Hyperspectral Microscopy
High Throughput Drug Delivery Development and Quality Control
White Paper 2017

Hyperspectral microscopy is rapidly developing as an effective tool for characterizing existing FDA approved APIs (active pharmaceutical ingredients) that are being incorporated with nano or micron-scale vectors for targeted drug delivery. It is also utilized extensively to image and characterize nanomaterials used in photothermal therapy and related theranostic studies as well as nanotoxicology and general nanomaterials synthesis and development studies.

Recently, the utilization of hyperspectral microscopy for these targeted drug delivery and related theranostic applications has been driven by studies at the FDA. These studies are being done to validate hyperspectral microscopy's efficacy for cross validation of data produced from established "black box" devices such as ICP-mass spectroscopy (ICP-MS), dynamic light scattering (DLS) and UV-Vis spectroscopy. While ICP-MS, DLS, and UV-Vis have been relied on for decades to provide important sample information such as chemical composition and particulate size distribution, they do not provide an appropriate level of spatial context and imaging. As such, these tools provide limited insight regarding how an active pharmaceutical ingredient (API) and drug vector are interacting across the sample during production and over time. They also provide limited insight regarding the interaction of drug delivery vectors and targeted cells or tissue in an in vitro or ex vivo environment.

The need to confirm the interaction of APIs and their delivery vectors is not easily accomplished by using traditional fluorescence or electron microscopy imaging. Often it is not feasible to attach fluorophores to these individual sample elements for fluorescence microscopy, and doing so can alter the chemical composition of the samples. Additionally it can be difficult to distinguish these individual elements visually, regardless of the image resolution with electron microscopy. The addition of energy dispersive x-ray spectroscopy to electron microscopy can be helpful, but its complexity combined with a very small field of view can be very limiting in its data production. One of the tools most often utilized for this application is Raman microscopy. While it has been proven effective for confirming the interaction between APIs and their delivery vectors, it is often considered to be time consuming and does not provide the level of imaging often desired. However, the quantitative nature of the mapped Raman images are of high value. As such, there is ongoing work to combine Raman imaging with high spatial imaging hyperspectral microscopy, which is discussed later in this document.

Hyperspectral imaging is rapidly developing as an established technique that can provide both high content optical imaging along with simultaneous pixel-level spatial / spectral data within the image. Hyperspectral imaging can be conducted with large scale "macro" images ranging from satellite to earth-scale (where it has its origins) down to micron or nanoscale imaging when utilized in combination with enhanced darkfield microscopy, such as that provided by CytoViva, Inc. https://cytoviva.com/products/microscopy-2/microscopy/

The spectral data provided by hyperspectral imaging is typically produced utilizing broadband illumination. With most drug delivery related applications, oblique angle darkfield microscopy is most often utilized with this broadband illumination for image creation. This oblique angle broadband illumination begins with 65° – 75° angle light produced by the condenser that is focused on the sample. The numerical aperture (NA) of this high angle light can be as high as 1.2 – 1.4 NA, which can be much higher than the NA of a high magnification microscope objective. As such, the direct source light will interact with the sample but will bypass the objective.

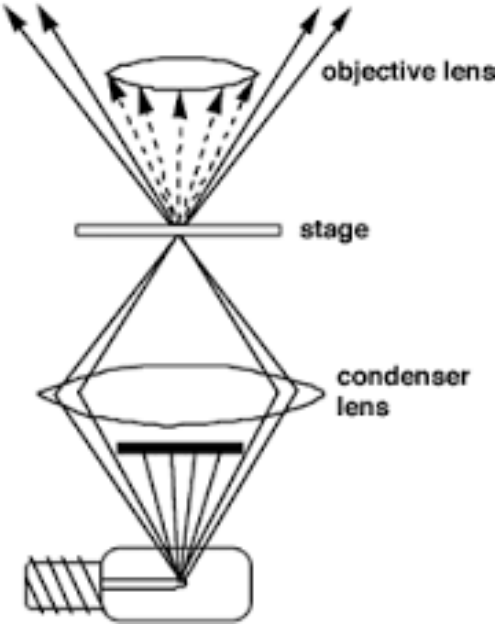

Figure 1: Diagram of Basic Darkfield Light Path.

