

# New Earth BioMed Activity Report

March 1, 2011 to June 30, 2011

## 1 Summary

This four month activity report describes activities and accomplishments of New Earth BioMed for the period 3/1/2011 to 6/30/2011. During this period, New Earth BioMed focused on two projects. These were: 1) assessment of synergism/antagonism/additivity within mixtures of various cytotoxic compounds, and 2) development of an imaging system based on laser light (coherent anti-Stokes Raman scattering, CARS). The two projects are related in that our lab is developing the CARS system in order to better assess the effects of drugs and natural compounds on cancer cells in culture. Our short-term goal is to improve the imaging system in order to allow quantification of cell viability based on image data alone, without the addition of dyes, molecular labels, or other agents.

In brief, the results developed in this four-month period are:

1. We have examined numerous mixtures of biologically active compounds, some of which demonstrate statistically significant synergism against A549 lung cancer cells in vitro.
2. We have performed our first spectral CARS scan of normal and treated cells, using indole-3-carbinol (I3C) as the treatment agent.

Both projects are discussed in more detail in the sections below. Also available at [www.NewEarthBioMed.org](http://www.NewEarthBioMed.org) is a short (1 minute) video showing how CARS images of a cell change as the spectral scan proceeds.

## 2 Cytotoxicity/Synergism Studies

### 2.1 Introduction

Our interest in synergism stems from the complex nature of a cancer cell, and our belief that mixtures of compounds should be better able to inhibit cancer cell proliferation than single agents used

alone. Dozens, if not hundreds, of proteins can be malformed, overproduced, or underproduced in cancer cells. Subsequently, numerous signaling pathways can be over- or under-activated. In general, these protein and signaling abnormalities provide cancer cells with a survival advantage, as well as an imperative to proliferate.

It is important to note that, in general, changes to protein composition, structure, and function tend to be minor compared with healthy protein counterparts. As such, drugs that target a specific protein used by a cancer cell are also likely to affect the same protein used by normal cells. Indeed, the differences between cancer cells and normal cells is often a matter of degree; a cancer cell is very much like a normal cell, only its many alterations causes it to behave differently. For these reasons, it does not seem likely that single agents (sometimes called *silver bullets*) will ever be developed to effectively and safely treat the majority of common solid cancers. Even if a single agent were highly effective when first administered, the genetic flexibility and population differences within a tumor would likely allow some cells to survive and form new, often more aggressive and drug-resistant tumors. This is why cancer drugs are rarely used alone in the clinic, even though they were developed and approved for market as single agents. A different approach is needed, one that embraces the complexity of the cancer cell. And one that embraces mixture design at an early stage of the drug discovery process, where optimizations can best be made.

At New Earth BioMed, we believe that a fruitful avenue for research is the study of large mixtures of natural products as anticancer agents. In particular, we focus on mildly potent compounds, such as might be found in foods or medicinal herbs. These compounds tend to have low systemic toxicity, which makes them safer to administer to humans, and yet they produce desirable biologic effects. Furthermore, large mixtures can take advantage of additivity and synergism to enhance a desired effect. The basic concept is that due to additivity and synergism large mixtures could allow each component to be used at a low, nontoxic dose, while still maintaining a robust therapeutic effect. By large mixtures, we mean mixtures that contain roughly six or more active compounds, perhaps as many as two dozen or more.

The important distinction between this and the more traditional silver bullet approach is that here the goal is not to affect a target protein—it is to affect the dynamics of the protein network as a whole. The dysfunctional signaling that occurs in a cancer cell is thus smothered under a therapeutic wet blanket. Mixtures would be designed to affect multiple signaling pathways, including those involved in proliferation, multidrug resistance, apoptosis, metastasis, invasion, and immune evasion. Under this type of therapy, cancer cells would find it difficult to exhibit their aggressive phenotype, and at the same time would be presented with stimulus to act more like normal cells.

The study of large mixtures is not easy. Indeed, the study of individual agents is difficult enough, and studying multiple agents complicates matters greatly. The biologic effects of each component in a mixture must be well-characterized, as must the effects of the mixture as a whole. Apart from cellular effects, later stages of discovery would require that pharmacokinetic and organ toxicity issues be addressed. Mixtures also present a combinatorial problem. Given only a small set of compounds for study, a very large number of mixtures could be created and tested. Putting these difficulties aside for the moment, the first step is to conduct traditional cytotoxicity/synergism studies using a small number of compounds. In this way, a body of evidence can be built up to guide the later steps and to serve as pilot data for securing necessary funding.

