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CHEMISTRY AND CHEMICAL ENGINEERING
DEPARTMENT OF MICROENCAPSULATION AND NANOMATERIALS

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Subject: SwRI® Project 01.15703 entitled *"Liposome Uptake Assay"* Report

Dear Ms. McKinnon:

The Southwest Research Institute (SwRI®) is pleased to submit this report for SwRI Project 01.15703.01.001

Objective of Project

As stated in the original proposal, the purpose of this study is to design a cellular uptake assay using LivOn's existing liposome formulation and collect images illustrating the uptake into cells in culture for the purpose of marketing by LivOn. To collect these images the following points must be addressed.

- Develop an assay using fluorescent imaging to visualize liposomal uptake in human cells
- Image the cells in the presence of the fluorescently-labeled liposomes using confocal microscopy.

Summary of Task Results

Below is a summary of results obtained for each of the above tasks.

1.1 Task 1: Develop an Assay with Relevant Cell Lines to Determine Liposomal Uptake

Previous work performed on this project (see Interim Report from August 2010) was done to develop a protocol for the uptake studies based on a method found in the literature.[1] The Hep(G2) cell line was used in conjunction with fluorescently-labeled liposomes (Peter Zhang-FormuMax). This previous work showed promising results indicating that the liposomes were possibly taken up by the cells and could be imaged after incubation with the cells. Using this same assay, confocal images were taken to confirm that the liposomes were within the interior of the cells. Two uptake assays were performed and imaged using the confocal microscope.

1.1.1 Optimization of Method

The previous report outlined areas that needed to be optimized in order to achieve better results. The main areas of optimization were the growth conditions of the cell line to achieve a monolayer of cells and the ratio of liposomes to cells in the uptake assay (for better visualization of cellular uptake). In order to address these issues the following strategies were employed.

[1] Mady, M.M. et al. "Efficiency of Cytoplasmic Delivery by Non-Cationic Liposomes to Cells in vitro: A Confocal Laser Scanning Microscopy Study" Physica Medica (2009) 25,88-93.

1.1.1.1 Liposome to Cell Ratio

The previous report (August 2010) showed very few liposomes interacting with the cells. In order to better visualize uptake of liposomes by the Hep(G2) cells, it was decided to repeat the imaging using a higher ratio of liposomes to cells. Dilutions of the liposomes were previously made between 1:100 and 1:6400. The imaging studies below focus on dilutions of 1:10 through 1:100 resulting in a much higher amount of liposomes present during incubation with the Hep(G2) cells.

1.1.1.2 Growth Conditions of Hep(G2) Cells

The images of cells from the aforementioned previous report indicated that the cells were not in a uniform monolayer on the surface of the slide. This "piling up" of cells makes confocal microscopy difficult, as the actual position of a liposome in relation to the cells may not be clear. Alternate sets of growth conditions were evaluated in an effort to produce a monolayer cell culture. Previous imaging studies were performed using Hep(G2) cells grown in a cell culture medium known as Eagle's Minimal Essential Medium (EMEM). A literature search revealed that a similar medium, Dulbecco/Vogt Modified Eagle's Minimal Essential Medium (DMEM) was also used with the Hep(G2) cell line. Both media types were evaluated for their ability to promote a monolayer of cells. Interestingly, the cells grown in DMEM did not grow as quickly, indicating less-than-ideal conditions. These cells appeared essentially dormant in the medium and were not imaged. Other parameters such as the addition/omission of antibiotics and another additive (Fetal Bovine Serum- a supplement) were explored. Because no improvement was seen when adjusting the above growth parameters, a review of the growth conditions recommended by the supplier of the cell line was conducted. It was found that the removal of the cells from the culture flasks could lead to the cells clumping if not performed in a specific manner. The latest sets of cells used in the uptake assays were processed by the supplier's specifications resulting in a morphology more amenable to microscopic imaging.

1.2 Task 2: Confocal Microscopy of Hep(G2) cells incubated with Fluorescently-Labeled Liposomes

Using the modifications described above, a protocol was generated to prepare the cells, incubate them with liposomes and image the samples as described below.

