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# Synchrotron-Based FTIR Spectromicroscopy: Cytotoxicity and Heating Considerations

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## Abstract

Synchrotron radiation-based Fourier transform infrared (SR-FTIR) spectromicroscopy is a newly emerging bioanalytical and imaging tool. This unique technique provides mid-infrared (IR) spectra, hence chemical information, with high signal-to-noise at spatial resolutions as fine as 3 to 10 microns. Thus it enables researchers to locate, identify, and track specific chemical events within an individual living mammalian cell. Mid-IR photons are too low in energy (0.05 - 0.5 eV) to either break bonds or to cause ionization. In this review, we show that the synchrotron IR beam has no detectable effects on the short- and long-term viability, reproductive integrity, cell-cycle progression, and mitochondrial metabolism in living human cells, and produces only minimal sample heating (< 0.5°C). These studies have established an important foundation for SR-FTIR spectromicroscopy in biological and biomedical research.

**Keywords:** Synchrotron, FTIR, spectromicroscopy, heating, cytotoxicity, viability, Alcian blue, colony formation, BrdU, MTT.

**Running Title:** SR-FTIR Spectromicroscopy: Cytotoxicity Studies

## **I. Introduction**

Diseases usually begin with a single cell. With the continuing successes in gene sequencing and protein structure identification, biomedical researchers are now increasingly focused on understanding the onset of disease and the functions of diseased cells. Imaging techniques which can simultaneously provide morphological and chemical information within living cells and tissues are very powerful unifying tools for meeting this scientific need. Today intensive research in experimental biology, spectroscopy, and analytical instrumentation is seeking new ways to image chemical information within living cells.

Infrared (IR) spectromicroscopy combines infrared spectroscopy, a sensitive analytical chemistry technique, with microscopy to enable detailed chemical analysis on a microscopic scale [1]. Many common biomolecules, such as nucleic acids, proteins, lipids, among others have characteristic and well-defined IR-active vibrational modes [2-4]. With appropriate interpretation of measured IR spectra one can detect, identify, and quantify many molecular species within a biological sample.

Sufficient signal to noise for detailed spectral interpretation of samples the size of individual human cells has only been very recently achieved by adding the brightness of synchrotron-based IR sources to the technique [5, 6]. Fourier transform infrared (FTIR) spectromicroscopy with a synchrotron radiation-based (SR) source is a newly emerging bioanalytical and imaging tool that can monitor biochemical events within different compartments of an individual living cell without the need for fixing, staining, or labeling. Recent uses of synchrotron infrared spectromicroscopy include the examination of biological samples such as individual living cells [7-13], tissue samples [14-17], microbial-chemical interactions in environmental settings [18, 19], protein conformations [20, 21], and plant-soil interactions [22].

In this overview, we describe briefly the SR-FTIR spectromicroscopy technique and demonstrate that this technique is a truly non-destructive chemical microprobe for biological and biomedical research. All the measurements presented here were performed at the Advanced Light Source (ALS) Beamline 1.4 at Lawrence Berkeley National Laboratory, Berkeley, CA, USA [23-25].

## **II. Synchrotron FTIR Spectromicroscopy**

Conventional mid-infrared sources used in FTIR instruments are thermal emission elements that produce a graybody spectrum from a filament heated to 1000 to 2000 K. These elements are physically large (at least several millimeters), and typically radiate in all directions. The optics of the FTIR bench collect and collimate the light, pass it through the scanning interferometer, and then on to the all-reflecting IR microscope where the modulated IR light is focused to a small spot on a sample. Finally, the light that the sample reflects or transmits is collected, focused onto an appropriate infrared detector, and processed by a computer via a Fourier transform to produce an infrared spectrum. The thermal emission sources can be focused to 75 – 100  $\mu\text{m}$  with an IR microscope. To measure something smaller, you must mask away part of the incoming light, significantly reducing the signal strength. A true point source could be focused to a diffraction-limited spot size; with  $f/1$  optics this is approximately the wavelength of the light [26]. This is where using a synchrotron as an IR source makes a big improvement.