## 2.2 Activities

As mentioned above, the cytotoxicity/synergism studies now being conducted at New Earth BioMed are designed in part to provide useful pilot data. To make these studies as interpretable and simple as possible, we have chosen to focus on water-soluble compounds. Lipophilic compounds require use of DMSO, cyclodextrin, or other solubilizing agents before they can be used in culture. Such solubilizing agents can have their own biologic effects, even at low concentrations. Thus we did not want to use any nonsoluble compounds until we can show that the solubilizing agents are not also having an effect. Lipophilic compounds could be tested in later studies, as appropriate.

Unfortunately, inexpensive, water-soluble compounds that show activity against cancer cells are not abundant. The compounds we have chosen to study in mixtures are epigallocatechin gallate (EGCG), dichloroacetate (DCA), indole-3-carbinol (I3C), dimethyl sulfoxide (DMSO), perillyl alcohol (POH), and doxorubicin (DOX). DMSO is not a naturally occurring compound in plants but was chosen for this study as it is used therapeutically and is a solubilizing agent. DOX was chosen as it is a natural compound (originally isolated from bacteria), and is highly potent against several cancer cell lines. A previous study by Boik and Newman indicated that several natural compounds were synergistic with DOX.[1] DCA is not naturally occurring; it is produced, for example, as a trace byproduct in the chlorination of drinking water. It was chosen here because of its low cost, interesting mechanism of action, and reported effects on cancer. In our preliminary work, we have also conducted cytotoxic studies on quercetin, resveratrol, cyclodextrin (also a solubilizing agent), limonene, and vitamin 1,25 D3. Thus in total, we have begun study on 11 compounds.

All have been tested using the A549 human adenocarcinoma lung cancer cell line. Every year, more people die of lung cancer in the United States than any other type of cancer. Nearly twice as many women die of lung cancer than breast cancer, and roughly three times as many men die of lung cancer than prostate cancer.[2] Adenocarcinoma, a subtype of non-small cell lung cancer (NSCLC), is the most common type of lung cancer, accounting for about 35 percent of all cases.

The first step in assessing synergism is to conduct traditional cytotoxicity studies on each single agent. The second step is to conduct cytotoxicity studies on each desired mixture. Generally, each cytotoxicity study is replicated in triplicate for statistical purposes. Given that each individual study is conducted using one 96-well incubation tray, the study of each single agent (and mixture) employs three trays.

The data gathered from cytotoxicity experiments are used as input to a mathematical model that estimates the degree of synergism/additivity/antagonism. First, a logistic model is fit to the concentration-response data. The response is an optical measurement indicating the change in color of a dye that is sensitive to cell respiration. It is an indirect measure of cell viability. The fitted parameters of the logistic model are used as input to a second model that estimates the Loewe interaction index. The set of models used here have been developed by Boik et al., code for which has been submitted to the Comprehensive R Archive Network (CRAN) for use by the public.[3, 4]

One advantage of this package is that it provides confidence intervals for the Loewe interaction index. Too often, authors of synergism papers only report an index value, and on that basis alone claim that a mixture is additive, synergistic, or antagonistic. The index is only an estimated value,

however, and as such it is fairly meaningless without its associated confidence intervals. If both the upper and lower confidence intervals are less than one, the mixture is synergistic. If both intervals are greater than one, the mixture is antagonistic. If the intervals span one, the mixture is additive. In common terms, additivity is the expected result that  $1+1=2$ , whereas synergism means that  $1+1=10$ , or some other number greater than 2. Antagonism means that  $1+1$  is less than 2. Note that while a goal of our studies is to identify synergistic mixtures, additivity is also a useful property and should not be overly discounted.

## 2.3 Results

As mentioned, we have assessed drug interactions in mixtures containing up to six compounds. Typical results are illustrated in the top panel of Figure 1. This mixture contained DCA, DOX, EGCG, I3C, and POH. As can be seen from the figure, at fraction affected values larger than 0.50 ( $x$ -axis), synergism is statistically significant. That is, both confidence intervals are below one. On the other hand, at fraction affected values smaller than 0.35, antagonism is statistically significant and both confidence intervals are greater than one. The fraction affected is an indicator of the portion of the cells still alive after treatment with a certain concentration of drug. It ranges from zero (at high drug concentrations) to one (at low or zero concentrations). Other mixtures tested showed more or less statistically significant synergism. Some showed no antagonism.

