Inform is a series of white papers designed to provide advice on material characterization issues

# Morphologically directed chemical identification – Coupling particle characterization by morphological imaging with Raman spectroscopy

Malvern

# Introduction

The ability to chemically characterize dispersed particles is important in a variety of application areas, from orally inhaled and nasal drug products (OINDP) and foreign/contaminant ID to bacteria identification. Dispersed particles can be referred to as “sparse samples,” where the sample of interest is spread over a large area, the majority of which is either empty or of no analytical interest.

Automated image analysis-based particle characterization provides an accurate and reproducible way to characterize the size, shape, and location of dispersed particles. But alone, it cannot deliver chemical ID. It does, however, provide an ideal environment to obtain this additional information. As the (x,y) coordinates of all characterized particles are stored, it is easy to return to those of interest and query their chemical ID using an additional chemical analysis tool.

For non-destructive chemical ID, Raman microspectroscopy has proven to be a powerful, adaptable and easy to use approach, with high molecular specificity. Given its attributes, Raman microscopy has been utilized as a workhorse analytical technique for decades. One of the benefits of Raman microspectroscopy, in comparison to the Mid-IR and Near-IR, is that very small samples can be characterized, as laser spot sizes under 5µm are routinely available. On the other hand, having such a small probe size can make it difficult and time consuming to sample large areas. Before the introduction in 2005 of a spectroscopic grade electron multiplying CCD (EMCCD), it could take hours or even days to completely map the area of a typical pharmaceutical tablet. The EMCCD has greatly improved data collection time for standard Raman mapping systems, and is well suited for solid (continuous) samples: tablets, polymers, etc.

For sparse samples though, where the time penalty is actually in identifying and locating the particles of interest, not in collecting their Raman spectra, simply turning up the speed with which Raman data can be collected provides less of a benefit. There are two approaches available to chemically characterize sparse samples, and they have not changed in 40 or more years:

1) Hunt and peck target selection
2) Brute force data collection over entire sample area, regardless of sample dispersion

Malvern Instruments have coupled automated image analysis-based particle characterization with Raman spectroscopy (chemical ID), providing (for the first time in decades) a new tool to characterize the chemical ID of sparse samples.

## Sparse Sample Characterization – time saving workflow

This approach is based on a novel paradigm in which results from a rapid visual/morphological analysis are used to target particles of interest for subsequent chemical ID. The steps are as follows:

- SOP-driven visual image capture of a sparse sample
- Morphological analysis and classification of particles
- Raman spectral acquisition of statistically significant number of representative particles
- Chemical ID and library classification of selected particles

This new paradigm offers significant advantages over the old way of analyzing sparse samples.

### "Morphologically guided" target selection vs "hunt and peck"

Because the (x,y) coordinates of all particles are recorded and saved this approach replaces the time consuming "hunt and peck" method of locating particles. Specific particles of interest can be reliably targeted for Raman spectroscopy, and returned to again and again. The task of identifying distinct morphological classes of particles is significantly simplified, using the tools provided by automated image analysis-based particle characterization instruments such as the Malvern Morphologi® G3. In setting up the data collection SOP, background contribution (SERS sample preparation contamination, dust, cellular components, morphologically irrelevant particles, etc.) can be excluded from the data record, greatly simplifying the process of targeting only relevant particles for further chemical ID analysis.

### Raman spectra of relevant particles vs "brute force" over entire area

Even with the use of EMCCDs for rapid data collection, the acquisition of Raman spectra is still considerably slower than acquiring visible images. Automated image analysis-based particle characterization provides the

means to intelligently limit the number of Raman spectra that subsequently need to be acquired. As collecting Raman spectra will always be the slower step, replacing brute force data collection over the entire sample area (99.7% of which may be background, or analytically irrelevant particles), with acquisition of the minimum number of Raman spectra needed to draw a relevant analytical conclusion can provide tremendous time savings.