Hep(G2) Preparation/Liposome Incubation Protocol for Confocal Imaging at Higher Liposome:Cell Ratio

Cell line preparation

1. Twenty thousand Hep-G2 cells were seeded in each well of an 8-well treated plate. Cells were grown overnight at 37 °C in cell culture media.

Liposome Preparation

2. Transferred 100 µL of prepared Liposome P16 (FormuMax-Zhang) into a sterile 1.5 mL microfuge tube. Washed with 1 mL sterile PBS (phosphate buffered saline pH 7.4). Liposomes were pelleted briefly by centrifugation and resuspended in 1 mL of cell culture medium.
3. Prepared serial dilutions of liposomes in cell culture medium at various ratios (**1:10 to 1:100)**.

Liposome/Hep-G2 cell incubation

4. Added 500 µL of serially diluted liposomes to designated wells on sterile 8-well plate and incubated cells in presence of liposomes overnight at 37 °C in a humidified incubator at 5% CO_2.
5. Removed growth media and washed twice with fresh media (removal of unincorporated liposomes)
6. Added 10 µL of Hoechst (blue nuclear stain) and/or Calcein stain (green cellular matrix stain) to each well and incubated cells for 30 minutes to stain.
7. Removed growth medium and washed cells twice with buffer (PBS) to remove unincorporated stains.

Preparation of cells for Imaging and Imaging of Hep-G2 cells/Liposomes

8. Fixed cells for 15 minutes at room temperature with a paraformaldehyde-based fixative solution and washed again with PBS twice.
9. Prepared imaging slide containing fixed cells according to manufacturer's recommendations.
10. Imaged cells on a Flowview FV1000 confocal fluorescent microscope. Imaged cells using the three different fluorescent filters:
    - Imaged the cell matrix itself using the green fluorescent filter(FITC)

- Imaged the cell nuclei using blue fluorescent filter(DAPI)
- Imaged the liposomes using the red fluorescent filter(TX2)

1.2.1 Imaging Runs

Two imaging runs are described below. Both runs utilized the protocol summarized above with specific modifications as described.

1.2.1.1 Imaging Run August 16, 2010

The set of images below represent the first images made using the confocal microscope of the Hep(G2) cells after incubation with liposomes. Dilutions of 1:10, 1:30, 1:50 and 1:100 were made of the liposomes prior to incubation with the cells. Due to poor signal from the prepared sample, only images of the 1:50 dilution of liposomes were recorded. These images are shown below. The confocal images contain images in both the normal XY plane as well as "cross sections" in the XZ and YZ planes. The cross sectional images were taken as indicated by the yellow lines in the main images.

Figure 1 illustrates a typical confocal image of Hep(G2) cells after incubation with liposomes. Note that some liposomes appear to be associated with the cells while others are not (red liposomes in dark areas around cells). However, some liposomes appear to be in very close association with and within the cells as indicated by the alphabetic labels. The cross sectional areas corresponding to the labels indicate that the liposome at A and B are very likely within the interior of the cell. The liposome at C may be within the cell or attached to the surface.