The estimated concentration-response curves for the five component drugs and Mixture 06 are shown in the bottom panel of Figure 1. As mentioned previously, the model is based on a logistic relationship between concentration and response. As such, very little effect is expected at low drug concentrations below a certain threshold; here the curve is almost flat. After the threshold is reached, cell death starts to become noticeable. The slope in the curve at this point can be either steep or mild, depending on the action of the drug. The IC<sub>50</sub> is the concentration at which a drug affects 50 percent of the cells (i.e., the response is half that of control wells). This is the point at which the slope is calculated, and it is the value often reported in the literature as the cytotoxic concentration.

The raw data are not as clean as the estimated curves shown in the bottom panel of Figure 1 might suggest. As an example, the raw data for one tray of I3C is shown in Figure 2. Clearly, the exact IC<sub>50</sub> and slope (the two parameters of the logistic curve) cannot be estimated with high precision when the data are noisy, as cytotoxicity data usually are. This means that the Loewe index will also be uncertain. Hence the need for confidence intervals. In Figure 2, the dotted line connects the means of the wells treated with identical concentrations of the drug. The red line signifies responses seen in wells that have only media and drug, but no cells.

Unfortunately, the simple logistic model is not always appropriate for cytotoxicity data. For example, in Figure 2 it appears that low concentrations of I3C might actually increase cell proliferation relative to control wells. (Control wells receive zero drug, but because the log of zero is not defined, they are shown in the graphs at a virtual low drug concentration.) Trays of data for several of our tested drugs appear to indicate a growth advantage at low drug concentrations. As such, the model of Boik et al could be expanded to include this possibility.

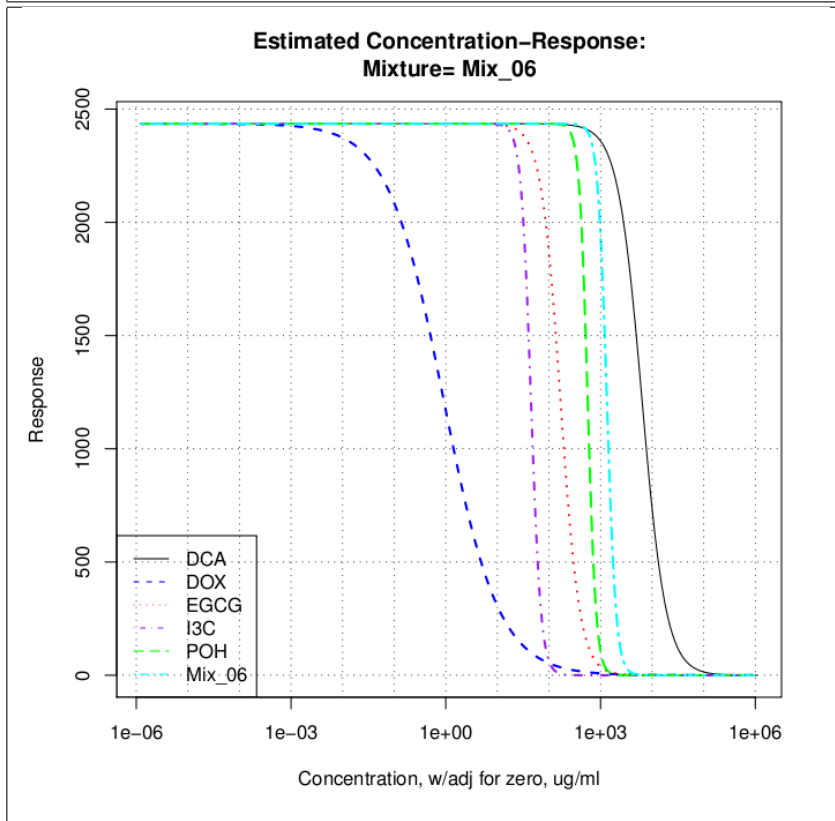
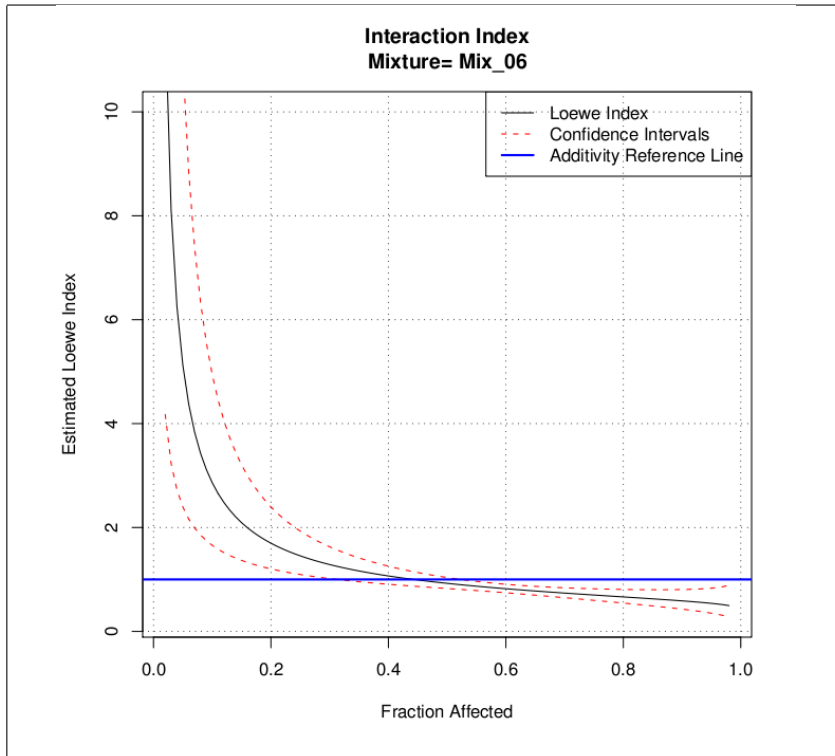