## Application examples

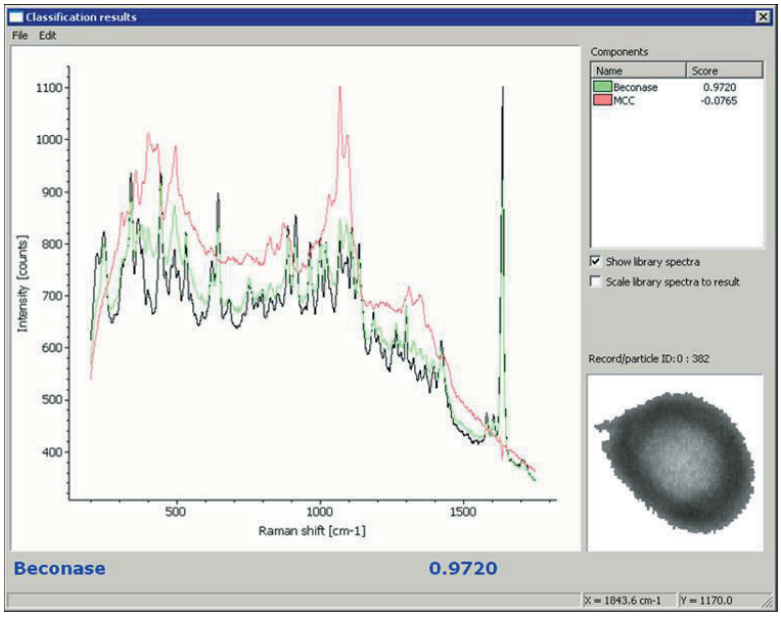



**Figure 1** – Representative Raman spectrum from particle (black trace) overlayed with library spectra from pure API and excipient (green and red traces, respectively). Best library match and corresponding score are shown in blue text below spectra. The particle thumbnail and particle ID measured by the Malvern Morphologi® G3 are included.

### Particle size distribution of API in nasal spray suspension

The performance of nasal sprays, and other OINDPs (orally inhaled and nasal drug products) is determined in part by the particle size distribution (PSD) of the active pharmaceutical ingredient (API). The ability to obtain

this information is currently limited by the difficulty in unequivocally distinguishing between the API and excipients with available analytical techniques. A morphologically guided Raman spectroscopic approach, by providing an unequivocal chemical ID, provides a potentially validatible method to non-destructively determine this information.

Basing an initial classification between API and excipient on particle brightness, the API specific PSD of 835 particles was subsequently determined. Raman chemical ID was employed for unequivocal determination of a subset of particles that were not clearly distinguished as either API or excipient using a morphological characterization only.

**Table 1**- API particle sizes by target range

| CE diam (µm) | # of API Particles | % total |
|---|---|---|
| >10 | 4 | 0.5 |
| 7-10 | 104 | 9.6 |
| 1-7 | 727 | 89.9 |

The overall API only PSD results are presented in table 2, showing both number and volume based distributions. The area occupied by the API particles is ~0.3% of the total sampled.

**Table 2** – API particle size distribution – number and volume based

| | $D_{10}$(µm) | $D_{50}$(µm) | $D_{90}$(µm) |
|---|---|---|---|
| CE diameter - number | 2.15 | 4.44 | 6.95 |
| CE diameter - volume | 4.18 | 6.31 | 8.87 |

## Molecular Pathology

The automatic segmentation of samples based on morphological features is a powerful tool in the field of pathology, where size and shape analysis form the basis of this field of study. The ability to generate molecular spectroscopic information, with the hope of seeing indications of disease state before gross morphology has been affected, is a long standing goal of the new relatively new field of molecular pathology. In a simple example presented below, the nuclei of squamous cells are identified, segmented, and the (x,y) location recorded for each.