A synchrotron is a high-energy electron storage ring optimized for the production and collection of the intense light radiated by the electrons upon acceleration. For mid-IR and longer wavelengths, the effective source size for a synchrotron light source is diffraction-limited. In other words, it is very close to an ideal point source. This means that in FTIR spectromicroscopy based on

synchrotron radiation (SR). The beam is focused to a spot with a diameter  $\leq 10$   $\mu\text{m}$  [25-27], smaller than a typical mammalian cell, and provides hundreds of times the brightness of conventional IR sources. The sample can be positioned using a computer-controlled x-y-z stage with 1  $\mu\text{m}$  precision, allowing mapping measurements of FTIR spectra as a function of x and y position on the sample. A schematic of a synchrotron infrared beamline is shown in Figure 1.

The high brightness of synchrotron IR spectromicroscopy facilities, with over 1000 times better signal to noise ratio compared to thermal IR sources for small samples, enables a multitude of new scientific applications where size matters. The SR-FTIR spectromicroscopy technique is therefore ideal for the study of small and/or heterogeneous samples, for example; individual living cells, microorganisms, and larger biological systems in which local biochemistry may have significant spatial variations.

### **III. No cytotoxic effects of SR-FTIR spectromicroscopy observed**

We report the results from a series of tests to explicitly quantify the non-destructive properties of SR-FTIR spectromicroscopy. Mid-infrared photons are significantly lower in energy (0.05 - 0.5 eV) than the excitation sources used in fluorescence probes including the newer two-photon techniques (photons energies of approximately 1 eV), implying that photo-induced effects will be minimal. Although mid-IR photons are too low in energy to directly break bonds or cause ionization, other effects from the SR-IR source may occur, including sample heating, drying, or other more subtle interactions which could influence long-term metabolic and other cellular physiological processes.

The following highlights the results of *in vitro* studies to determine if the SR-IR beam causes any detectable effects on living cells [28]. Two classes of effects were measured in this study: (1) immediate and/or short-term effects in

cell viability, cell-cycle progression, cell metabolism, and (2) long-term effects on the proliferative/metabolic capacity of exposed cells. Four widely accepted cellular and molecular assays were selected to measure these potentially deleterious effects on cells subjected to different doses of the SR-IR beam. Finally, we determined the steady-state temperature rise in a typical biological sample continuously illuminated by the SR-IR beam to quantify any sample heating. Information on the temperature rise is important because many biochemical processes are temperature-sensitive.

A human T-1 cell-line from an established aneuploid cell-line derived from human kidney tissue was used in this study [29]. They were selected partly because of their high plating efficiency (> 90%), and partly because they have been previously used as a model biological system in studies of the effects of radiation and oxygen on human cells [30-34]. Most importantly the size of T-1 cells is typically ~10 micron in the G<sub>0</sub> and G<sub>1</sub> phases, which is similar to the size of the synchrotron IR beam.

A custom on-stage mini-incubator was used to maintain the proper moisture and growth environment for the cells while allowing *in situ* SR-FTIR spectromicroscopy measurements. The mini-incubator was temperature controlled via circulating water from a water bath, and infrared transparent CaF<sub>2</sub> windows on the top cover were separately temperature-controlled to avoid condensation. The location of the synchrotron infrared beam within the field of the microscope was fiducialized to approximately one micron by mapping a titanium on silicon calibration target [35]. Every exposure experiment was conducted at 37°C and lasted for less than one hour. Complete experimental details are found in reference [28].

We employed the Alcian blue dye exclusion assay to measure short-term cell viability [36]. As shown in Figure 2-i, neither cells exposed to up to 20

minutes of synchrotron IR beam nor nearby non-exposed cells retained the blue dye 6 hours after exposure. This indicates that the SR-IR beam did not produce detectable effects on the viability of exposed cells. Other exposed cells remained free of stain 12 and 24 hours after exposure indicating that their membranes still remained intact.

While this short-term test has revealed that cells were able to exclude the Alcian blue dye, a long-term colony-forming assay demonstrates that exposed and non-exposed cells continue to proliferate into colonies of similar size after 10 days (Figure 2-ii). Since none of the 46 SR-IR exposed test cells developed into colonies with less than 50 cells (each colony was counted), we interpret this as an indication that SR-IR beam does not impact cell survival and proliferative activities.