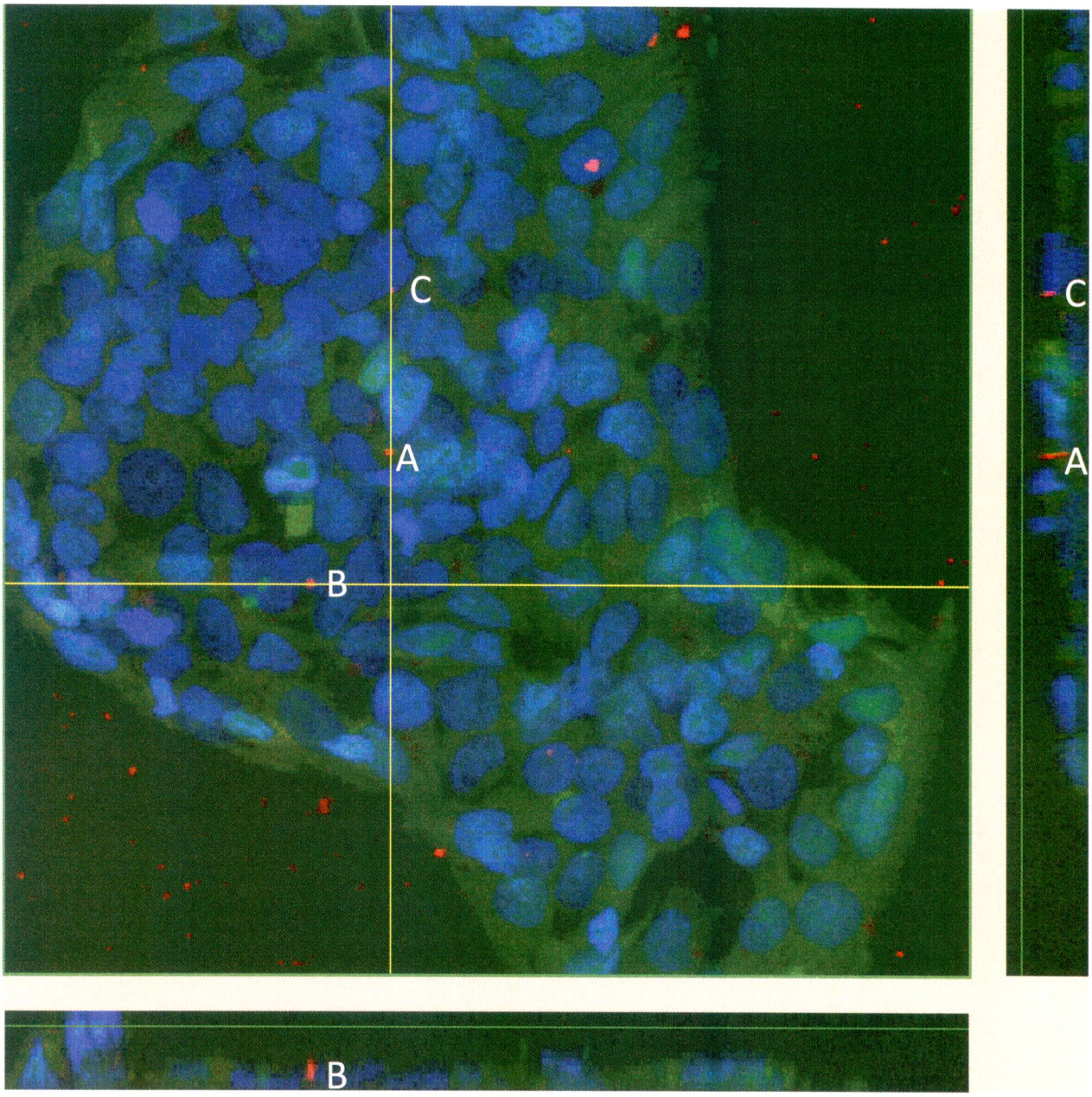

Figure 1: Confocal Fluorescent images of Hep(G2) cells incubated with fluorescently labeled liposomes diluted 1:50. Images at right and bottom are cross sections of YZ plane and XZ plane respectively. Cross sections were taken from main image at yellow lines. Labels A, B, and C indicate liposomes in close proximity to cells. Cross sections of labeled areas indicate liposomes are within interior of cells. Magnification 40X