Figure 2A

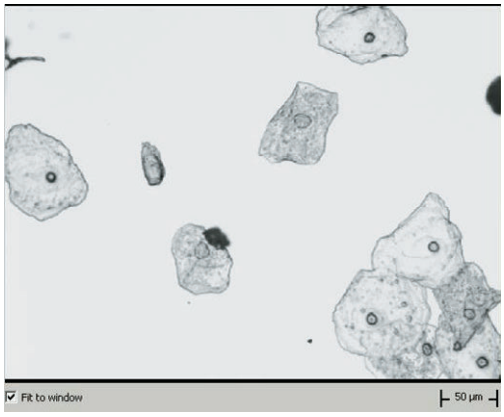



Figure 2B

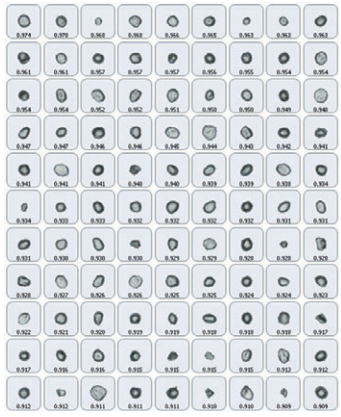


**Figure 2** – Single visible image plane (2A) from morphological data set showing squamous cells. Morphologically segmented cell nuclei (2B) isolated from cell images.

Figure 2A shows a visible image of a collection of squamous cells, with the cell nuclei clearly evident as localized regions within the cells. A 3.5 x 4.0 mm region of the sample was analyzed using the Malvern Morphologi® G3. Across this sample area, 471 cell nuclei with a mean area of 63µm$^2$ were identified. The percentage of the sample area occupied by these cell nuclei is 0.2% of the entire area. Figure 2b shows ~100 of these thumbnails to highlight the capabilities of the visible image segmentation.

Figure 3B

Figure 3A

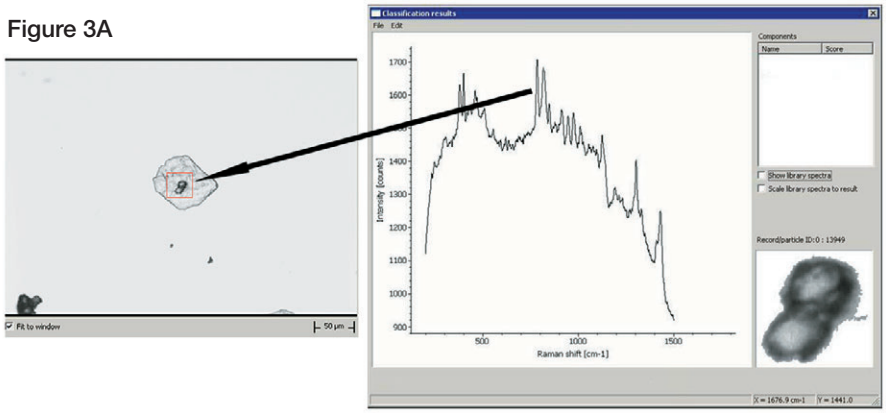



**Figure 3** – Visible image plane of a single squamous cell (3A) with box showing the location of the nucleus subsequently targeted for Raman spectroscopy. Raman spectrum and particle thumbnail (3B) of the nuclei.

Once the nuclei are segmented, and all (x,y) coordinates determined, it is possible to obtain the Raman micro-spectra of individual nuclei. Figure 3 shows a visible image of a single cell, along with the corresponding Raman spectrum and visible thumbnail of the optically isolated nuclei.

The cell was apparently in one of the final phases of mitosis, hence what appears to be a double nucleus, and appearance of a strong Raman band at 785cm$^{-1}$[Ref 1]. Figure 4 shows the Raman spectra of a cell nucleus that is not actively dividing. The spectral differences between these two cells highlight the type of molecular information that may be obtained from Raman spectroscopy of cells or tissue.

Figure 4A

Figure 4B

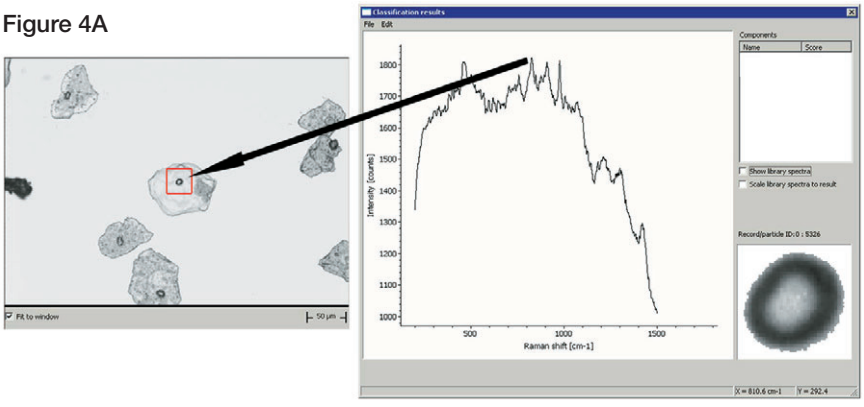



**Figure 4** – Visible image plane of a squamous cell (4A) with box showing the location of the nucleus subsequently targeted for Raman spectroscopy. Raman spectrum and particle thumbnail (4B) of the nucleus.