The oblique angle light that does interact with the sample creates scatter from the sample. More specifically, this is typically Rayleigh or Mie scattering, depending on the size of the objects scattering light. Because this scattered light from the sample enters the objective without any source light interference, it appears bright against a dark background. This is why the technique is referred to as darkfield (see Figure 1). With this technique, the scatter from sample elements down to the nanoscale can often be easily observed directly from the microscope oculars or captured via a standard optical camera. To improve on the performance of darkfield microscopy, CytoViva Inc. has commercialized a patented enhanced darkfield illumination system. This enhanced darkfield illumination serves to further improve the signal-to-noise as much as 10x over standard darkfield. This enhancement has been documented to enable scatter detection from nanoparticles as small as 10 – 20 nm. It is also much easier to align and use than standard darkfield optics. By integrating hyperspectral imaging onto this enhanced darkfield microscope, hyperspectral images of nano to micron-scale drug delivery vectors can be easily captured with this technique, enabling individual sample elements to be identified and then spectrally mapped across the high resolution images.

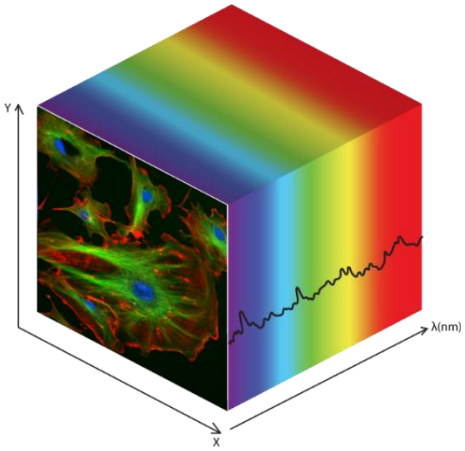

Figure 2: Hyperspectral Data Cube Illustration.

A hyperspectral image is often referred to as a datacube. A single image can contain as many as 500,000 pixels, with pixel size varying from microns down to 100 – 200 nm each, depending on magnification. These hyperspectral datacubes are considered 3D images with the X and Y axis being spatial dimensions and the Z axis containing spectral data (see Figure 2).

Each pixel of the image contains the spectral response in the Vis-NIR spectral range from 400 – 1,000 nm or in the SWIR range from 900 – 1,700 nm. Spectral resolution can be as high as 2 nm in the Vis-NIR range and 5 nm in the SWIR range.

When captured using a microscope, these hyperspectral images are typically created in a line scan or “pushbroom” fashion by moving the sample across the field of view of the microscope and spectrograph via an automated translational microscope stage. Typically these hyperspectral images are created in seconds or minutes, depending on the required exposure and the area of the sample being imaged.

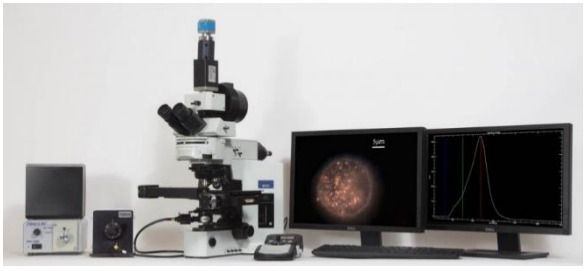

Figure 3: Enhanced Darkfield Hyperspectral Microscope.

Components for the microscopy-based hyperspectral imaging system include a specialized broadband light source, automated translational stage, transmission diffraction grating spectrograph and camera (see example in Figure 3). These components are integrated to work together with highly functional image capture and analysis software. This proprietary software provides the ability to compare spectra within a sample image or among large numbers of samples. It can also build a spectral library of unique sample elements, and using this spectral library these sample elements can then be mapped in subsequent samples.

RESTASIS® API Delivery Vector Example Application

A specific API delivery vector example that was recently tested by the FDA with CytoViva's enhanced darkfield hyperspectral microscopy was a cyclosporine and emulsion sample. This sample is already in the market and is branded as RESTASIS® by Allergan, Inc. A detailed poster demonstrating results from this test can be found here: http://cytoviva.com/wp-content/uploads/2016/07/FDA-AAPS-CytoViva-2015-imaging-poster.pdf

Detailed data from the FDA test focused primarily on the ability to spectrally identify and map the API cyclosporine in the emulsion vector. The FDA also produced data using the system to spectrally identify and map other elements within the sample.