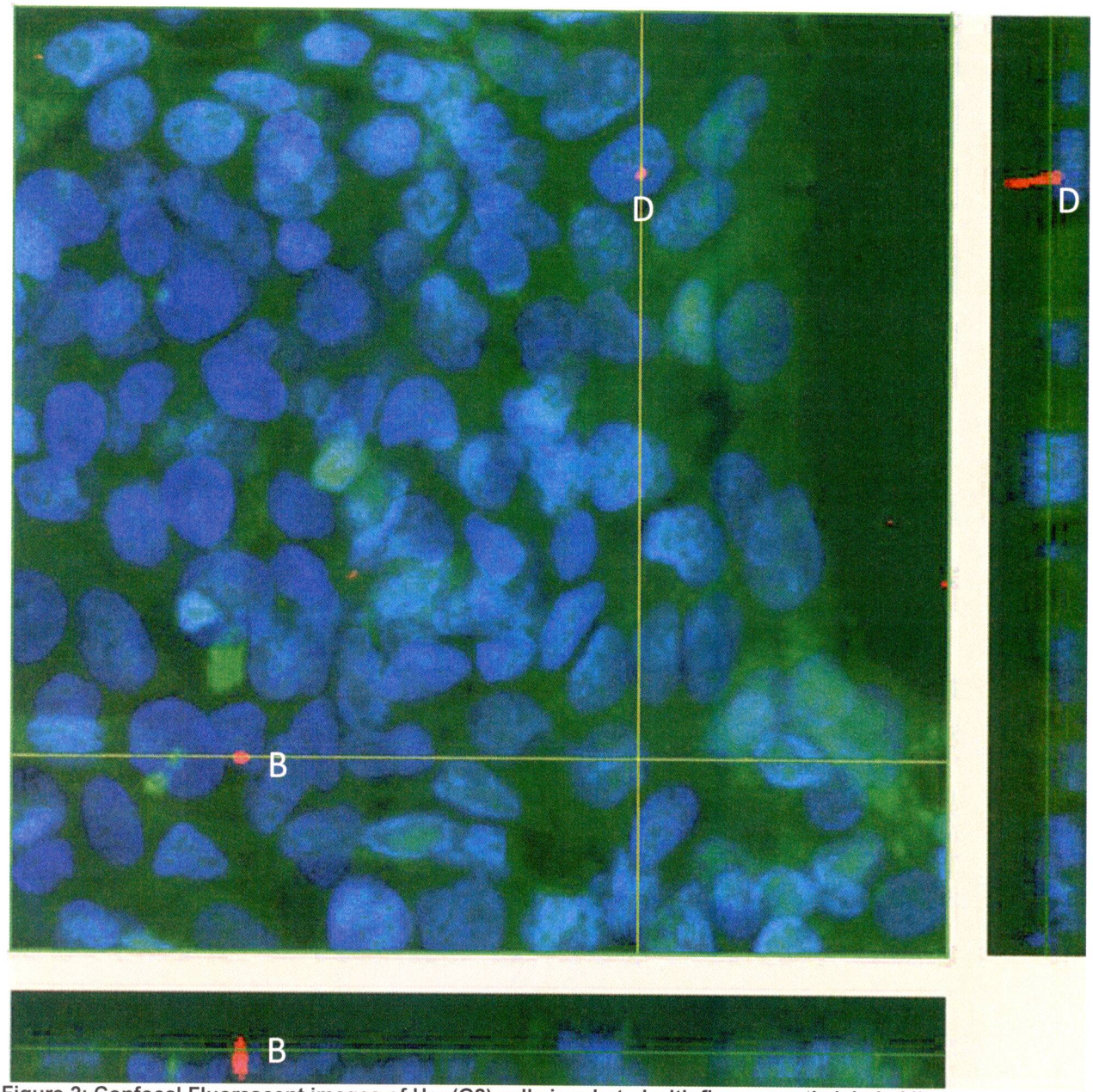

Figure 2: Confocal Fluorescent images of Hep(G2) cells incubated with fluorescently labeled liposomes diluted 1:50. Images at right and bottom are cross sections of YZ plane and XZ plane respectively. . Labels B and D indicate liposomes in close proximity to cells. Liposome at B was previously seen in Figure 1. Liposome at D may be in interior of cell or bound to surface directly above the nucleus of the cell (blue). Cross sections were taken from main image at yellow lines. Magnification 40X. Digitally magnified field from Figure 1.

1.2.1.2 Imaging Run September 28, 2010

This set of images is of a second preparation of cells and liposomes performed while studying various growth conditions. In this sample set, the cells were exposed to liposomes diluted to 1:10. These images are shown below. The confocal images contain images in both the normal XY plane as well as "cross sections" in the XZ and YZ planes. These cross sections indicate the presence of liposomes within the cells.

