*SPIE Proceedings 1854, Los Angles, 1993*

# FEL-microscopy for the investigation of transient local heating in single living cells

[1]B. J. Tromberg, [2]K. A. Peterson, [1]T. B. Krasieva, [1]S. Shimizu, [2]C. A. Rella, [2]A. Jeung, [1]C. F. Chapman, [4]D. D. Dlott, [3]M. D. Fayer, [2]H. A. Schwettman, and [1]M. W. Berns

[1]Beckman Laser Institute and Medical Clinic, University of California, Irvine 92717
[2]Hansen Experimental Physics Labs, Stanford University, Stanford, CA 94305
[3]Department of Chemistry, Stanford University, Stanford, CA 94305
[4]School of Chemical Sciences, University of Illinois at Urbana-Champaign, 61801

## ABSTRACT

A free-electron laser (FEL) microscope has been constructed to perform spatially- and spectrally-resolved pump/probe experiments in single living cells. Picosecond infrared FEL pulses are absorbed by the sample and rapidly converted to heat. Excitation of localized fluorescent reporter molecules using a UV/VIS probe beam leads to thermally-induced alterations in the radiative signal. Fluorescence-detected infrared (FDIR) spectra are generated from regions proximal to reporter molecules by varying the FEL pump wavelength. Sub-wavelength spatial resolution is a composite function of media thermal properties and probe selectivity.

## 2. INTRODUCTION

Free electron lasers are unique sources of tunable, pulsed, high-power, infrared radiation.[1] Although they have been used widely in molecular spectroscopy[2], there are few accounts of FEL studies of cellular systems.[3] In contrast, conventional lasers have been extensively applied to cell biology, particularly in the development of laser-based microscopy methods.[4]

In this report , we describe our efforts to develop a novel form of FEL microscopy for the investigatation of molecular structure in living cells. The microscope employs FEL pump and a UV/VIS probe beams coupled to a modified, inverted, fluorescence microscope. Energy from the FEL is absorbed by structures in the cell and dissipated as heat. The probe beam excites fluorescence of a temperature-sensitive reporter molecule residing in specific cellular compartments. Fluorescence intensity and lifetime diminish with increased reporter proximity to IR absorption sites. Since organelle-localizing fluorescent probes are used, variations in the fluorescence-detected infrared (FDIR) signal can provide IR spectra of the probe's cellular environment.

Infrared spectroscopy has been employed to characterize the structure and dynamics of cellular proteins and membranes.[5, 6, 7] More recently, Fourier Transform Infrared (FTIR) microscopy techniques have been developed and applied to IR structural mapping of various biological materials.[8, 9, 10] These methods suffer from poor sensitivity due to path length and size limitations in single cell systems. For example, obtaining spatially-resolved spectral information from a 5nm-thick lipid bilayer in a 10-μm diameter cell would require overcoming diffraction and signal-to-noise (S/N) constraints. FDIR improves upon these limitations by employing a highly sensitive radiative process (fluorescence) as an indicator of IR absorption. Using this approach, spatial resolution is a function of the chemical localizing properties of the probe and the thermal properties of the medium.

In this work, we summarize our ongoing efforts to construct an FDIR microscope and report preliminary results of FDIR signals and spectra acquired in polystyrene films. In addition, temperature-dependent fluorescence lifetime data are presented for a membrane-localizing probe in a cellular system. Although


0-8194-1080-2/93/$6.00

our cellular data were not acquired using FEL-induced heat generation, these results are presented to highlight factors which effect sensitivity and spatial resolution in single-cell FDIR measurements.

## 3. THEORY

Fluorescence intensity, $I^{fl}$, decays as a function of time, t, and can be described by:

$$I^{fl} = I_0^{fl} \exp(-t/\tau) \tag{1}$$

where $\tau$ is the radiative lifetime and $I_0^{fl}$ corresponds to the t=0 signal. The fluorescence lifetime $\tau$, diminishes with temperature in a linear fashion (for certain probes):

$$\tau = mT + b \tag{2}$$

The terms m and b are determined empirically and describe $-\Delta\tau/\Delta T$ and the initial $\tau$, respectively.

