens center. Following observations were made.

1. As the new (wet) lens is exposed to the air and the water starts evaporating, the lens starts shrinking from the exposed top surface and hence the fluorescence intensity starts to show up at lower height (z position) as the exposure time increases. On the other hand, since the bottom surface, which is in contact with the PS mount, does not lose appreciable amount of water within the experimental time scale, the fluorescence intensity does not change much.
2. The new (wet) lenses of lot number 991204 do not have uniform fluorescence intensity distribution across the lens thickness, which is indicative of that the dyeing process was stopped before it reached the uniform equilibrium state. The fluorescence intensity was higher near the lens surfaces and lower at the middle part of the lens thickness.
3. The observations with the new (wet) lenses at different locations at about the same exposure time can be summarized as following:
    1) As the radial distance from the lens center increases, the lens thickness increases.
    2) The fluorescence intensity distribution is not uniform across the lens thickness, which is increasingly more evident as the area of the interest is further away from the lens center and hence the lens thickness increases.
    3) As the area of interest is moved from the center (A→B→C→D or A→E→F→G in **Figure 7**), the fluorescence intensity at the middle of the lens thickness, $I_m$, becomes lower, indicative of the less amount of dye diffusion through the thicker part of the lens.
    4) The fluorescence intensity profile is not quite the same at different locations of the lens with the same lens thickness.
4. The observations with a new dry lenses at different locations can be summarized as following, in comparison with the new wet lenses:
    1) The dried lens has much smaller thickness.
    2) The overall fluorescence intensity is lower when compared at the same lens thickness.
    3) The fluorescence intensity distribution is much more homogeneous across the lens thickness.
5. In the case of worn lenses:
    1) The status of drying, distortion and deformation was different from sample to sample, and hence it was not possible to look at the lens center area or at the general same area from the lens center.

2) Except for 991204-131-OS and 991204-131-OD worn lenses, the imaging of the rest 3 worn lenses and hence their fluorescence intensity profiles were probably not across the lens thickness in right angle, because they were not imaged vertically thoroughly the lens thickness (see **Figures 22, Figures 24** and **Figures 26**). This is because the samples were highly distorted.

3) The z resolution of the confocal microscope is around 0.5 μm, and it is not possible to differentiate the fluorescence intensity profile very close to the lens surface within 1 μm thickness.

## V. RECOMMENDATION FOR FURTHER STUDY ON WORN LENSES

For better controlled study of the worn lens samples, it is recommended to apply following approaches.

1) To maximize the possibility of relative comparison with new (unworn) lenses, the worm samples need to be stored in an air-tight container to keep the shape and the water content as much as possible until they are analyzed.

2) If the practice suggested above is not possible, the worn samples may be immersed in a saline solution and examined with waster immersion objectives. The diffusion of PVP-dye molecules, which probably have high molecular weight, may be not so fast to affect the fluorescence intensity profile within the experimental time scale of less than 10 min.

3) It will be also useful to immerse the worn lenses in a fluid, which is not a solvent either for the lens material or for the fluorescent molecules. In order to greatly improve the imaging at the lens boundary, this fluid may contain a dye with different excitation wavelength from the fluorescein type that was used in the lens manufacturing.

4) If the desorption/diffusion loss of the fluorescent molecules needs to be studies within several hundred nanometer range from the lens surface, it is necessary to observe the difference in the fluorescence intensity profile in this small scale distance. It is recommended that one should pursue the photon tunneling microscopy (evanescent wave microscopy).