A final data set produced by this FDA study and included in the poster was a demonstration of the benefits of the high resolution imaging produced by the enhanced darkfield microscopy optics from the CytoViva system. These images enabled the ability to conduct counts and size distribution of the <1 um size emulsion vectors. By producing histogram outputs at different concentrations, comparative data was established to test against similar data from dynamic light scattering (DLS) techniques. DLS is often considered a standard for these size distribution measurements. Unlike DLS, measurements from these enhanced darkfield images allow you to "see" and observe the sample from which the data is produced, providing a high level of qualitative confidence in the data production.

Outlined below are some example hyperspectral images and associated data from a RESTASIS® sample. This data was captured separately from the FDA work but is illustrative of the ability to capture hyperspectral images and conduct image analysis as well as identify and spectrally map the API within the emulsion vector.

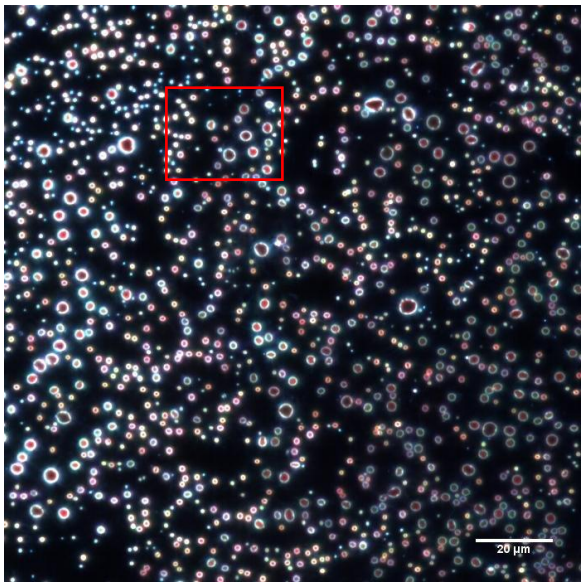

Figure 4: 60x Hyperspectral Image of RESTASIS Sample.

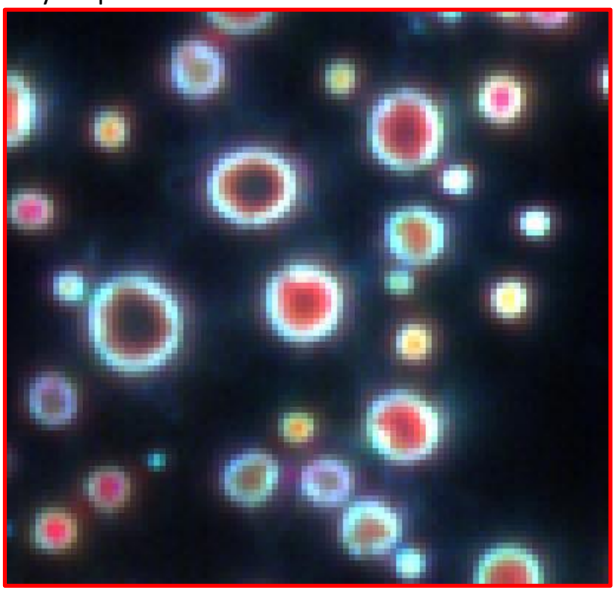

Figure 5: 4X Digital Zoom Hyperspectral Image.

Figure 4 above is a full hyperspectral image of the RESTASIS® sample captured with a line scan hyperspectral imaging system mounted on a CytoViva enhanced darkfield equipped optical microscope. Notice the very bright image of the emulsion against a dark background, which is indicative of the high signal-to-noise image production possible with these enhanced darkfield optics. This hyperspectral image is ~700 x 700 pixel resolution and individual pixels sizes (that can be seen in the digital zoom image of Figure 5) are ~210 nm square. This image was captured at 0.8 seconds exposure setting for each pixel row captured, so the entire image was captured in a matter of minutes. Each pixel of this hyperspectral image contains the complete 400 – 1,000 nm spectral response of that pixel's spatial area. The spectral resolution across the entire wavelength range was ~2nm.