A two-antibody bromodeoxyuridine (BrdU) assay was designed to specifically answer the question, "Do SR-IR beam exposed cells progress into S-phase at the same time as unexposed control cells?" The incorporation of BrdU into newly synthesized DNA at 11 hours after cell setup and 10 hours post SR-IR exposure was used to monitor cell-cycle progression. Both exposed cells and non-exposed controls had reached the DNA synthetic phase (S-phase) of cell-cycle at this 12-hour observation point (Figure 2-iii). The similarities among these immunofluorescent BrdU and DAPI (4,6-diamidino-2-phenylindole) labeled cells indicate that the exposed cells are not compromised in their ability to enter their S-phase after exposure to the SR-IR beam. Furthermore, the lack of detectable uptake of BrdU into DNA in exposed and control cells at 6 or 24 hours after their release (not shown) demonstrates that the cell-cycle progression of SR-IR exposed cells remains uninterrupted.

We have shown that the cell cycle progresses uninterrupted, and now use a MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay to



test for any SR-IR beam effect on ATP and NAD<sup>+</sup>-associated metabolic activity. Representative photos of the MTT assay results are shown in Figure 2-iv, with exposed and nearby non-exposed controls showing a similar purple-blue stain. This shows that both the exposed and negative control cells produced mitochondrial dehydrogenases during the two-hour MTT assay. Mitochondrial dehydrogenases are associated with the ubiquitous metabolic pathway of glycolysis [37] that generates the critical biomolecules of ATP and NAD<sup>+</sup>. Thus these results indicate that the SR-IR beam has negligible effects on this important metabolic pathway which provides energy to cells.

We also compared SR-FTIR spectra of cells as a function of time looking for any biochemical changes induced by the beam. SR-FTIR measurements performed repeatedly on one living cell for thirty minutes showed an unchanging IR spectrum to within 0.005 absorbance units across the entire mid-IR spectral range. Longer exposure times can be tested; however the living cell continues growing through its cell cycle, which results in other spectral changes as previously described [13].

In all 5 assays studied (Alcian blue, colony formation, BrdU, MTT, and SR-IR spectra) we found no detectable changes between cells exposed for 5, 10, and 20 minutes to the synchrotron infrared beam and nearby non-exposed controls. 267 individual cells were tested using standard biochemical assays with zero tests showing any measurable cytotoxic effects (counting statistics error is 6.1%), with over 1000 control cells used (Figure 3). Additionally, infrared spectra that are a measure of the overall biochemistry within a cell were obtained from test and control cells, and showed no spectral changes. These results show that the high-brightness mid-IR synchrotron beam is not just non-destructive, but causes no detectable effects on the short- and long-term viability, proliferation, and metabolism within living human T-1 cells. Although the present study has focused on only one established human cell line, we

anticipate that these results will be generally applicable to most, if not all, living biological systems.

#### **IV. Negligible heating detected**

The power levels in the mid-IR spectral region of the SR beam are fairly modest ( $\sim 1$  mW integrated power [28]), however this power is focused onto one cell. Since all cellular processes are sensitive to temperature, we must determine the extent of heating by the intense synchrotron beam.

The phospholipid dipalmitoylphosphatidylcholine (DPPC) was utilized as an internal thermometer to determine the steady state temperature rise due to the continuous exposure to the SR-IR beam [38]. DPPC, when dispersed in water, forms bilayers which exist in at least two different states, depending on the temperature. These states are separated by a phase transition temperature ( $T_m$ ) at around 315 K, when the bilayers are converted from a gel into a liquid-crystalline state [39, 40].

The inset to Figure 4 shows the measured infrared spectrum of a 20:l dilution of hydrated DPPC dispersion at  $T=328$  K (above  $T_m$ ). As has been shown before [39, 40], the methylene ( $\text{CH}_2$ ) symmetric stretch vibration at around  $2850\text{ cm}^{-1}$  and the phosphate asymmetric stretching mode around  $1240\text{ cm}^{-1}$  shift when the sample passes through the phase transition. For this study we monitored the  $\text{CH}_2$  symmetric stretch mode (labeled in Fig. 4).

The  $\text{CH}_2$  symmetric stretch mode's center frequency was measured using the less bright thermal Globar<sup>TM</sup> IR source as the sample temperature was increased and decreased through  $T_m$ . The results are displayed in main part of Figure 4 along with a best fit to the data using a Boltzman expression with a linear background. The transition temperature was determined by this fit to be  $T_m = 316.7 \pm 0.1$  K.

