Fast Confocal Raman Imaging Using a 2-D Multifocal Array for Parallel Hyperspectral Detection

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ABSTRACT: We present the development of a novel confocal hyperspectral Raman microscope capable of imaging at speeds up to 100 times faster than conventional point-scan Raman microscopy under high noise conditions. The microscope utilizes scanning galvomirrors to generate a two-dimensional (2-D) multifocal array at the sample plane, generating Raman signals simultaneously at each focus of the array pattern. The signals are combined into a single beam and delivered through a confocal pinhole before being focused through the slit of a spectrometer. To separate the signals from each row of the array, a synchronized scan mirror placed in front of the spectrometer slit positions the Raman signals onto different pixel rows of the detector. We devised an approach to deconvolve the superimposed signals and retrieve the individual spectra at each focal position within a given row. The galvomirrors were programmed to scan different focal arrays following Hadamard encoding patterns. A key feature of the Hadamard detection is the reconstruction of individual spectra with improved signal-to-noise ratio. Using polystyrene beads as test samples, we demonstrated not only that our system images faster than a conventional point-scan method but that it is especially advantageous under noisy conditions, such as when the CCD detector operates at fast read-out rates and high temperatures. This is the first demonstration of multifocal confocal Raman imaging in which parallel spectral detection is implemented along both axes of the CCD detector chip. We envision this novel 2-D multifocal spectral detection technique can be used to develop faster imaging spontaneous Raman microscopes with lower cost detectors.

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projected to different vertical positions on the charge-coupled device (CCD) camera for simultaneous collection of multiple spectra.\textsuperscript{6−8} Avoidance of crosstalk can be further aided by the use of a synchronized galvomirror placed in front of the entrance slit of the spectrograph that positions each spectra to different pixel rows on the camera.\textsuperscript{7,8} This time multiplexing technique not only makes the multifocal confocal Raman microscope more compact and cost-effective, it also offers a major advantage for imaging small samples because the foci can be positioned much closer to each other. The acquisition of a Raman image (30 × 30 pixels) in 33 s has been demonstrated, which allowed for Raman imaging of fast biological dynamics of a single bacterial spore.\textsuperscript{6}

However, the vertical dimension of the CCD detector ultimately limits further improvement of the imaging speed. The number of pixel rows on the camera determines the maximum number of spectra that can be acquired in parallel and limits the size of the multifocal array that can be used. We recently developed a new multifocal detection scheme that circumvents this limitation, allowing for the spectra from a 2-D multifocal array to be collected in parallel.\textsuperscript{9} In this approach, the horizontal dimension (along the dispersion direction) of the CCD detector is also used for parallel spectral detection. A two-dimensional multifocal array was created at the specimen plane using a spatial light modulator (SLM). The 2-D array of Raman signals was delivered simultaneously to the CCD detector. Spectra from a given row of foci were projected to the same horizontal row of pixels on the CCD detector, resulting in overlapped spectra. We devised an encoding, or modulation, technique to resolve the superimposed spectra and retrieve the individual Raman spectra. Using this modulated multifocal detection scheme, both the vertical and horizontal dimensions of the CCD chip were used to acquire spectra from a 2-D multifocal array of optically trapped particles.\textsuperscript{9}

In this article, we report on the development of a new time-sharing 2-D multifocal Raman microscope for confocal hyperspectral Raman imaging that combines a galvomirror-based parallel spectral detection along the CCD’s vertical dimension and a Hadamard transform detection along the CCD’s horizontal dimension. After the Hadamard transform process, individual Raman spectra from each focus in a horizontal row of the multifocal array can be reconstructed from the superimposed spectral data. Hadamard transform has been widely used for Raman spectroscopic applications, such as Hadamard coded masks for improving the signal-to-noise ratio (SNR) and throughput of a spectrometer,\textsuperscript{10} Hadamard transform Raman imaging with global illumination from a defocused laser beam\textsuperscript{11,12} and Hadamard coded apertures for Raman detection of diffuse samples.\textsuperscript{13} However, its implementation in confocal Raman microscopy as demonstrated in this work has not yet been reported. We demonstrate that the capability for parallel detection along both vertical and horizontal dimensions of the CCD detector in our 2-D multifocal Raman microscope significantly improves the confocal imaging speed when the CCD detector noise is significant, such as when the detector is operated with a fast read-out rate or at high temperatures.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Figure1}
\caption{(a) Schematic of the confocal Raman imaging system by using 2-D multifocus array for parallel hyperspectral detection. Abbreviations: GM, Galvomirror; SM, Steering mirror; Ph, Pinhole. (b) 2-D 5 × 10 multifocus array generated by XY galvomirrors of GM\textsubscript{1} and GM\textsubscript{2}. (c) Measured Raman signals from a polystyrene plate excited by the 5 × 10 multifocus array shown in part b.}
\end{figure}