The temperature rise, $\Delta T$, due to dissipation of heat from a light pulse absorbed by the sample is a function of the incident intensity, $I_0$, the attenuation coefficient, $\alpha$, density, $\rho$, and heat capacity, $C$:

$$\Delta T = \frac{I_0 \alpha}{\rho C} \tag{3}$$

The time-dependent temperature (T) decay following absorption of a pulse by a thin slab can be approximated by:

$$T = T_0 \exp(-t/\tau^*) \tag{4}$$

where the thermal decay time, $\tau^* \approx \rho Cd/A$. The parameters d and A are the sample thickness, and heat transfer efficiency (Watts/$m^2$degree), respectively. Here we assume: 1) the diameter of the pump beam is much greater than the sample thickness, 2) the precise relationship is a complex function of sample geometry and boundaries, and 3) the interval between FEL pulses is greater than the thermal relaxation time, $t_r$ . The thermal relaxation time is defined as the average time required to achieve $T_0$ at the surface of the absorbing object:

$$t_r \propto d^2/4\kappa \tag{5}$$

where d is the sample thickness and $\kappa$ is the thermal diffusion constant (typically on the order of $10^{-7}$ $m^2/s$).

In order to acquire localized IR absorption information from, for example, the plasma membrane, we incubate the cell with a membrane-localizing fluorescent probe. The cell is exposed to the FEL pump beam and cellular temperature rises according to equation (3). Assuming IR-absorbing species are distributed throughout the cell, the primary challenge is to determined the relative abundance of that particular functional group in the membrane. This is accomplished by varying the delay time between pump and probe beams.

For example, according to equation (5), a 5-nm-thick lipid bilayer will achieve $T_0$ in about 60 ps. Each structure, in turn, will exhibit different thermal relaxation characteristics. The temperature decay time

$\tau^*$, however, may be on the order of 10 ms. This ensures that by 60 ps, reporter molecules will essentially experience a constant temperature environment. As a result, fluorescence lifetime measurements will be perturbed throughout the radiative decay process. The critical determinant of spatial resolution is, therefore, the temporal delay between pump and probe. The shorter the delay, the more likely we are to sample heat generated from sources proximal to the probe. Thus, spatial resoultion, $\sigma$, can be estimated from the Stokes-Einstein relation:

$$\sigma = \sqrt{2\kappa t} \tag{6}$$

Assuming $k \approx 10^{-7}$ $m^2/s$, the first 125 ps of the fluorescence decay curve will contain information confined entirely to the 5-nm membrane. At longer times, heat may diffuse to the membrane from more distal locations and contribute to the reduction in $I^{\prime\prime}$. However, if the probe beam is timed substantially prior to $t_r$ for other strucutures, more of radiative decay profile will reflect the fluorophore's local environment. Since the plasma membrane is substantially thinner than other organelles commonly investigated with fluorescent probes, we expect the entire decay curve will be indicative of membrane absorption. For 1-μm-diameter structures, up to 250 ns are available for acquiring site-specific information.

## 4. EXPERIMENTAL

### 4.1. FEL Microscope

The FEL microscope system, illustrated in Figure 1, consists of a standard Zeiss Axiovert-35 inverted fluorescence microscope modified to accomodate the FEL pump beam.