Figure 1: Loewe index and concentration-response for Mixture 07

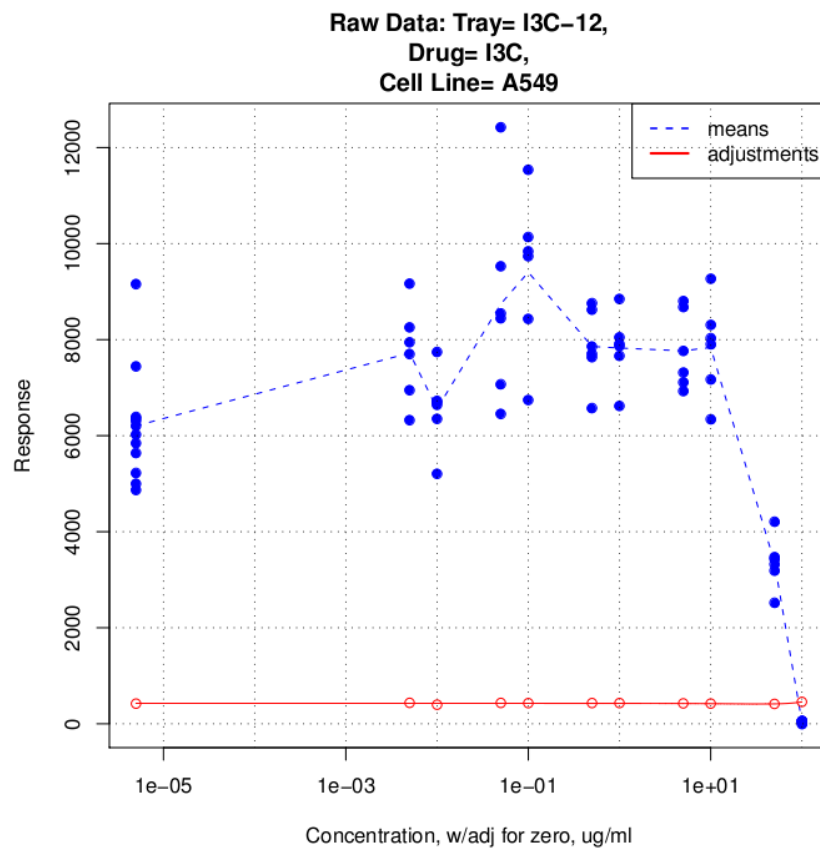


Figure 2: Raw cytotoxicity data for one tray of I3C

## 3 CARS Imaging

### 3.1 Introduction

Our CARS project addresses a significant need in the research community: to develop improved methods to assess the effects of drugs and natural compounds in complex cell cultures. The methods in current use, such as molecular labeling with fluorescent tags, and use of dissolved dyes that change color on cell respiration, are suboptimal in that many are not designed to allow collection of time-series data, or are not amenable to the study of thick, three-dimensional cultures, or the study of cultures containing cells of mixed types. Furthermore, labels and dyes can themselves alter cell function. And when using molecular labels, the investigator must know before the experiment which molecules are of interest. There is also some limit to the number of molecular labels that can be used in a given experiment. CARS holds promise to revolutionize biological assays by providing a means of label-free, non-destructive molecular-contrast imaging that is applicable to three dimensional complex tissues. If we are successful in developing an improved assay based on CARS or related nonlinear optical processes, our ability to study the effects of natural compounds on cancer will be greatly enhanced. The long-term goal is to use this assay in a sophisticated natural product mixture-screening program, where culture conditions resemble those of a real tissue (e.g., co-cultures of normal and cancer cells in a three-dimensional matrix).

In brief, CARS provides molecular (vibrational) contrast imaging by measuring the intensity of the light scattered by molecules. A molecule, such as water, contains chemical bonds. In the case of water, two bonds connect the oxygen molecule to the two hydrogen molecules. These bonds vibrate at a natural frequency, much as a guitar string vibrates at a natural frequency. Light from two or more lasers is used to excite molecules in a sample. The lasers are tuned such that the difference in their frequencies match the natural frequency of the bonds under study. When this occurs under the right focusing conditions, the bonds vibrate and the beat frequency is strongly and coherently emitted. It is similar in some ways to the beat frequency (a third tone) that can be produced when two guitar strings are plucked. If a CARS signal exists, it indicates that a particular bond has been set into motion. Because each bond vibrates at a unique frequency, the CARS signal allows molecular contrast imaging. Not all bonds produce a strong CARS signal, however. The CH<sub>2</sub> bonds, found in lipids, provide particularly strong signals. For this reason, many of the papers published on CARS are studies on lipids.