**EXPERIMENTAL SECTION**

A schematic of the experimental setup is shown in Figure 1a. A high-powered (>800 mW) 785 nm diode laser (Sacher Lasertechnik, TEC-510-0785-1000) was used as the excitation source. The laser beam was rapidly steered by a pair of XY galvomirrors GM\textsubscript{1} and GM\textsubscript{2} (Cambridge Technology, 6220H) to generate a 2-D laser focal array at the sample plane after passing through a water immersion objective (Olympus, 60X/1.2 W). For example, Figure 1b shows a 5 × 10 multifocal array when the XY galvomirrors were scanned at a rate of 5 kHz. Backward propagating Raman scattering signals generated at each focus of the 2-D multifocal array were collected by the objective and then combined into a single, descanned beam after being reflected by the XY galvomirrors GM\textsubscript{1} and GM\textsubscript{2}. A dichroic mirror separated the incident 785 nm laser from the Raman signals. The Raman beam was focused through a 100-μm pinhole for confocality. Polystyrene beads (200 nm) were used to measure the spatial resolution of the confocal system. The instrument was measured to have a lateral resolution of 570 nm and an axial resolution of 1.6 μm. More information on the spatial resolution measurement can be found in the Supporting Information. Another fast steering mirror SM\textsubscript{1} (Newport, FSM-300-01) was placed in front of the entrance slit of the spectrograph (Princeton Instruments, LS785) to synchronously steer the Raman signals from each row of foci onto different rows of pixels on the thermoelectrically cooled CCD camera (Princeton Instruments, PIXIS 100BR, 1340 × 100 pixels) of the spectrograph. The spectral resolution of the spectrograph is 5 cm\textsuperscript{-1}. Figure 1c shows a CCD image of the Raman signals from the 5 × 10 array that illuminated a bulk polystyrene sample. The steering mirror SM\textsubscript{1} directed the Raman signals from each of the 10 rows to different positions along the vertical dimension of the CCD camera to avoid crosstalk between adjacent spectra. Note that the spectrum of each row is comprised of five individual spectra (i.e., from the five foci) that, at this point, are superimposed on top of each other and, therefore, unresolvable.

The superimposed Raman spectra along the horizontal dimension of the multifocal array were decomposed by applying Hadamard encoding patterns to the multifocal array. See the Supporting Information for more details on the Hadamard coded pattern measurement. In this study, we used an order-31 S-matrix,\textsuperscript{14} a 31 × 31 matrix, as the Hadamard...
coded patterns. Figure 2a shows a part of the S-matrix, where 0 and 1 indicate positions in the multifocal array where the laser was “off” and “on”, respectively. Each row of the S-matrix is a linear array in which 16 foci are “on” and 15 foci are “off”. Experimentally, this was achieved by programming the X galvomirror to generate the coded pattern by raster scanning the laser focus along the horizontal direction to the 16 “on” positions according to each row of the S-matrix while skipping the 15 “off” positions. The Y galvomirror was used to scan the Hadamard encoding pattern for each of the 10 rows in the multifocal array. In this manner, 2-D 16 × 10 “on” foci arrays were generated with the XY galvomirrors. Figure 2b,c illustrate the first and second arrays that were generated, which correspond to the patterns of the first and second rows of the S-matrix in Figure 2a. The steering mirror SM3 was synchronized with the Y galvo-mirror to project the 10 coded Raman spectra (i.e., rows in each of these array patterns) to 10 different rows of pixels on the CCD chip for parallel spectral detection. In all, a total of 31 of these 16 × 10 coded patterns were acquired. This entire acquisition process allowed us to acquire Raman spectra from a 31 × 10 multifocal encoded pattern was 0.1 s. In order to make a fair comparison of the 2-D multifocal patterned array with the point scan approach, the total laser power was set at 320 mW, which distributed over the 160 “on” foci is equivalent to 0.2 mJ of energy deposited at each focus. The image shown in Figure 3d is a mosaic of two 31 × 10 pixels images. Each image took 3.1 s to acquire for a total acquisition time of 6.2 s, 10 times faster than the point scan method. This speed improvement is attributed to the parallel spectral detection in the vertical dimension of the camera, since 10 spectra are being acquired simultaneously.

