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In the first grant period we proposed to develop Hadamard transform imaging and apply these techniques to problems of chemical interest. Our intent was to develop imaging techniques which used high power but defocused laser beams. By keeping power density low, we hoped to achieve high signal/noise ratios and short acquisition times while avoiding damage to delicate samples. Low power density was to provide a spatial multiplex advantage, even when the imaging technique possessed no classical multiplex (Fellgett's) advantage. Although the primary focus was to be Raman imaging, exploration of other contrast principles was proposed. These goals have been achieved.

We have constructed a Hadamard transform Raman microscopic imaging system, and have developed it to a high definition (64k pixel) technique. We have demonstrated multispectral Raman imaging and developed the first three-dimensional (digital confocal) Raman imaging. We have explored the systematic errors in Hadamard multiplexing techniques and developed corrections. We have used our Raman microscope techniques to explore defect distributions on graphite electrodes and damage effects on SERS-activated silver electrodes. We have used the microprobe capabilities of our instrument to investigate the kinetics of polyacrylamide formation in electrolysis capillaries. We have worked closely with a manufacturer of holographic displays to develop and incorporate holographic filters and holographic beam splitters into Raman spectrographs and microscopes. Finally, we have developed Hadamard multiplexing techniques for densitometric measurements of protein or nucleic acid blots. The project has been described in twelve original communications, a review article, two book chapters and one edited book, as well as almost forty invited lectures and contributed conference papers.

A. Hadamard Raman microscopes. Our first Hadamard Raman imaging microscope employed a 255-element mask arranged in a 15x17 rectangle and provided only coarse maps [1]. Although the images generated were coarse, they demonstrated the spatial multiplex advantage, in that they allowed us to obtain images of crystals of low-melting compounds such as N,N-dimethyl

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nitroaniline. They also demonstrated that a combined direct imaging/multiplexed imaging approach would be needed to provide truly high definition.

Our approach to high definition imaging has been to use a charge-coupled device detector (CCD) and imaging spectrograph to simultaneously record spectral dispersion in one dimension and spatially resolved signals in the other direction (parallel to the slits). The second spatial dimension is provided by a Hadamard mask, placed at an intermediate image plane in the spectrograph fore-optics, and used to multiplex spatial information parallel to the grating dispersion direction. Cylindrical optics are used to compress the multiplexed signal to approximately match the slit dimensions [2]. This general approach has remained unchanged throughout the project period, although the instrument has been refined[3,4]. While the original microscope generated 127x128 pixel images, the later versions [3,4] generated 255x256 pixel images. A careful redesign of the optics in 1991 improved the throughput of the device substantially [4].

Most Hadamard Raman images contain echoes. We have identified three sources of echo formation. These are poor alignment of the optical train between the Hadamard mask and the spectrograph, mask imperfections and diffraction at the spectrograph entrance slits. All three errors were present in early images, but could be corrected with a simple mathematical procedure [3]. More recently images contain diffraction errors only, and then only when the spectrograph entrance slits are closed to below about 200 μm.

Multispectral imaging, that is imaging on several bands simultaneously, is inherent in Hadamard Raman microscopy [4,5]. The CCD actually acquires in each column of pixels illuminated by Raman scatter. Multiple images are taken simultaneously, although the CCD data must be rearranged to disentangle them. In favorable cases, bands characteristic of individual chemical species of a multi-component material can be identified. More commonly, bands overlap and chemometric methods must be used to distinguish individual components. In a polyethylene/polystyrene laminate, for example [4], the strong phenyl ring stretch of polystyrene at 1600 cm^{-1} identifies that polymer, but polyethylene is visible only as methylene bands, e.g. the CH_2 wagging mode at ca. 1450 cm^{-1}, which is not resolved from the similar backbone vibration of polystyrene. At this writing, programming of a 2-D principal components analysis is almost complete. Our approach is an extension to two-dimensions of this standard chemometric technique.