CARS is not without its serious challenges. As mentioned, some bonds are more active than others. Furthermore, the CARS signal contains both resonant and non-resonant (noise) components. Lastly, while CARS is orders of magnitude faster than Raman scattering (a related, commonly-used parent technology), it is still too slow to image large tissue areas, as would be required in a practical cellular assay system. Other challenges also exist, such as obtaining a sufficient signal at low enough power to prevent sample damage.

CARS itself is rather new, with most work done only in the last 10 years. The first CARS images of live cells were published in 1999 by Zumbusch et al.[5] At that time, it took about 30 minutes to acquire an image. Since then, developments in laser engineering and microscope optimization

have progressed rapidly. Our collaborator, Eric Potma, PhD, at the University of California, Irvine, was a co-author on the first paper to demonstrate video-rate (30 images/second) imaging of live tissue. [6]. Thus CARS and related technologies are still at the early stages of investigation. Due to the complex equipment and setup requirements, most CARS work is still limited to special physics/chemistry laboratories. Simple CARS microscopes are, however, beginning to appear on the market. The field of nonlinear optical processes offers great promise of one day providing radically improved methods for biophysical investigations.

## 3.2 Activities

CARS imaging technologies can be divided into two categories, narrowband and broadband. In narrowband CARS, the sample is excited with two lasers (pump and Stokes beam), each at a different wavelength as noted above. The combined laser light is raster-scanned across a sample and a photodiode is used to measure the light emitted. In contrast, broadband CARS uses one laser at a fixed frequency and one broadband laser containing a spectrum of frequencies. The broadband setup allows investigators to capture CARS images over a full spectrum at once. Each type of CARS has drawbacks and advantages. For example, narrowband CARS tends to produce a stronger, more clear signal. Whereas broadband CARS offers the advantage of signal acquisition over a spectrum of frequencies. Further, narrowband CARS is faster at acquiring a single image, and broadband CARS is much slower. Speed is a critical factor if the goal is to image many cells in a culture.