The sample temperature was continuously held at 317.0 K, where we would have maximum sensitivity to temperature changes as it is the point of greatest slope in Fig. 4. The IR source was then switched to the brighter synchrotron source and infrared spectra were acquired every minute for 30 minutes to look for any heating that occurred instantaneously or more slowly over time. The temperature of the sample within the beam spot was measured by monitoring the position of the CH<sub>2</sub> symmetric stretch mode as previously calibrated. The sample temperature fluctuated slightly over the 30 minutes, however no discernable longer-term trends were observed.

Statistical analysis of the measured temperature fluctuations determined that the average temperature rise due to the SR beam is a very modest  $0.5 \pm 0.2$  K. We therefore conclude that the synchrotron IR source does not appreciably heat the sample under investigation. Since the test sample is mainly water, 98% by weight, we are confident that our result generalizes to most biological samples.

## **V. Conclusions**

In summary, we have introduced the newly emerging synchrotron-based FTIR spectromicroscopy technique and shown that it is truly non-destructive for biological systems. Future contribution of this new bioanalytical imaging technique will build upon previous IR studies. Most importantly it will complement other microscopy and biochemistry techniques to investigate changes in many different types of cells, as well as cellular biochemical processes resulting from a variety of agents. While the infrared spectra of whole cells are quite complex, the use of cell lines which are defective in a single process or pathway may allow the identification of key spectral features associated with important biochemical and physiological mechanisms. With

sufficient development infrared spectromicroscopy may become a rapid and inexpensive diagnostic tool for medical screening applications. In addition, the single cell nature of the SR-FTIR technique will allow reliable detection and identification of a small number of cells within a sample that are different from the others, potentially opening new areas of research in environmental health and biomedicine.

The results reported here established an important foundation for future biomedical and biological applications of synchrotron infrared spectromicroscopy, which will complement other biochemistry and microscopy techniques. SR-FTIR spectromicroscopy enables the successive monitoring of biochemical changes in individual cells non-destructively without having to treat cells with exogenous dyes, fluorescent labels, or stains, or to resort to destructive techniques. The non-invasive and non-destructive nature of the technique allows each cell in a population to be studied sequentially over a period extending to hours or even days. By monitoring individual cells over time it will be possible to detect the onset of disease and other cellular changes, and to probe the heterogeneity of responses to various treatments or insults within a population of living cells. The development of SR-FTIR spectromicroscopy will result in a broadly applicable and powerful research tool available to the scientific community.

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## Figure Captions

**Figure 1.** Schematic diagram of a synchrotron-based FTIR spectromicroscopy experimental setup. Synchrotron radiation from a bending magnet is collected, collimated, and transported to a commercial FTIR interferometer bench. After modulation by the interferometer, a commercial infrared microscope focuses the beam onto the sample using all-reflecting optics. Biological samples can be placed in an on-stage mini-incubator with environmental controls. The sample stage position is computer controlled with  $\pm 1 \mu\text{m}$  precision. The reflected light from the sample is collected by the microscope optics and sent to an IR detector. A computer performs a Fourier transform on the measured interferogram to obtain an infrared spectrum for each sample location.

### **Figure 2.**

**(i)** Photograph showing results from Alcian blue assays of cells exposed to the SR-IR beam for (a) 5 minutes, (b and c) 10 minutes, and (d) 20 minutes. Other cells in the field were not exposed and were used as negative controls. No cells show retention of the blue dye demonstrating that no immediate cytotoxicity is observed.

(ii) Photographs showing typical results for colony forming from (a) a negative control cell and (b) a test cell that had been exposed to the SR-IR beam for 20 minutes. Both cells proliferated into similar sized colonies after 10 days.

(iii) Photographs showing BrdU assay results for cells exposed to the SR-IR beam for (a) 5 minutes, (b) 10 minutes, and (c) 20 minutes. Two other cells in the field were unexposed and used as negative controls. In the lower panel, the blue color indicates DNA and the red color indicates BrdU incorporation during DNA synthesis. All test and control cells show the same incorporation of BrdU into the DNA.

(iv) Photograph showing typical MTT assay results for control and test cells that had been exposed to the SR-IR beam for (a) 5 minutes, (b) 10 minutes, and (c) 20 minutes. Other cells in the field were unexposed and are used as controls. All test and control cells show the same blue color indicating the same level of metabolic activity.

**Figure 3.** Summary of cytotoxicity tests. 267 cells were tested with over 1000 control cells studied. 100% of exposed cells tested showed no difference in response to non-exposed control cells. Error bars are from counting statistics ( $\sqrt{N}$ ) for each assay.