## Foreign Contaminant ID

Finding and identifying contaminant particles amongst thousands of "expected" particles can be a daunting task. Automated image analysis-based particle characterization is a powerful tool for rapid classification of morphologically unique contaminant particles. With the addition of Raman spectroscopy, it is possible to establish the chemical ID of these morphological outliers. The example shown here is a sample of polymer beads with highly regular size and shape, with some morphologically unique contaminants. Sorted by aspect ratio, the first five particle thumbnails in Figure 5 are obviously quite different from the spherical particles that make up the bulk of the sample.

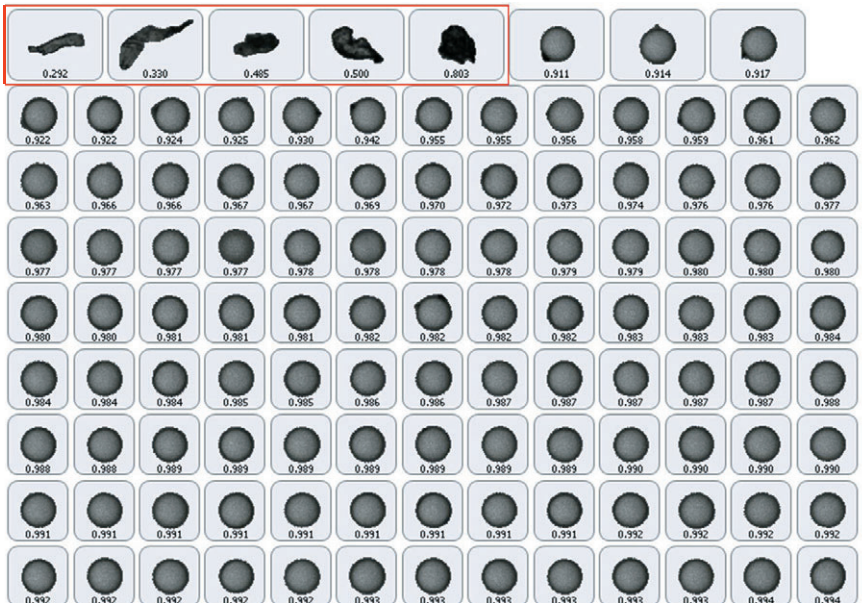



**Figure 5** – Typical polymer microspheres are the dominant particle type in this sample. Low aspect ratio contaminant particles appear amongst the spheres, highlighted by the red box at the top of the image.

**Table 3** - Shows the chemical ID and corresponding score for the morphological outliers in this data set.

| Particle ID | Aspect Ratio | Chemical ID | Score |
|---|---|---|---|
| 494 | 0.292 | Cellulose | 0.9591 |
| 1951 | 0.330 | Cellulose | 0.9742 |
| 2068 | 0.485 | Cellulose | 0.9606 |
| 2069 | 0.500 | Cellulose | 0.9970 |
| 1670 | 0.803 | Graphite | 0.9650 |

The results displayed in Table 3 show that the bulk of the contaminant particles belong to a similar chemical class, whereas one has an altogether unique Raman spectrum.

The spectrum shown in figure a shown in Figure 6 was taken from the polymer microsphere, the majority particle in the sample. As expected, there is a strong correlation between the particle spectrum and the corresponding library component.