The Potma lab is among three in the world (the others are in the Netherlands and France) that is pursuing an approach that lies between narrowband and broadband CARS. We use narrowband CARS multiple times, each at a different pump beam frequency. In this way, results over a (limited) spectrum can be obtained more quickly than they can using broadband CARS. Early results examining tissue samples have already been published by the Potma lab, putting us in the lead at applying this technology to biology.[7]

Until recently the laser scans needed to be preformed manually. That is, the laser equipment was adjusted by hand each time a new frequency was desired. Work done under the current project has changed that. Our group has now developed software that interconnects the various pieces of equipment (optical parametric oscillator (OPO), power meter, half-wave plate, and microscope control software), allowing an automated scan to be performed. Rather than taking several hours per manual scan of 50 frequencies, the automated scan takes about 20 minutes. While the Netherlands group has also developed such software, ours will be the first to be applied to biological samples. Ours also provides controls over wavelength and power that the Netherlands group might not have. Considerable effort was spent developing this integrated software product. It will be useful for a number of different studies planned in our lab.

### 3.3 Results

By using the automation software developed in our lab, we were able to obtain several series of CARS images, each over a 50-frequency spectrum. As mentioned, each series takes about 20 minutes to collect. This is too long of a period to image live cells, as cellular components and position would not remain steady. Therefore, it was necessary to fix the cells. Paraformaldehyde was used as the fixing agent in our studies.

As an example, one image taken at a pump beam of 810.8 nm is shown in the top panel of Figure 3. For all scans, the Stokes beam was tuned to 1064 nm. The cell in Figure 3 was treated with 0.1  $\mu\text{g}/\text{ml}$  of I3C. This drug concentration is not expected to produce a significant effect on cell viability. Submitted with this report is a one-minute movie (mp4 file) that shows the cell of Figure 3 as the CARS scan progresses.

The bottom image in Figure 3 was obtained by mathematical manipulation of the 50 images in this series. The raw data provide a multidimensional matrix of size  $512 \times 512 \times 50$  (each image is  $512 \times 512$  pixels). We used principal component analysis to compress the 50 dimensional signal down to 3 dimensions, which we then used as red, blue, and green levels to create the image. Thus the image shown in the bottom panel is of size  $512 \times 512 \times 3$ , and contains an optimally compressed signal, based on linear PCA. The compressed image clearly distinguishes the cytoplasm from the nucleus, and as well clearly distinguishes lipid bodies (in red) from other cellular components.

The raw data provide a 50-value spectrum for each pixel in an image. An example of such a spectrum is shown in Figure 4. This spectra contains a peak at about 821 nm, which corresponds to 2780 wavenumbers. It is likely due to the C-H stretch mode.

As mentioned, a goal of this work is to relate the spectra of a cell to its viability. For example, a cell that is under stress might metabolize and synthesize compounds differently, and thus would contain a different makeup of lipids, proteins, and other cellular components. These differences should be observable in a CARS image. Our preliminary data suggest that this is indeed the case. As an example, a cell treated with 100  $\mu\text{g}/\text{ml}$  of I3C is shown in Figure 5. This concentration is sufficient to cause massive cell death, as indicated in Figure 2. In comparing Figure 5 and the bottom panel of Figure 3, several differences are immediately apparent. First, the yellow and red-colored areas are nearly absent from Figure 5. Second, the cytoplasm in Figure 5 shows more blebbing and an indistinct border. The cells shown in Figure 5 were likely dead at the time of fixation. Although the contrast between Figure 5 and the bottom panel of Figure 3 is striking, not all images taken show such a clear distinction. PCA images from all series are provided in Appendix B.

To relate the spectra of a cell to its viability, quantification is necessary, not just visual examination. Useful features must be extracted from images for use as input to a mathematical model that estimates cell viability. Cell viability information can be obtained from cytotoxicity studies. As a simple example, suppose that the level of red in an image was predictive of cell viability. This features would already be present in the PCA data, and no further extraction would be necessary. However, cell viability might also be related to nuclear volume, cytoplasmic volume, the roughness of the plasma membrane, blebbing within the cytoplasm, or a host of other characteristics that are more difficult to extract as features. Quite likely, a linear model of the features will prove too