**Figure 4.** Plot of the measured CH<sub>2</sub> symmetric stretch mode center frequency as a function of temperature. Data was obtained as the temperature was raised and lowered. The solid line is a best fit to the data using a Boltzman function with a linear background. (Inset) Infrared reflectance spectrum of hydrated DPPC at T = 328 K which is above the melting transition temperature, T<sub>m</sub>. The largest absorptions are due to water; the CH<sub>2</sub> symmetric stretch vibrational mode around 2850 cm<sup>-1</sup> used as a temperature probe in this study is labeled.

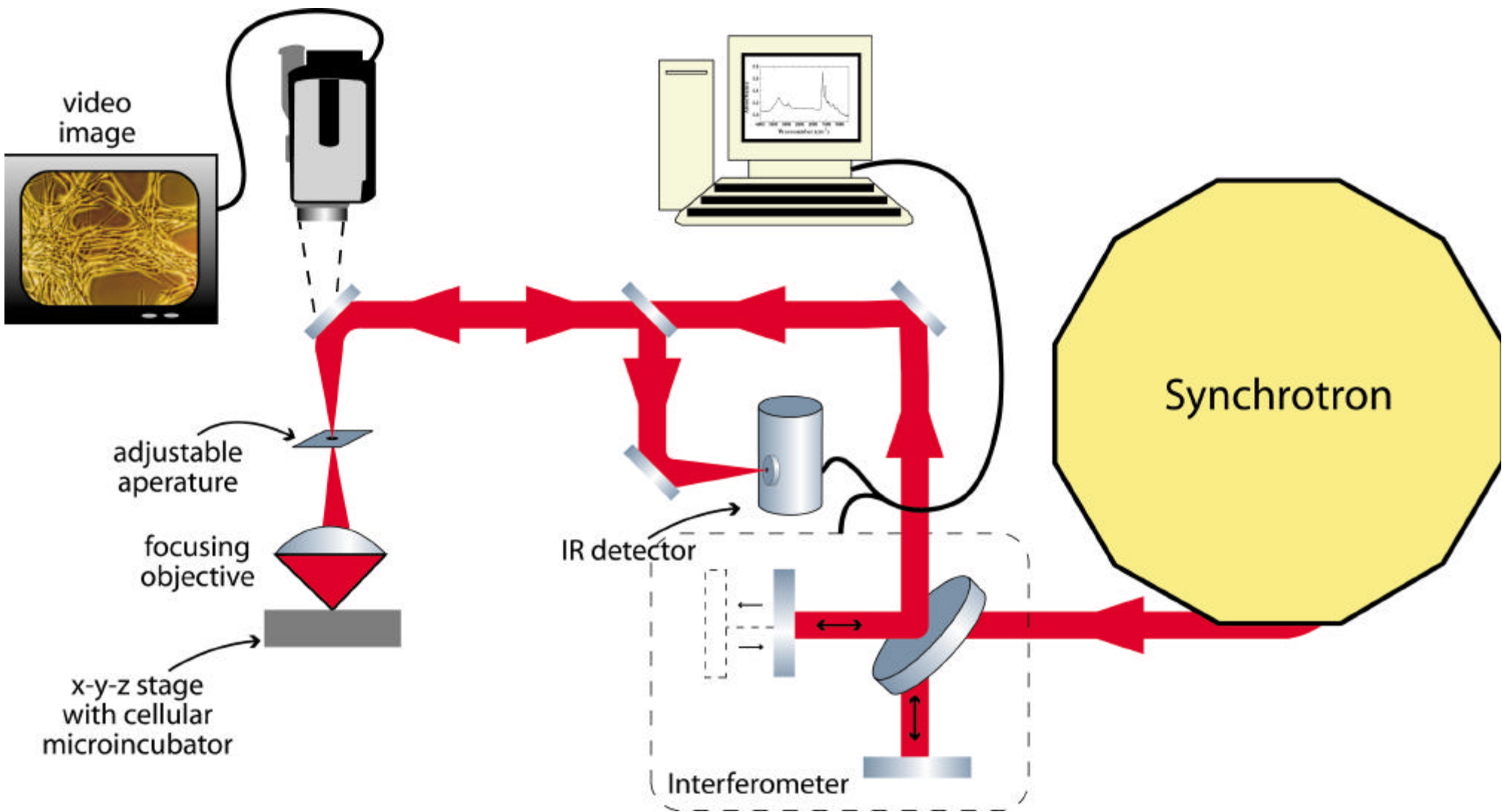
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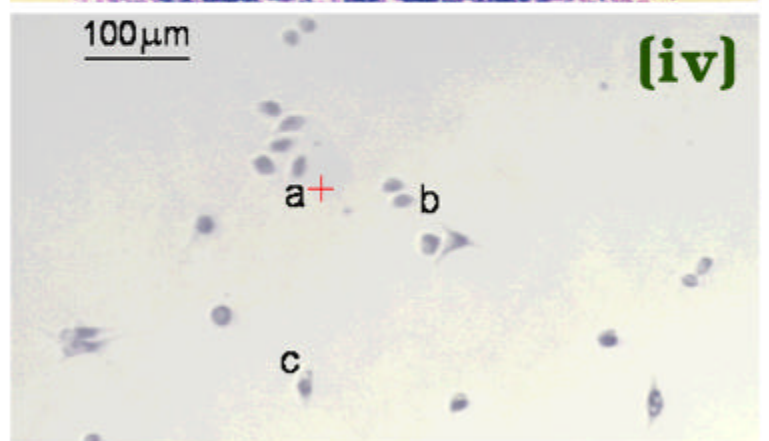
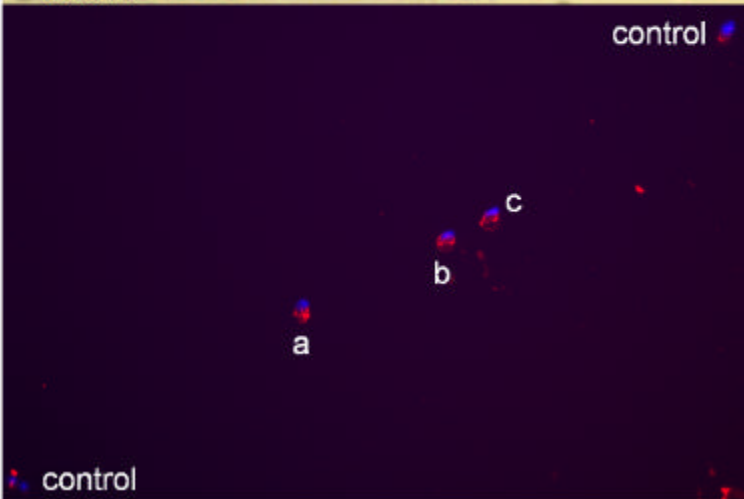
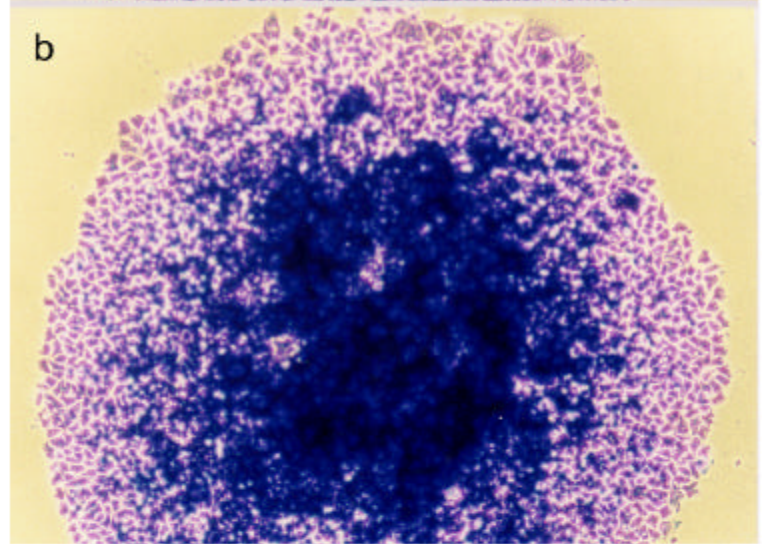
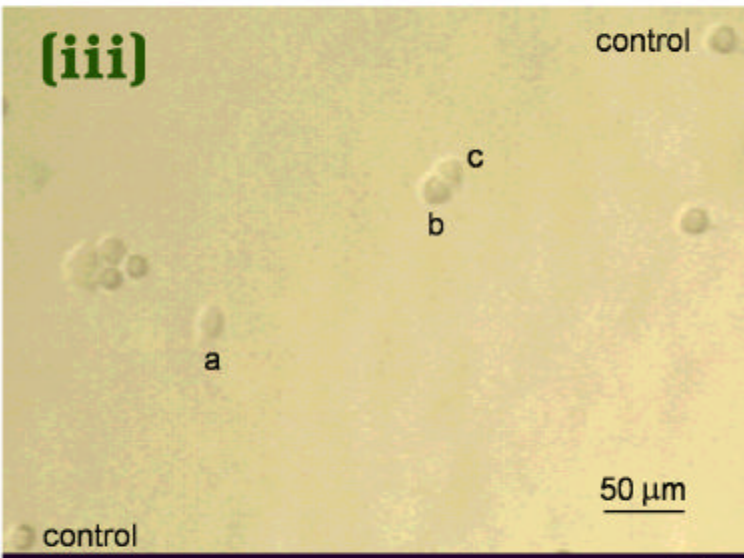
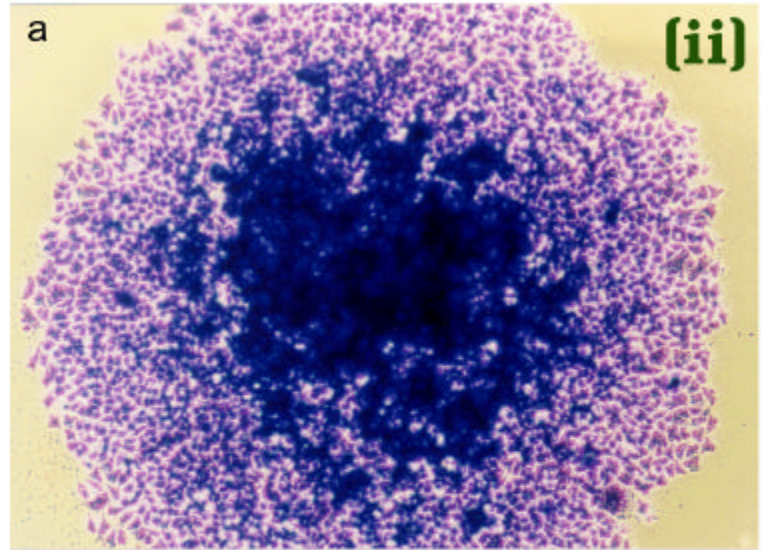
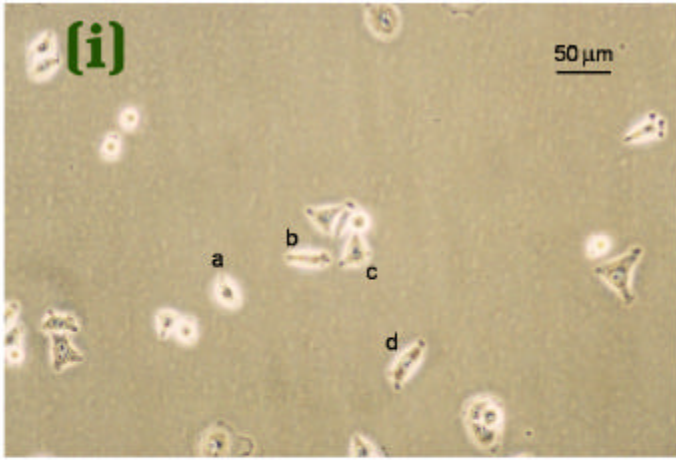
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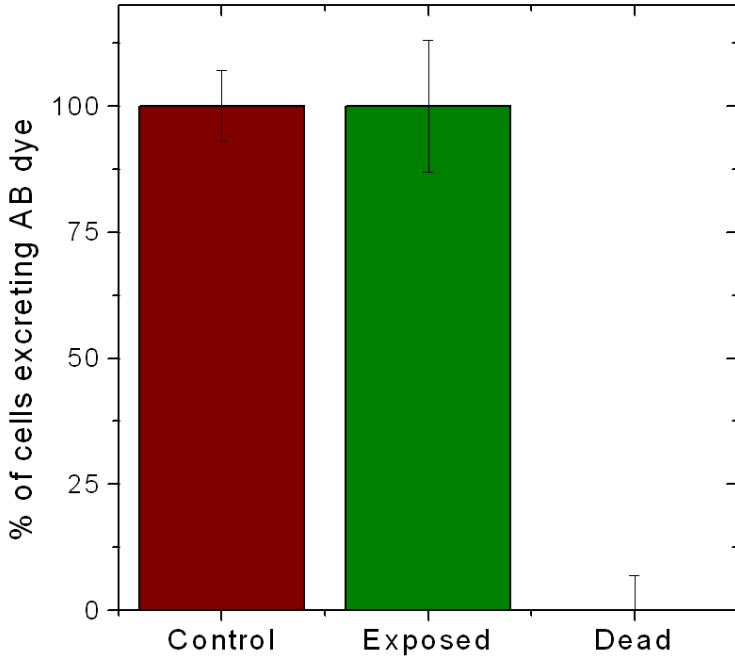