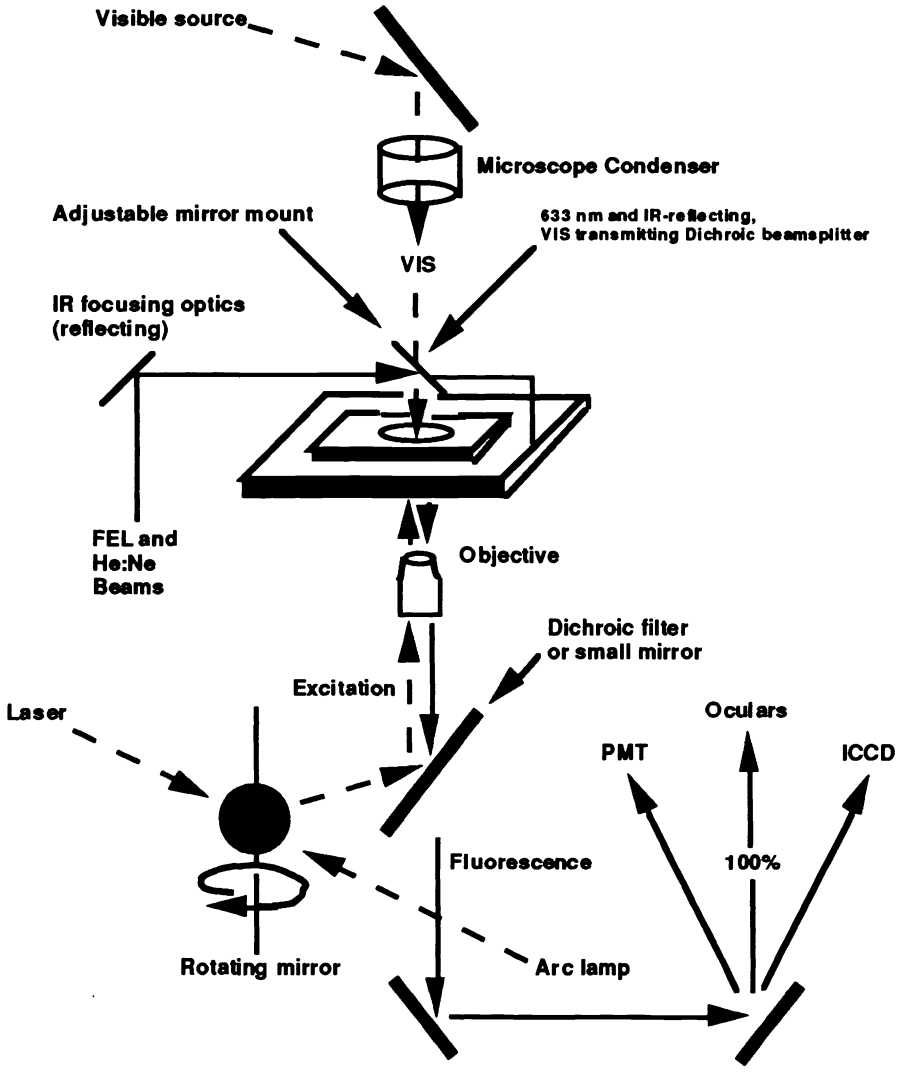


Figure 1. FEL microscope

The FEL (Superconducting Accelerator/Free Electron Laser, Stanford University) is coupled to the sample via reflecting optics and a dichroic beam splitter. This permits alignment and sample visualization using the colinear HeNe beam. FEL spot size at the sample can be varied from 100-400 μm using external optics. Typical FEL operating parameters are: 3 ps, 11.8 MHz micropulses; 2 ms, 10 Hz, 10 mJ macropulses; $\lambda$ = 5.0-5.3 μm. Average powers at the sample are approximately 5 mW.

Fluorescence exciation is provided by either a 100 W Hg-arc lamp or a CW Argon-ion laser (Spectra-Physics, model 162A-07). Using external optics, the 2 mW-sample-plane Argon spot size is variable from approximately 0.5 μm to 400 μm. A 100% beam splitter directs the emission onto a photomultiplier tube (PMT, Thorn EMI), an intensified CCD camera (Hamamatsu) or the oculars for viewing. The PMT photocurrent is either sampled directly using a digital oscilliscope (LeCroy model 9310) or by dual gated integrators (SRS model SR 250) prior to on-line oscilliscope processing. During gated experiments, gate B (500 ns duration) is set 40 μs prior to the macropulse rising edge and gate A (500 ns) is set 500 μs after the macrospulse rising edge. Gated integrator outputs are processed in the scope according to: (A-B)/B, where B corresponds to pre-macropulse fluorescence and A is the signal

acquired during macropulse heating. A shutter was employed to gate the Argon laser during A and B sampling intervals in order to minimize photobleaching effects.

4.2. Fluoresecence Lifetimes

All fluorescence lifetimes were measured using a multi-frequency phase and modulation fluorometer (SLM-Aminco model 48000 MHF, Champaign, IL). The fluorometer sample chamber was heated using a circulating water bath in order to acquire temperature-dependent data.

4.3. Samples and measurements

Fluorescence-detected infrared (FDIR) measurements were conducted on 300-400 μm-thick polystyrene films with two different embedded fluorophores: 1) Anthracene and 2) NBD-conjugated fatty acid (#N-678, Molecular Probes, Inc. Eugene, OR), a commonly-used anionic membrane probe. Dye concentration was adjusted so that optical densities were ≈ 0.5 @ 360 nm for Anthracene and 475 nm for N-678. All measurements were recorded using the FEL microscope system.