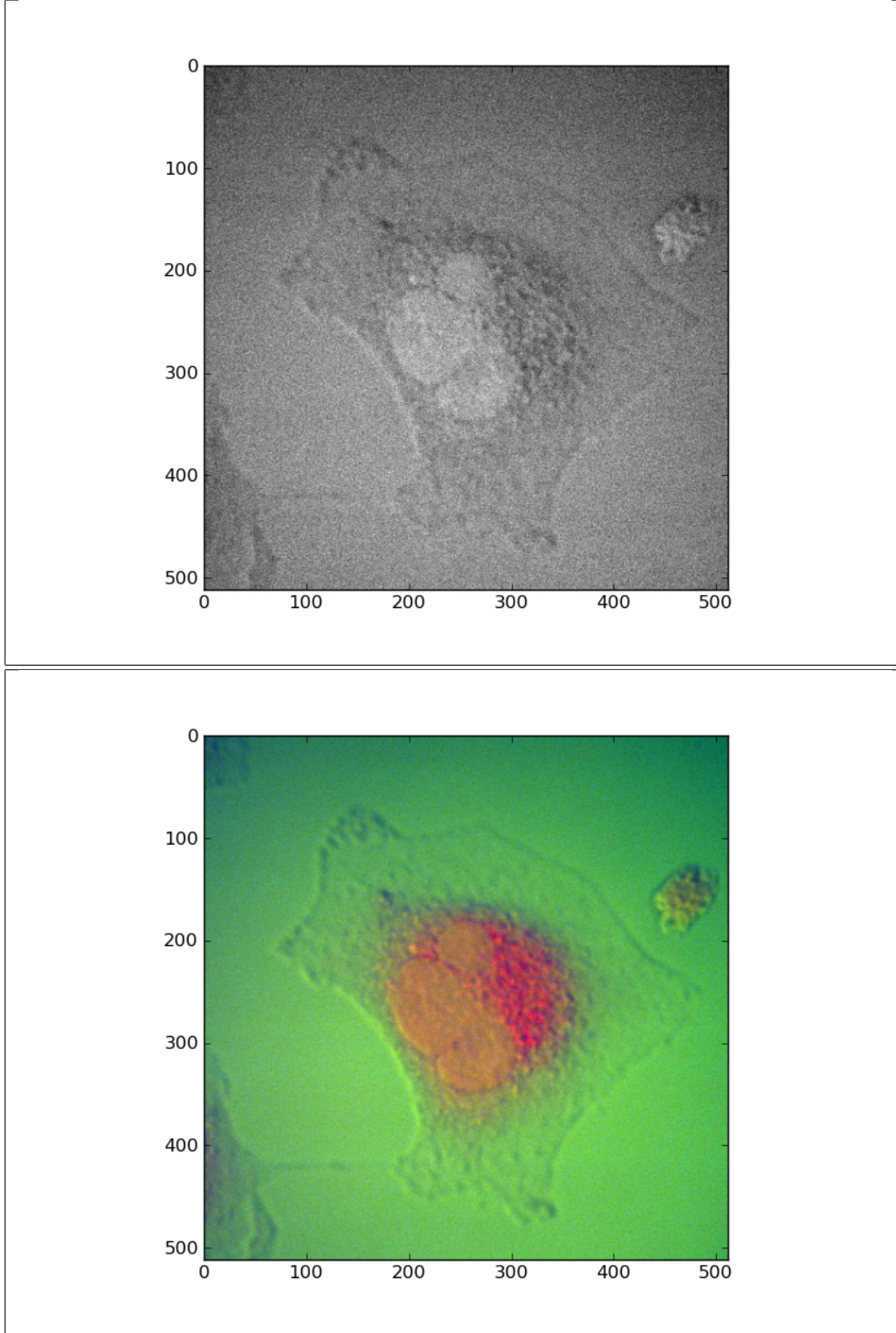


Figure 3: Cell imaged at 810.8 nm and PCA over spectrum after treatment with 0.1 ug/ml I3C