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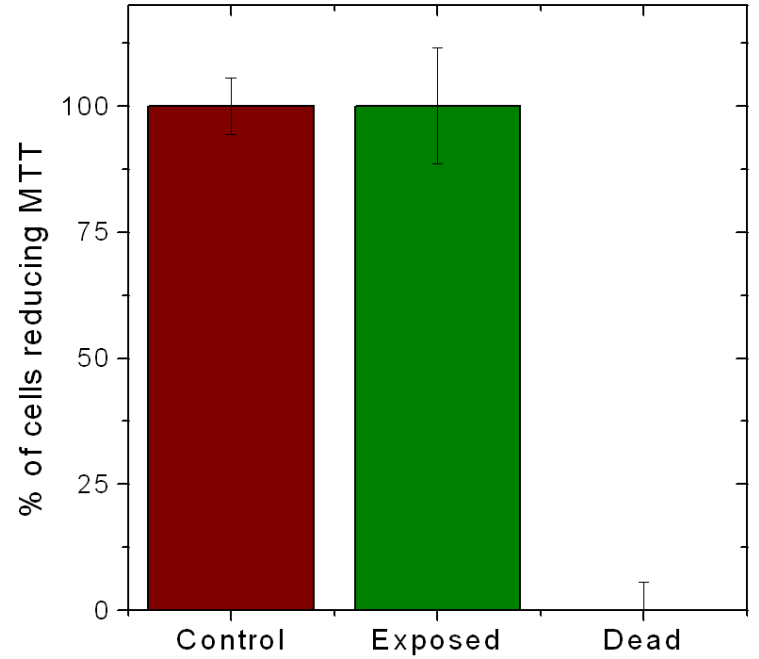