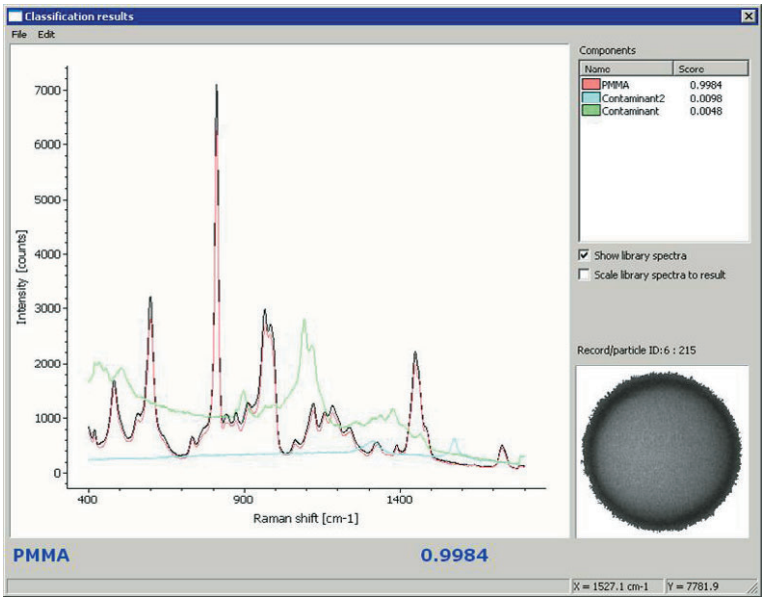



**Figure 6** – Raman spectrum, library classification hit, and corresponding score for sample majority particle. The particle spectrum is shown in black, and the library spectra are in red (polymer microsphere), green (cellulosic contaminant) and blue (contaminant 2).

Figure 7A shows the spectrum and library fit of one of the cellulose particles, and 7B show the spectrum of the second contaminant, identified as a graphite particle [Ref 2].

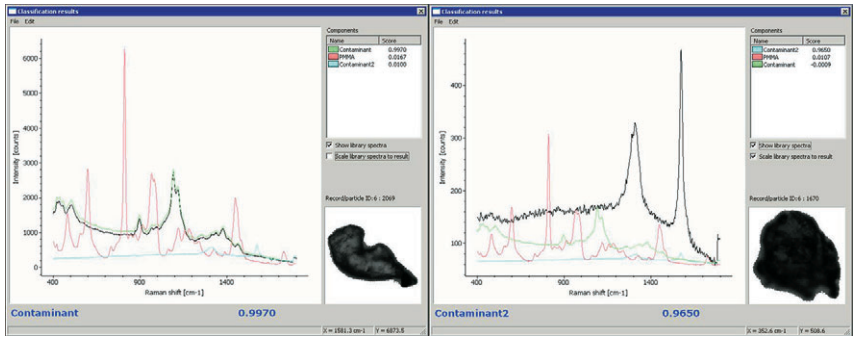



**Figure 7** – Raman spectrum, library classification hit, and corresponding scores for two types of contaminant particles. Particle spectra are shown in black, and the expected polymer library spectrum is in red, and the contaminant library spectra are shown in green and blue.