In order to explore the temperature dependence of NBD fluorescence, lifetime measurements were acquired from Chinese Hamster Ovary (CHO) cells incubated with NBD-conjugated phospholipid (N-3787, Molecular Probes, Inc. Eugene) and N-678 polystyrene films. Sample chamber temperatures were varied from 20-70 °C.

## 5. RESULTS AND DISCUSSION

In order to demonstrate fluorescence sensitivity to IR absorption, an anthracene film was placed on the FEL microscope and irradiated with 5.15 μm light. The arc lamp was used to continuously excite fluorescence. A combination of excitation bandpass (365 nm), dichroic (395 nm), and long pass (410 nm) filters were used to isolate the emission. The PMT output, illustrated in Figure 2, shows a reduction in fluorescence during the FEL macropulse.

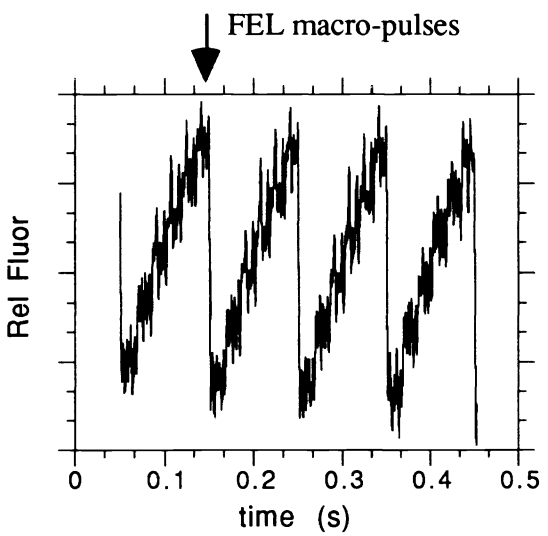

Figure 2. Fluorescence-detected IR absorption.

The sample cools between pulses and fluorescence levels nearly return to baseline. The gradual drop in fluorescence at the end of each recovery cycle is indicative of bulk heating effects.

Figures 3a-c illustrate the temperature-dependent lifetime of NBD membrane probes in various systems.