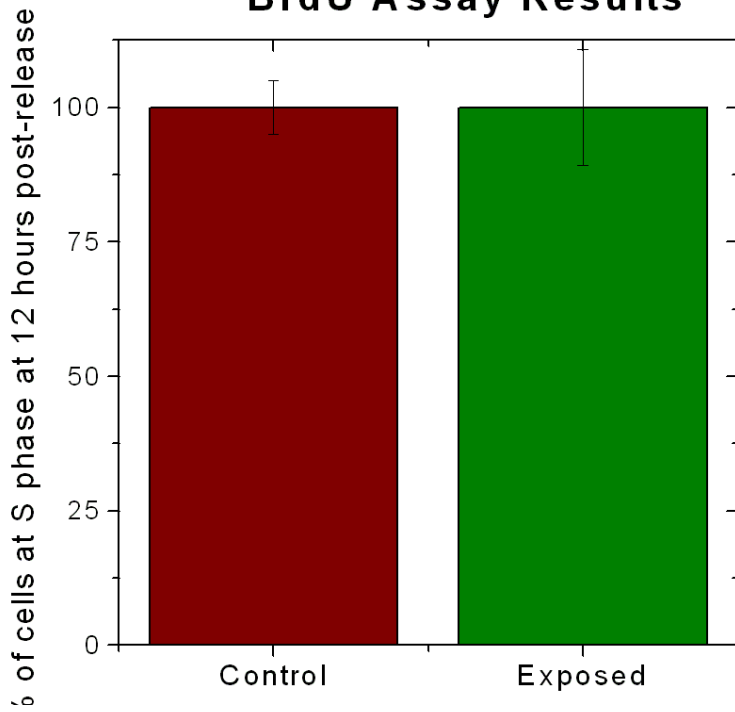
### Alcian Blue Assay Results



### MTT Assay Results



### BrdU Assay Results



### CFU Assay Results