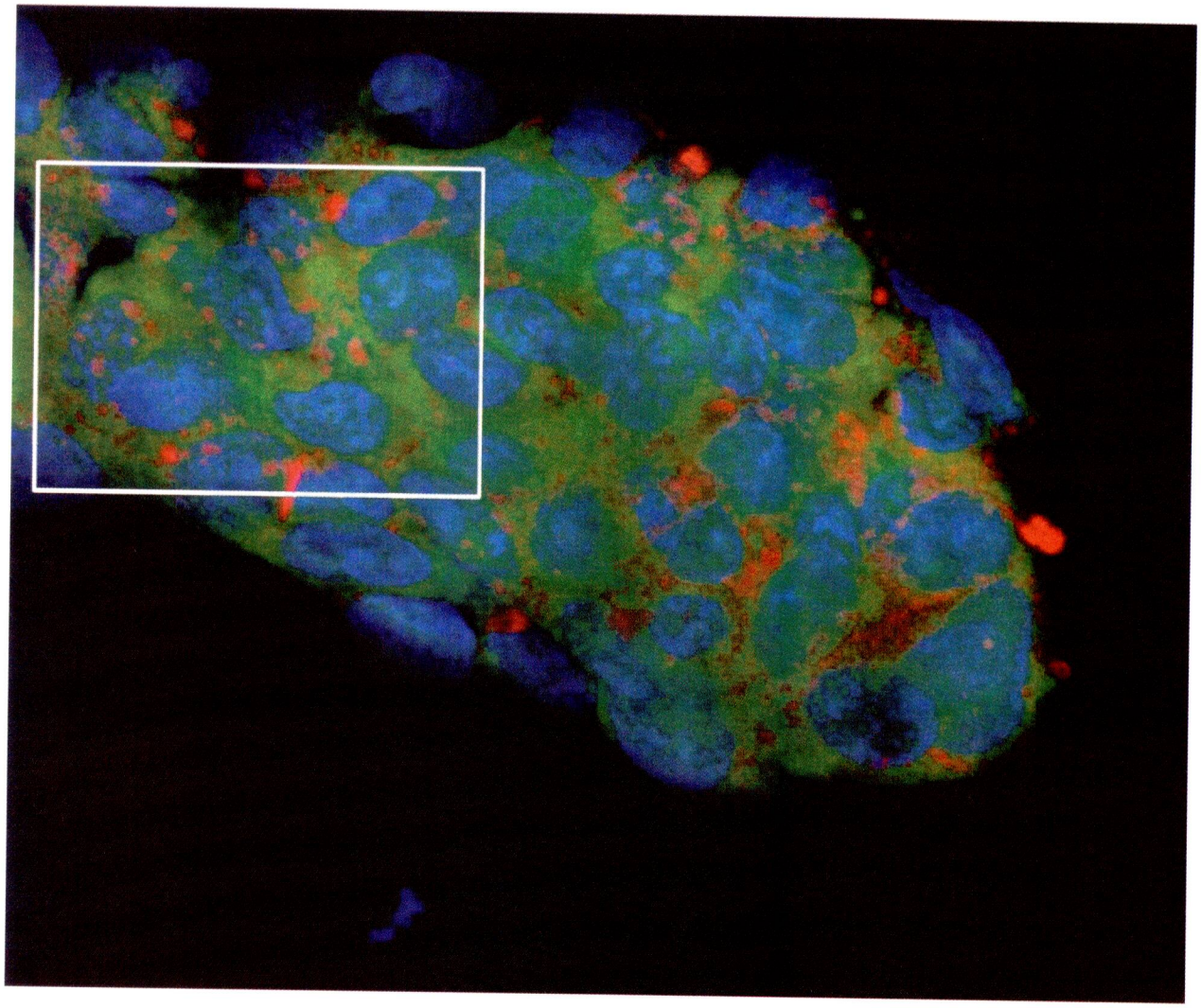

Figure 3: Fluorescent images of Hep(G2) cells incubated with fluorescently labeled liposomes diluted 1:10. Box indicates area enlarged in Figure 4. Magnification 40X

Figure 3 is a normal (not confocal) image of the Hep(G2) cells (stained green and blue) and the red fluorescently-labeled liposomes. By using a 1:10 dilution of liposomes, there are many more liposomes visible in the image. The liposomes appear to have incorporated into the space occupied by the cells. Below are confocal images of the same sample.