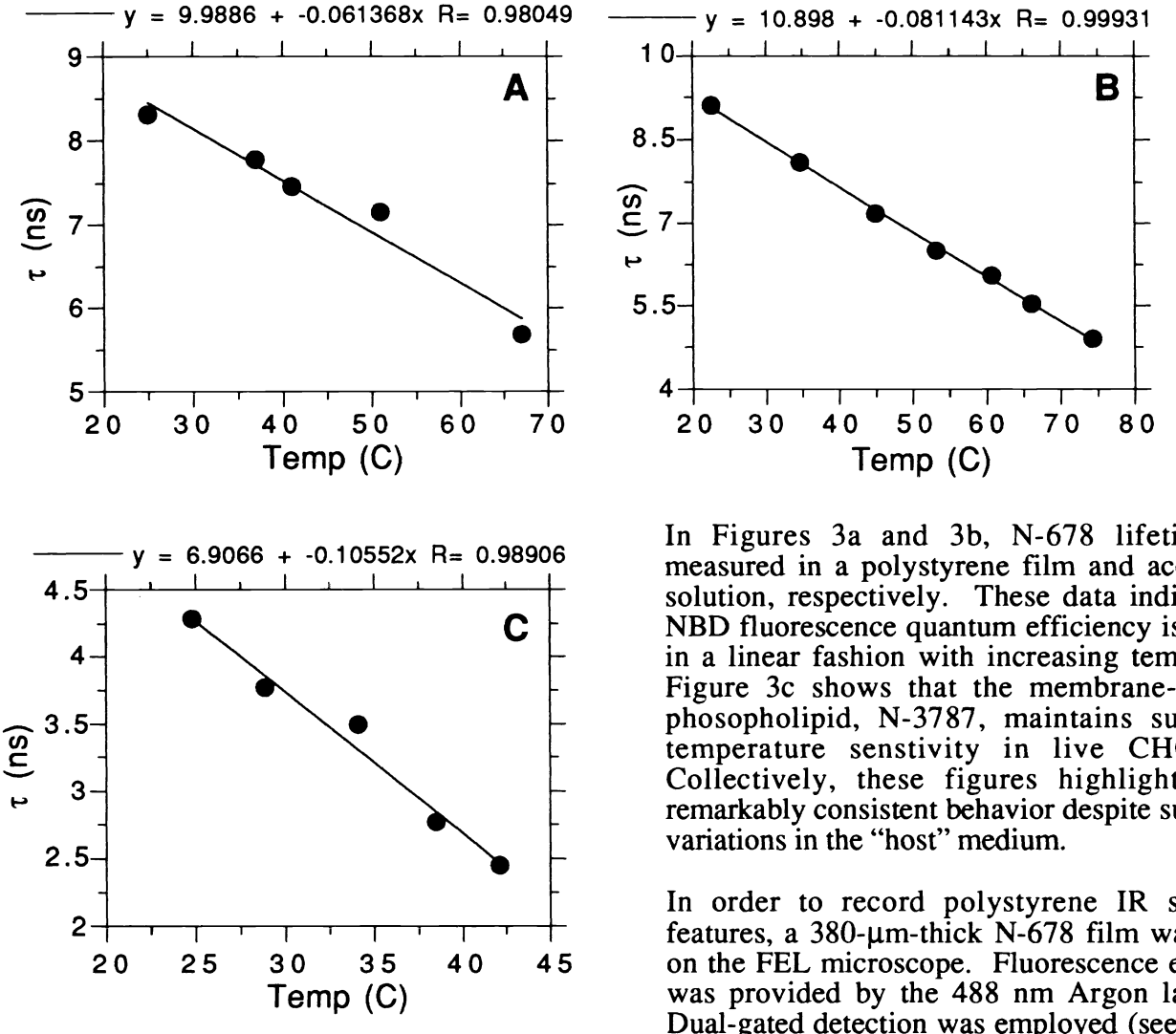


Figure 3. Temperature-dependent NBD lifetimes in polystyrene film (A), acetonitrile (B), and live CHO cells (C).

In Figures 3a and 3b, N-678 lifetime was measured in a polystyrene film and acetonitrile solution, respectively. These data indicate that NBD fluorescence quantum efficiency is reduced in a linear fashion with increasing temperature. Figure 3c shows that the membrane-inserting phosopholipid, N-3787, maintains substantial temperature senstivity in live CHO cells. Collectively, these figures highlight NBD's remarkably consistent behavior despite substantial variations in the "host" medium.

In order to record polystyrene IR structural features, a 380-μm-thick N-678 film was placed on the FEL microscope. Fluorescence excitation was provided by the 488 nm Argon laser line. Dual-gated detection was employed (see methods section) as the FEL was tuned between 5.00 and 5.26 μm (1900-2000 $cm^{-1}$). Figure 4a shows the N-678 film FTIR spectrum (although not shown, pure polystyrene films have identical features). In figure 4b, the change in fluorescence is greatest in spectral regions which correspond to the highest film absorption. Each point in the fluorescence-detected infrared (FDIR) spectrum is the mean response of 100 macropulses with an average error of ±0.005.