An important advantage of the coded Hadamard 2-D pattern measurement for parallel detection along the horizontal dimension of the camera is the improvement of the signal-to-noise ratio (SNR) by a factor as high as \( \left( \frac{N+1}{2} \right)^2 /2 \), where \( N \) is the order of the S-matrix.14 Figure 3b shows Raman spectra from the center of the left polystyrene bead in Figure 3a for both the point-scan method and from the 2-D encoded pattern method after spectral reconstruction. The SNR calculated for the 1001 cm\(^{-1}\) band is 14 and 22 for the point scan and multifocal encoded pattern scan, respectively. It should be noted that the spectra displayed in Figure 3b (also Figures 4d and 5d presented later in the article) are raw data without using any noise reduction or smoothing process. Similar images can be generated using the weaker Raman peaks in the spectrum, such as the 1602 cm\(^{-1}\) band (see Supporting Information, Figure S3). The SNR for the 1602 cm\(^{-1}\) band is 4.7 and 6.4 for the point scan and multifocal pattern scan images, respectively. This slight improvement in SNR is similar to that of the 1001 cm\(^{-1}\) images.

The modest improvement in SNR by a factor of only 1.6 is attributed to the optimal detector conditions that were used to acquire the images in Figure 3. The CCD detector was cooled to −80 °C, and the read out rate was set at 100 kHz, which provided a low dark current noise of 0.03 e−/p/s and a low spectral noise was reduced by singular value decomposition (SVD), and a Gaussian smoothing filter was applied to the final Raman images.

RESULTS AND DISCUSSION

Figure 3a is a bright-field image of three 3 μm polystyrene beads used as a test sample in our study. Raman images based on the 1001 cm\(^{-1}\) band were generated by operating our microscope in a conventional point-by-point scanning method and also the multifocal encoded pattern approach, as shown in parts c and d of Figure 3, respectively. For the point scan image, the laser power was set at 2 mW and the integration time of each pixel was 0.1 s. Thus, the energy deposited at each pixel was 0.2 mJ. The total acquisition time was 62 s for a 31 × 20 pixel image. With the multifocal approach, the acquisition time for each 16 × 10 multifocal encoded pattern was 0.1 s. In order to make a fair comparison of the 2-D multifocal patterned array with the point scan approach, the total laser power was set at 320 mW, which distributed over the 160 “on” foci is equivalent to 0.2 mJ of energy deposited at each focus. The image shown in Figure 3d is a mosaic of two 31 × 10 pixels images. Each image took 3.1 s to acquire for a total acquisition time of 6.2 s, 10 times faster than the point scan method. This speed improvement is attributed to the parallel spectral detection in the vertical dimension of the camera, since 10 spectra are being acquired simultaneously.

Figure 2. (a) Portion of the order-31 S-matrix showing the first 13 rows. (b) First and (c) second patterns of 2-D coded 31 × 10 multifocal arrays based on the first two rows of the S-matrix. A red dot denotes an “on” focus while a blank space denotes an “off” focal position.

Figure 3. (a) Bright-field image of three 3 μm polystyrene beads. (b) Measured Raman spectrum with point scan (top) and reconstructed Raman spectrum from Hadamard S-matrix coded 2-D pattern scan (bottom). These two Raman spectra are from the same central point of the left polystyrene bead; the peak intensities of the 1001 cm\(^{-1}\) band are normalized to the same level. Raman image of 1001 cm\(^{-1}\) band (c) using point scan with a total integration time of 62 s, and (d) using coded 2-D pattern scan with a total integration time of 6.2 s. (CCD detector is cooled to −80 °C and read-out rate is 100 kHz).
If the read-out rate of the detector is increased to 2 MHz, for a faster read-out speed, the read-out noise of the CCD detector increases to 11 e− rms. Under this condition, the point scan method could not generate a Raman image with 2 mW laser power and an integration time of 0.1 s/pixel, as shown in Figure 4a. Spectrum I shown in Figure 4d is an example of a Raman spectrum from Figure 4a (the central region of the left polystyrene bead). The spectrum has a poor SNR of only 4. A longer integration time was needed to increase the SNR enough to generate a Raman image. By increasing the integration time to 0.3 s/pixel, a Raman image (Figure 4b) could be generated using the point scan method with a total acquisition time of 186 s. The corresponding raw Raman spectrum (spectrum II in Figure 4d) has a SNR of 9. When the 2-D multifocal encoded pattern method was used with an integration time of 0.1 s/pattern and 0.2 mJ energy at each focus (i.e., the same experimental parameters that were used for Figure 4a), a Raman image could be formed with a total acquisition time of 6.2 s, which is shown in Figure 4c. Spectrum III in Figure 4d is a reconstructed Raman spectrum from this multifocal Raman image, which has a SNR of 9. Both Figure 4b,c yield Raman spectra with a SNR of 9, but the imaging speed of the 2-D multifocal method is 30 times faster than the point scan method (i.e., 186 s vs 6.2 s). See the Supporting Information, Figure S4, for equivalent images based on the 1602 cm\(^{-1}\) Raman band.