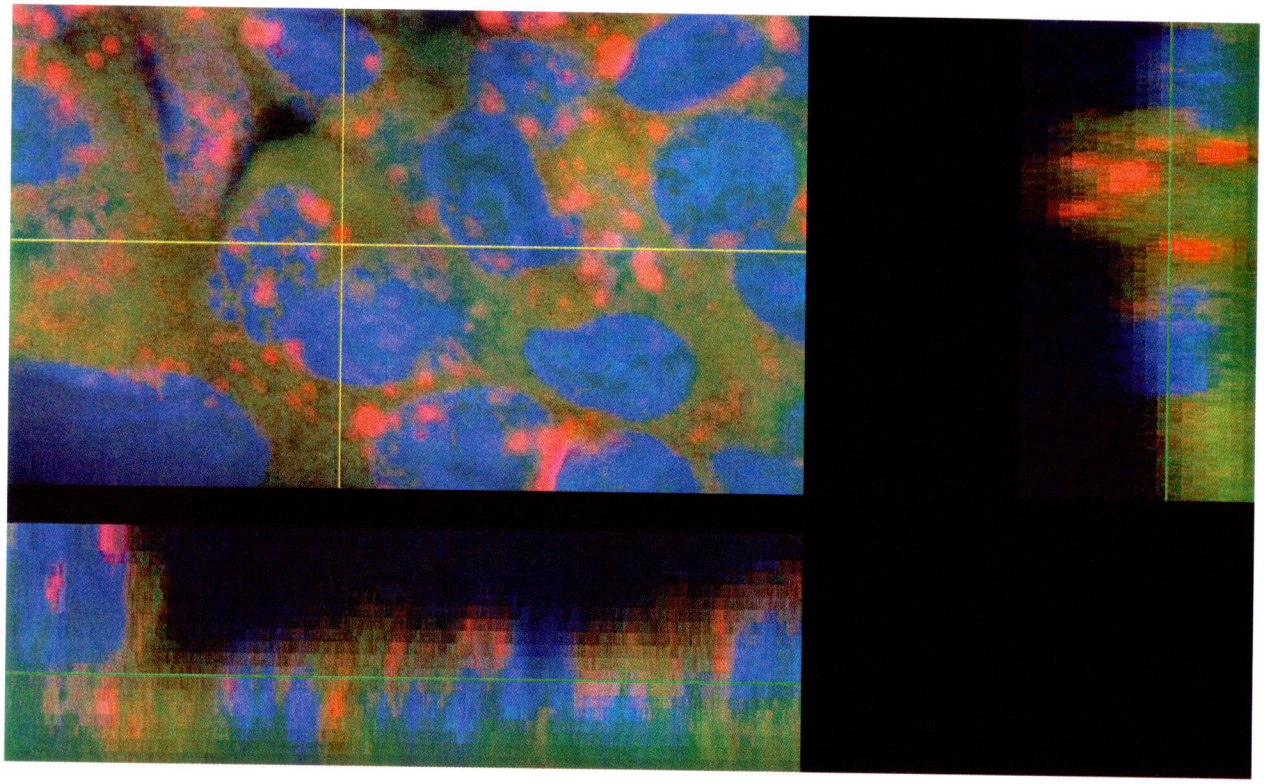

Figure 4: Confocal Fluorescent images of Hep(G2) cells incubated with fluorescently labeled liposomes diluted 1:10. Images at right and bottom are cross sections of YZ plane and XZ plane respectively. Cross sections were taken from main image at yellow lines. Magnification of objective was 40X. Image was then digitally magnified.

Figure 4 is a confocal image of the sample. The image is a digitally enlarged view of the previous image in Figure 3. The enlarged section is indicated by a box in Figure 3. In this confocal image, liposomes are clearly seen to be within the membranes of the Hep(G2) cells. This is most easily seen by looking at the cross sectional views which show the red liposomes surrounded by the green-stained cell cytoplasm.