We have used nearest-neighbor deblurring to implement a digital version of confocal Raman microscopy [5,6]. The nearest-neighbor algorithm is computationally simple and yields images about as sharp as obtained with a conventional confocal microscope. However, as we have

pointed out, nearest-neighbor deblurring degrades signal/noise ratios in the restored images, and is really only useful for high signal/noise ratio images. But Raman images are inherently weak. Conventional confocal microscopy would attenuate them even more than deblurring, reducing the initial intensity by a factor of 10 or more. Image restoration appears to be the more practical alternative when a set of sections through an object is available. However, if only a single section (image) of an object is needed, then a conventional hardware confocal microscopy approach will usually be faster.

Sophisticated image restoration algorithms are have been proposed for deconvolution of microscope point spread functions from fluorescence images. These algorithms are essentially three-dimensional extensions of the maximum likelihood deconvolution techniques widely used in infrared spectroscopy. We have very recently completed implementation of a maximum-likelihood image restoration procedure for Raman microscopy. A typical original and restored image of suspended 10 μm polystyrene beads are shown as Figure 1. A set of higher quality phototypesetter reproductions of several original and restored images from the same experiment is presented as an appendix to this proposal. The work is now being prepared for publication.

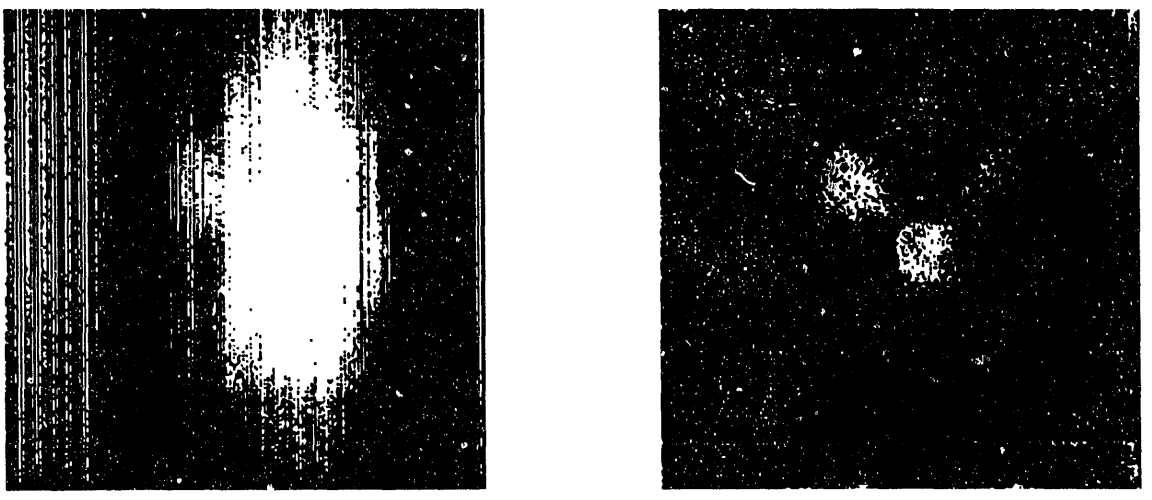

Figure 1. Image of suspension of polystyrene beads (phenyl ring, 1600 cm^{-1}), 50 mW 532 nm, 50X/1.0 NA water-immersion objective. Left, original image. Right, restored image.

Our procedure restores a stack of 12 256x256 point images in about 15 minutes on a low-end RS-6000 computer. The code can doubtless be tweaked to improve this time somewhat. In any event, newer single-user workstations can run the same code about 5X faster.

B. Raman imaging applications. We have examined the distribution of defects in laser-activated (i.e., damaged) pyrolitic graphite electrodes [3]. The electrodes had been activated by irradiation

with a pulsed Nd-YAG laser, which forms craters containing microcrystalline graphitic carbon. The microcrystallites can be identified by a broad Raman band at 1360 cm^{-1}. Imaging on this band demonstrated that the microcrystallites are uniformly distributed, as proposed from electrochemical and SEM evidence. Although we image at ca. 0.6 cm^{-1} resolution, no structure is visible in the defect distribution.