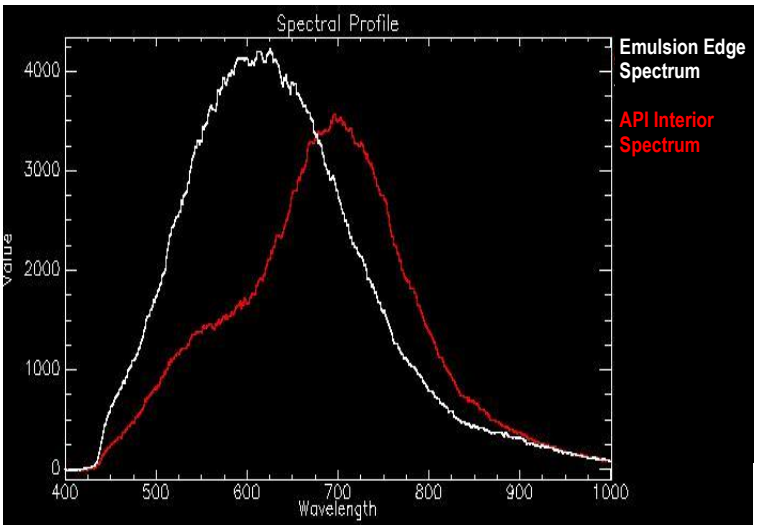

Figure 6: Example Spectrum of the API (red) and Emulsion (white).

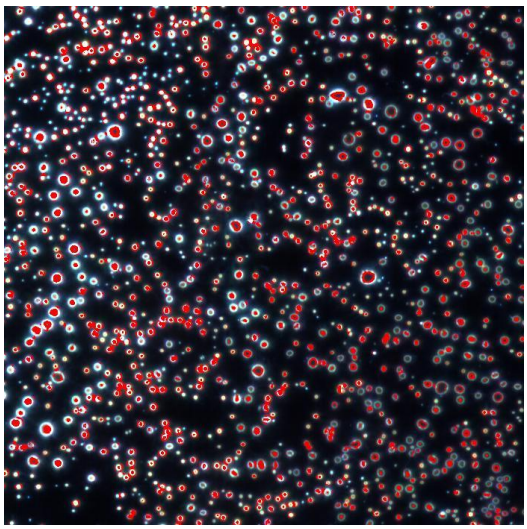

Figure 7: Spectral Mapping of the API Spectra Throughout the Entire Image. Note That Most Mapping of the API Occurs Inside of the Emulsion.

In Figure 6 above, example spectrum from pixel areas of the emulsion chemistry and API are illustrated. There is a distinct red-shift of the API spectrum, which is ~100 nm difference at the peak wavelength versus that of the emulsion edge spectrum. Based on this difference, a spectral library of the API spectrum is created and tested against a negative control and is then used to conduct spectral mapping of all pixels matching the spectrum of the API in the image shown in Figure 7.

The application above is just one of many successful examples of drug delivery vector analysis that has been conducted utilizing CytoViva's enhanced darkfield hyperspectral microscopy system to provide visual spatial and spectral context concerning the interaction between an API and its delivery vector.

Beyond this specific application of measuring the interaction of APIs and delivery vectors, enhanced darkfield hyperspectral microscopy is utilized extensively in many other areas where nano to micron-scale materials need to be quickly visualized, spectrally characterized and mapped in the spatial context of almost any substrate. Examples include biological targets, such as live cells or tissue, or materials-based matrices, such as films, fibers or any substrate enhanced with nano to micron-scale materials.

Finally, CytoViva's enhanced darkfield hyperspectral imaging has recently been integrated with full-featured Raman imaging on the same microscope platform. This enables cross-correlation between the two techniques of an identical area of a sample. More information regarding this integration of fully featured Raman and broadband hyperspectral microscopy will be outlined in a separate white paper.

To learn more about enhanced darkfield hyperspectral microscopy and how it can improve the development of APIs and nano to micron-scale delivery vectors or provide an effective quality control tool for existing formulations, you can contact CytoViva at info@cytoviva.com or 1-888-737-3130.