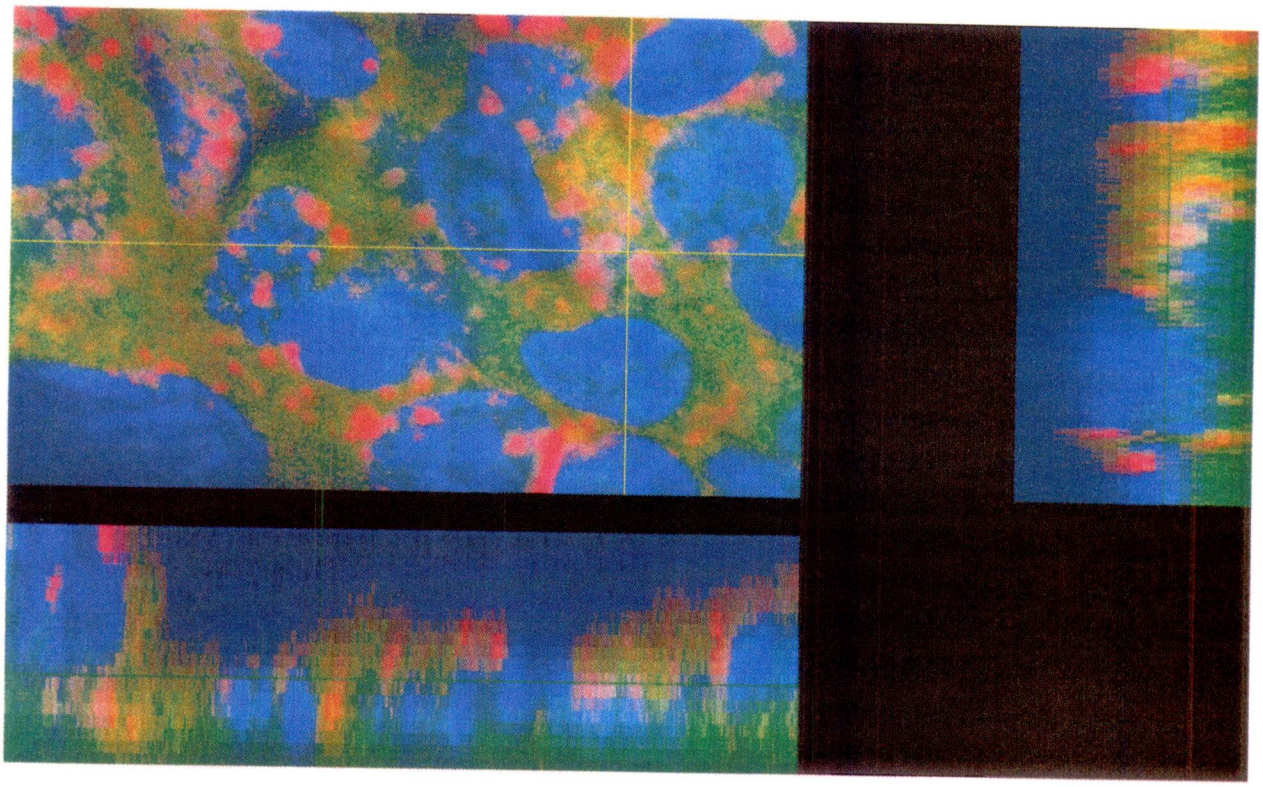

Figure 5: Fluorescent Confocal Fluorescent images of Hep(G2) cells incubated with fluorescently labeled liposomes diluted 1:10. Images at right and bottom are cross sections of YZ plane and XZ plane respectively. Cross sections were taken from main image at yellow lines. Magnification 40X

Figure 5 is a second confocal image of the same field of view of Figure 4. The cross sectional images are taken at different locations as indicated by the yellow lines. As in Figure 4, liposomes are clearly seen below the surface of the cells indicating uptake of the liposomes by the cells.

1.3 Future work and Conclusion of Imaging Studies

At this point, we have collected compelling evidence that LivOn's liposomal formulation can be taken up by the Hep(G2) human hepatocyte cell line in culture. It is advised that a discussion of the current results be held to determine if any further studies should be attempted.

Sincerely,

Kenneth H. Carson

Kenneth H. Carson, Ph.D.
Research Scientist

Approved by:

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KHC:mp