## Conclusions

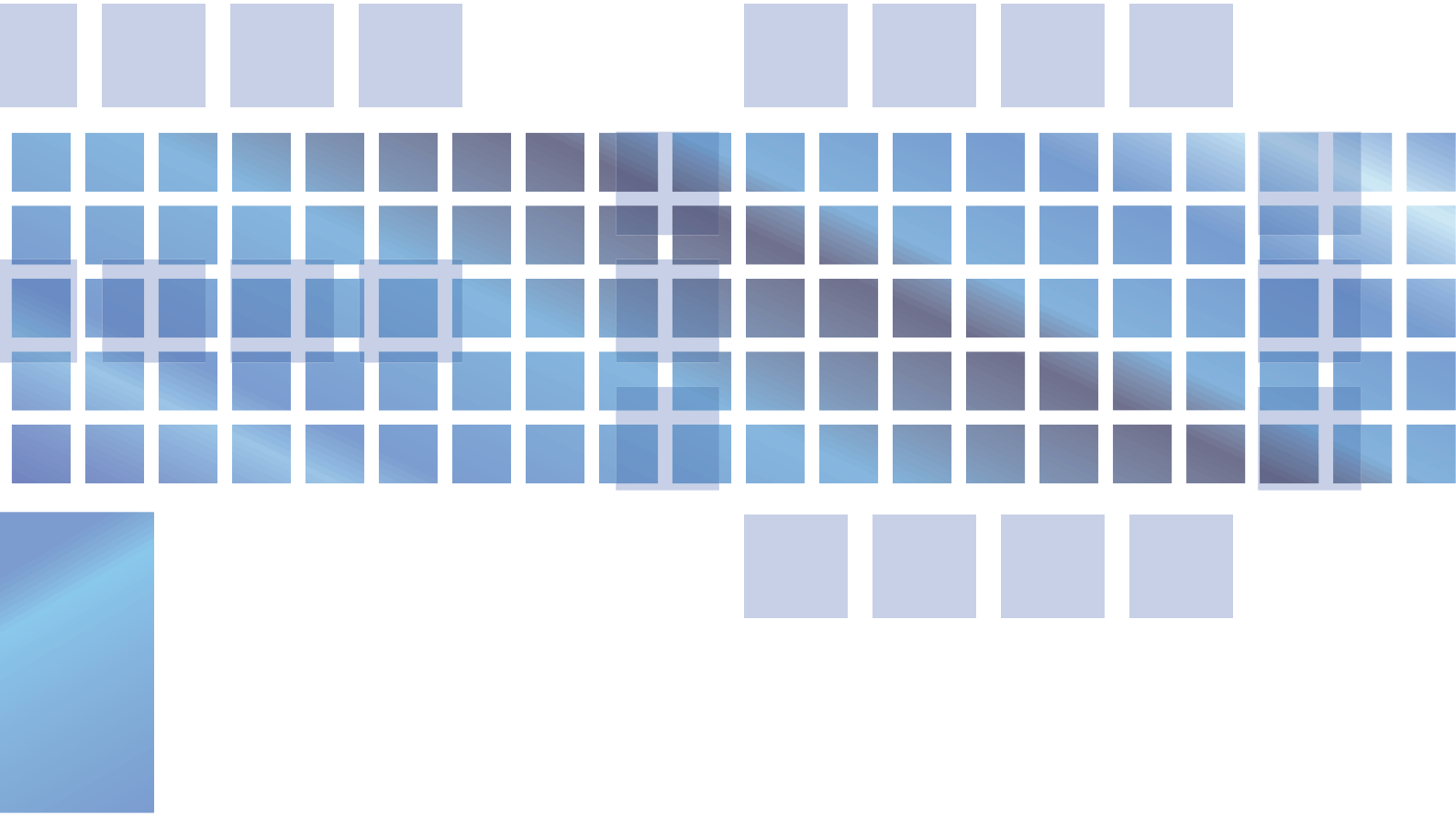
There is a growing requirement to obtain physical and chemical characteristics of materials simultaneously. Advances in chemical imaging and mapping techniques such as Raman, Near-infrared and Mid-infrared have greatly expanded the tools available to explore contiguous solid samples such as pharmaceutical tablets, solid materials composites, polymers, etc. However, the application of traditional mapping and chemical imaging to so-called sparse samples (or dispersed samples), is time consuming and inefficient. For samples in which the material of interest covers as little as 0.5% of the overall sampling area, the traditional approach may be simply unworkable.

Automated image analysis-based particle characterization provides a rapid, accurate and reproducible way to morphologically characterize sparse samples, recording relevant physical characteristics of the particles, and simultaneously storing sample locations so that selected ones can be re-visited to obtain chemical ID information. This problem oriented approach is fast, statistically directed, non-subjective, minimizes redundancy and reduces time taken on the rate-limiting step – generating spectroscopic chemical IDs of the sample.

### References

[1] Matthaus, C., Boydston-White, S., Miljkovic, M., Romeo, M., and Diem, M., "Raman and Infrared Microspectral Imaging of Mitotic Cells," Applied Spectroscopy, 60, 1-8, (2006).

[2] Larsen, K. L. and Nielsee, O. F., "Micro-Raman spectroscopic investigations of graphite in the carbonaceous meteorites Allende, Axtell and Murchison," Journal of Raman Spectroscopy, 37, 217-222, (2006).



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