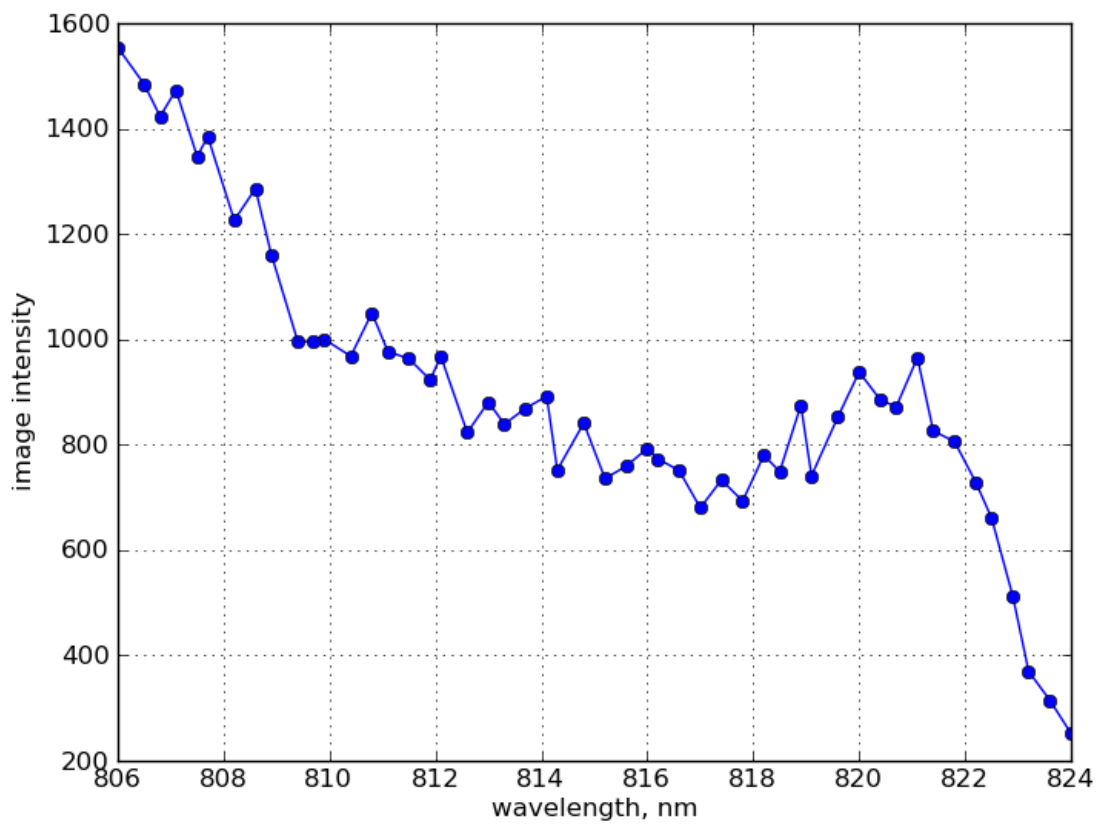


Figure 4: Spectra for one pixel of an image

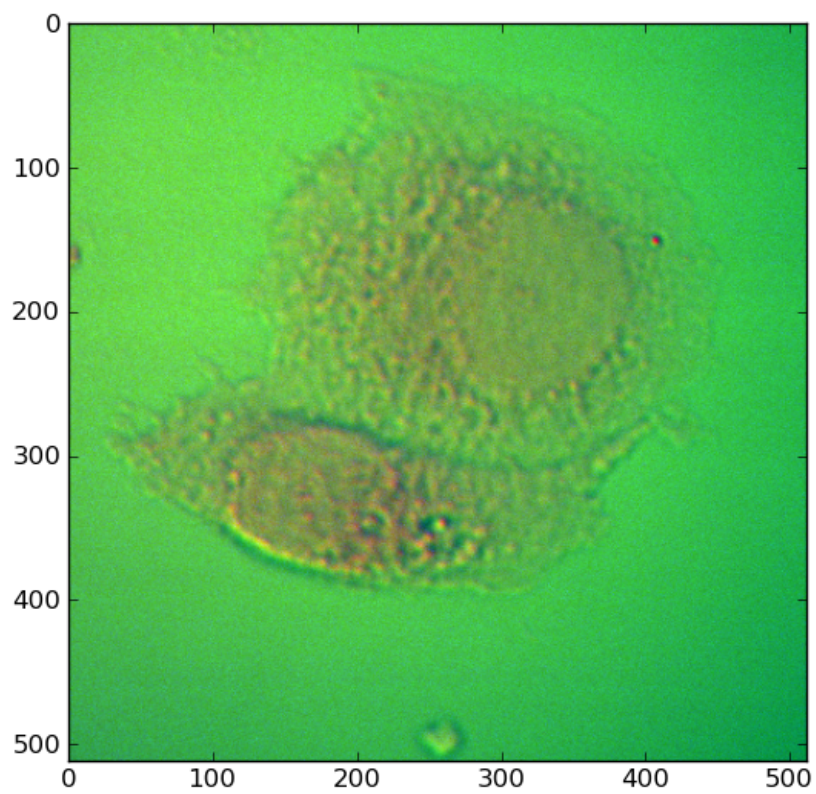


Figure 5: PCA over spectrum after treatment with 100 ug/ml I3C

simplistic, and a more complicated nonlinear model will be necessary. Work will soon begin on feature extraction and model development.

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