Another example of the advantage of the 2-D multifocal Hadamard coded pattern technique for Raman imaging was demonstrated by intentionally increasing the working temperature of the CCD chip to increase noise. With the CCD detector cooled only to \(-15^\circ \text{C}\), and operating at a 2 MHz read-out rate, Raman images (Figure 5a) were obtained by point scan technique using 2 mW laser power and 1 s/pixel integration time, for a total acquisition time of 620 s. After increasing the working temperature of the CCD detector to \(-10^\circ\text{C}\) and keeping all other parameters the same, no Raman image could be formed, as shown in Figure 5b, due to the large noise. However, when the 2-D Hadamard coded pattern technique was implemented under the exact same sensor temperature and read-out rate conditions, a Raman image of the beads could be generated (Figure 5c). The total acquisition time for generating the image in Figure 5c was 6.2 s. Spectra I–III in Figure 5d are raw Raman data corresponding to the images in Figure 5a–c. The calculated SNRs of spectra I–III are 5, 3 and 5, respectively. These results demonstrate a 100 times improvement in the imaging speed by using the 2-D multifocal array patterned detection method compared to a conventional point scan imaging approach. See Supporting Information, Figure S5, for equivalent images based on the 1602 cm\(^{-1}\) Raman band.

**CONCLUSIONS**

We have demonstrated a technique for improving the imaging speed of confocal Raman microscopy that uses a 2-D multifocal array. While polystyrene beads were used as a test sample in our experiments, this technique can also work with other specimens as well (see the Supporting Information, Figure S6, for Raman images of yeast cells). Because the system uses the same confocal pinhole design as in a conventional single focus confocal Raman microscope system, the multifocal patterned detection technique does not compromise the lateral and axial spatial resolutions of the Raman images. Unlike other systems that rely on a rigid multifocal array design, the system we
developed is highly versatile and flexible for generating different sized array patterns with different interfocal spacings by simply reprogramming the scanning galvomirrors. The use of a descanned beam as well as the steering mirror is simpler than using fiber bundle arrays to couple the light into discrete locations on the CCD camera. Our system is unique in that parallel detection is demonstrated along the horizontal dimension of the CCD detector (i.e., along the dispersion direction), whereas many multifocal Raman systems to date have shown parallel detection only along the vertical axis. We showed that a rapidly modulated detection scheme based on Hadamard encoding patterns could be implemented by programming the galvomirrors to scan the beam to “on” and “off” positions in the multifocal array, which is different than masks and apertures that have been used previously. This scheme was used to deconvolve the superimposed Raman spectra, allowing for the reconstruction and retrieval of the individual Raman spectra from each focus in a horizontal row of the multifocal array. Our results indicate that the 2-D multifocal Raman microscope could be used to increase imaging speeds, in particular, in cases where noise plays a significant role. An improvement of as much as 100× faster than a conventional single focus point scan approach was demonstrated for a condition in which the CCD detector was operated at high temperature and a fast read out rate to increase noise. In principle, larger multifocal arrays can be generated to further increase the imaging speed. However, the maximum number of foci will eventually be limited by the scan speed of the galvomirrors. As the number of foci increases, the dwell time of the laser beam at each focus will decrease and at some point will approach the response time of the mirrors.

This present work has several important implications for spontaneous Raman imaging. Total imaging time for Raman microscopy can be significantly decreased with our method thanks to the improved signal-to-noise ratio of the reconstructed spectra, the faster image acquisition times, and the ability to operate cameras at fast read out rates without suffering the adverse effects of the increased noise. Our method also shows the potential of using less expensive cameras for Raman imaging as we were able to image samples without needing to cool the camera down to −80 °C. We envision this novel imaging technique can be used to develop faster spontaneous Raman microscopes that use lower cost detectors.

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**ASSOCIATED CONTENT**

* Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b03707.

Description of the Hadamard coded pattern measurement, determination of spatial resolution of the system, Raman images of polystyrene beads based on the 1602 cm⁻¹ Raman peak, and Raman images of yeast cells based on the 1001 cm⁻¹ Raman peak (PDF)

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**Notes**

The authors declare no competing financial interest.