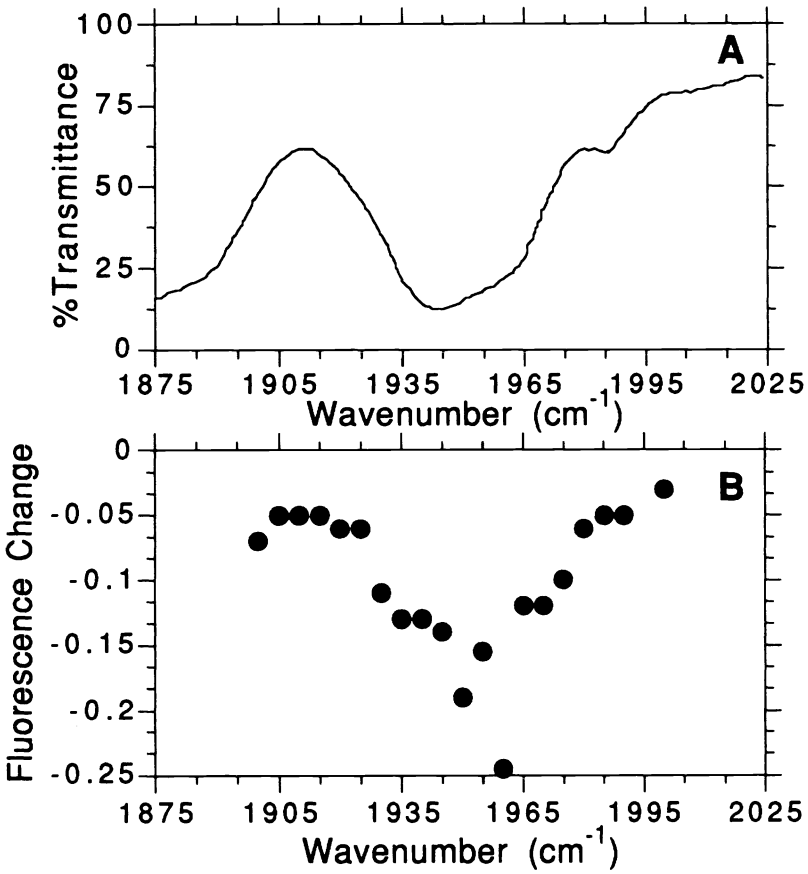


Figure 4. %T vs. wavenumber for FTIR (A) and FDIR (B) of N-678 polystyrene film.

## 6. CONCLUSIONS

We have demonstrated that fluorescence can be used as a sensitive probe of thermal events in organic films and cells. Varying the wavelength of the heat-generating pump beam permits acquisition of fluorescence-detected infrared (FDIR) spectra. In order to record spatially-resolved FDIR spectra in single living cells, ultrafast pump-probe experiments must be conducted. We are currently modifying our FEL microscope system in order to accomplish this goal.

## 7. ACKNOWLEDGEMENTS

The authors wish to acknowledge the Office of Naval Research/MFEL program for support of this work. BJT thanks Tom Milner and Lars Svaasand for helpful discussions.

## 8. LITERATURE CITED

1. T.I. Smith, B.A. Richman, R.L.Swent, H.A. Schwettman, "Facilities at the Stanford Picosecond FEL Center".Proc. SPIE 1854, Los Angeles (1993).

2. D.D. Dlott, M.D. Fayer "Application of a two-color free-electron laser to condensed-matter molecular dynamics" J. Opt. Soc. Am. B., 6, 977-994 (1989).

3. M.W. Berns ; W. Bewley ; C.-H.Sun ; P.Templin "Free electron laser irradiation at 200 microns affects DNA synthesis in living cells" PNAS, 87, 2810-2812 (1990)

4. M.W. Berns , W. H. Wright ; R. Wiegand-Steubing "Laser microbeam as a tool in cell biology." International Review of Cytology, 129, 1-44 (1991).

5. D. Chapman; PI Haris, "Biomembrane structures. Fourier transform infrared spectroscopy and biomembrane technology" Biochemical Society Transactions, 17, 951-3 (1989).

6. RW Sarver Jr; WC Krueger . "Protein secondary structure from Fourier transform infrared spectroscopy: a data base analysis." Analytical Biochemistry, 194, 89-100 (1991).

7. J.H. Crowe ; F.A. Hoekstra ; L.M. Crowe ; TJ Anchordoguy ; E. Drobnis "Lipid phase transitions measured in intact cells with Fourier transform infrared spectroscopy." Cryobiology, 26, 76-84 (1989).

8. M Daudon; C. Marfisi; B. Lacour; C. Bader . "Investigation of urinary crystals by Fourier transform infrared microscopy." Clinical Chemistry, 37, 83-7 (1991) .

9. R. Mendelsohn;A. Hassankhani; E. DiCarlo; A. Boskey "FT-IR microscopy of endochondral ossification at 20 mu spatial resolution" Calcified Tissue International, 44, 20-4 (1989).

10. J.A. Centeno; V.F. Kalasinsky; F.B. Johnson ; T.N. Vinh ; T.J. O'Leary "Fourier transform infrared microscopic identification of foreign materials in tissue sections." Laboratory Investigation, 66, 123-31 (1992).