Because it is an almost ideal blackbody absorber, microcrystalline graphite is a particularly difficult sample for Raman imaging. We do no damage to graphite electrodes by irradiating with 250 mW, approximately 1.3 kW/cm^2. However, we do heat them, and it is necessary to bring the sample to a steady-state temperature in order to obtain images which remain in focus throughout the data acquisition cycle.

Laser-induced rearrangement of surface-enhanced Raman spectroscopy (SERS) electrodes has been examined by comparison of laser reflectance images to pyridine SERS images [7]. We find that the apparent diameter of SERS images from focused 514 nm laser beams is approximately 4 μm greater than the reflectance image diameter. This apparent increase results from rearrangement of the silver surface induced by the laser beam itself. The damaged surface has a lower SERS activity than the unirradiated electrode, whose preparation had been optimized for acquisition of single point spectra at low incident power. Because the incident beam has a Gaussian spatial distribution, the power density and resulting damage are higher at the center than at the edges. Consequently, the SERS images have larger apparent diameters than reflectance images. Valid SERS images can be obtained only if red (He-Ne) illumination is used, although weak (5-6 mW) green illumination does not do much damage over the few minutes required for image acquisition.

C. Raman microprobe experiments. Our microscope can be operated as a microprobe, of course, and we have used it to study the kinetics of formation of cross-linked polyacrylamide in 75 μm i.d. electrophoresis capillaries [8]. We monitor the disappearance of the 1292 cm^{-1} acrylamide CH_2 wag, which we can follow to almost 99% disappearance. We find that the reaction obeys a second-order kinetic equation well. However, when the same reaction is monitored in bulk, it is faster and does not follow second-order equations. Direct thermocouple measurements demonstrate that the bulk system undergoes a 5° C temperature rise caused by the exothermic reaction. The temperature rise in the capillary is much smaller and the system can be regarded as isothermal. It is not the case, therefore, that the final structures of the cross-linked polymers are identical in the bulk and capillary systems. Consequently, one can not uncritically transfer the structural or performance data from classical polyacrylamide gel electrophoresis to its capillary version. In general, one should exercise caution in preparing gels for such critical applications as

nucleic acid sequencing. In the classical slab format, controlled temperature synthesis may be more difficult than is commonly recognized.

D. Hadamard Transform Photothermal Deflection Imaging. We have formed images of spinach plastocyanin and molecular weight standard proteins electrophoretically separated and then blotted onto nitrocellulose [9]. Plastocyanin was stained with the leuco-dye NBT-BCIP, a nitroblue tetrazolium derivative which generates purple stains. The molecular weight standards were stained with India ink. One dimensional transverse photothermal deflection (mirage effect) imaging was performed with 20-25 mW Nd-YAG 532 nm illumination encoded with a 127-element Hadamard mask. The system is only 2-X noisier than a 14-bit CCD camera. Detection limits were 5 ng protein for the photothermal system and 3 ng for the video camera. There is no need for laser illumination in this work, and a system based on incoherent illumination, e.g. an arc lamp or sodium vapor lamp would probably perform as well.

E. Holographic Optics. As a side project, we have developed Raman microprobe systems using holographic optical elements in place of conventional filters and beam splitters [10,11]. We have demonstrated that recently developed holographic notch filters allow acquisition of Raman spectra about 60 cm^{-1} from the exciting line with a single stage spectrograph. There are no polarization artifacts associated with these filters when they are operated at normal incidence. A holographic beam splitter, essentially a modified notch filter designed and constructed to our specifications (and now available commercially), can replace the conventional dichroic beam splitter in the epi-illumination system of a Raman microscope or microprobe of any sort. The holographic device allows insertion of the laser beam with 90% efficiency and collection of Raman scatter as close as 50 cm^{-1} from the exciting line on both the Stokes and anti-Stokes side. The Stokes/anti-Stokes performance is especially important, because it opens the way for efficient Raman microthermometry.

F. Review literature. We have written reviews on Hadamard techniques [12] and Raman microscopy [13,14] and have edited a book on spectroscopic imaging, which includes a Raman microscopy review [15].

G. Patents. Aspects of the Hadamard imaging technology developed under this grant have been awarded U. S. patent number 5,048,959 (issued September 17, 1991.

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