Portrait of a Dying Cell

Confocal Raman microscopy is a new, noninvasive way of obtaining morphological and chemical information about cells that may lead to better cancer research

Katherine Lau, Frederick Coffman and Bhagavathi Ramamurthy

Autophagy is normally a good thing in a healthy host – it's a self-degradative process important for homeostasis and stress survival. Cells begin by sequestering intracellular components like proteins, lipids, micronuclei and damaged organelles in double-membraned structures known as autophagosomes; then, the autophagosomes fuse with lysosomes in the cell, allowing lysozyme to digest those components into their basic units. Autophagy recycles the basic units of intracellular components to aid in cell

At a Glance

- *Autophagy normally helps healthy cells survive – but in cancer, it can aid in the survival of malignant cells*
- *An essential part of understanding cell death processes in cancer is understanding the nature of autophagy and apoptosis, and their effects on the cell*
- *Raman spectroscopic imaging measures molecular light scattering to provide a rich variety of morphological and chemical information*
- *By applying Raman imaging to autophagic and apoptotic cells, we were able to observe their features in a label-free, non-contact, nondestructive way*

survival, and helps to suppress cancer and promote genome stability by removing damaged DNA, protein and organelles. But what happens when cancer is already present? In that case, the ideal scenario is to remove the tumor with minimal cytotoxicity imposed on the surrounding healthy cells – for instance, by inducing apoptosis, a kind of cell death that minimizes cytotoxicity to surrounding healthy cells.

Malignant tumor tissue is a stressful environment for several reasons: rapid proliferation makes high metabolic demands on the cells, the tumor interior is hypoxic, and cells far from the vasculature have little access to nutrients. This stress can induce autophagy – but in this case, it's bad news; autophagy can aid the survival of malignant cells by exerting cytoprotective effects and antagonizing chemotherapy-induced damage (1). That's why many successful cancer treatment regimens involve combining chemotherapy drugs with autophagy-inhibiting agents. But those are just the first steps toward addressing the complex relationship between autophagy and cancer, and we need to learn a lot more about the interplay between them (2) – so it comes as no surprise that autophagy is a hot topic in cancer research.

Raman explained

Raman spectroscopy is a technique that provides chemical information by measuring light scattering. How does it work? Molecules vibrate at a particular set of frequencies. When light interacts with a molecule, it's usually scattered without any change in energy – called "elastic scattering." But one in a million photons scatters with an energy change, and when that happens, it's known as "inelastic" or "Raman scattering." This change in energy is specific to the individual molecules, so by measuring it, we can identify the materials in a sample. And to generate Raman images, we take the technique one

step further – collecting spectra for an array of points on or in a sample and plotting pictures based on the chemical information in those spectra.

Raman images offer numerous opportunities for spatial analysis. We can use them to determine the presence of a particular material in a sample, detect unknown materials, examine the variation in a sample, or analyze the size, distribution or relative concentration of any given material. The images are powerful communication tools, too; not only can they clearly display complex visual information, but that information can be

> *"Not only can [the images] clearly display complex visual information, but that information can be used for quantitative measurements as well."*

used for quantitative measurements as well.

We wanted to explore the possibilities of Raman imaging in understanding autophagy, apoptosis and cancer. There are several advantages to examining tissues with Raman imaging. For instance, it requires minimal sample preparation, you can use it on live cells to obtain both chemical and spatial information simultaneously. It reveals the complete chemical profile of the sample, meaning that there's no need to select specific targets.
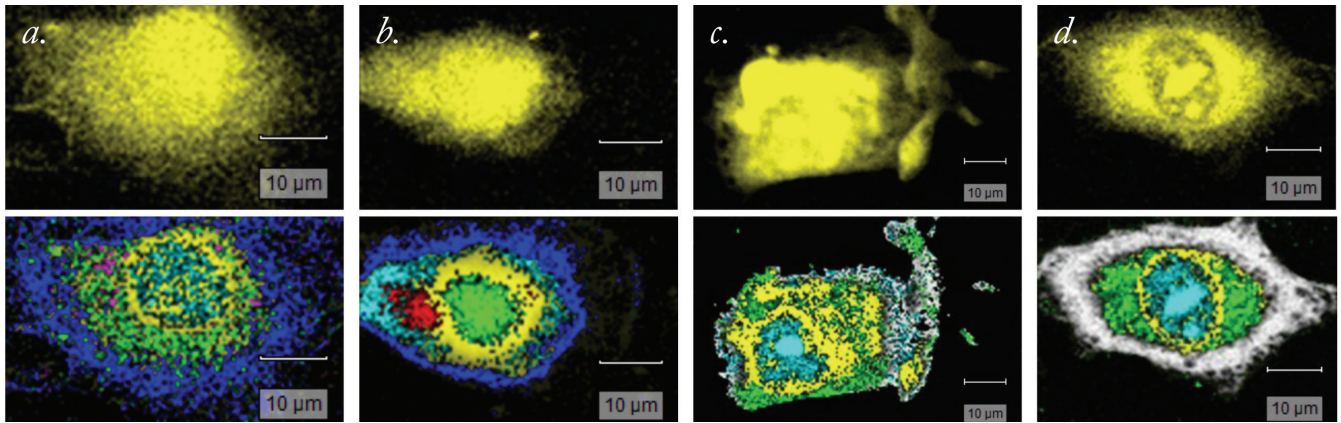
Figure 1. Phenylalanine intensity (top) and principal component analysis (bottom) images for a. normal, b. autophagic, c. early (4-hour) and d. late (24-hour) apoptotic MG-63 cells. Cellular components are shown in different false colors (cyan: nucleic acids, green: membrane-rich organelles, magenta: lipid droplets, blue: membranous areas).

It's a label-free technique, which reduces the potential for artifacts. And because it's a non-contact, nondestructive method, it's possible to analyze samples multiple times – allowing the use of downstream parallel techniques to generate correlative and complementary information. Because of its many benefits, we hoped to use this relatively unknown technique to examine the morphological and chemical changes associated with autophagy and apoptosis, revealing new information on the life and death of a cancer cell.

To represent our Raman data visually, we use a technique called principal component analysis (PCA). We established the outlines of the cells and then indicated each subcellular component – nucleus, nucleoli, membrane-rich organelles and lipid vesicles – using a different color.

Autophagy or apoptosis?

In autophagic cells, we were able to spot an increased number of vesicles in the membranous areas and identify them as putative autophagosomes. Raman imaging also allowed us to examine the contents of those vesicles without extraction or staining. We were able to see autophagosomes whose color was the same as that of the cytoplasm (due to containing mainly proteins and lipids) and those that were the same color as the nuclei or nucleoli (due to the presence of nucleic acids) – results we confirmed by analyzing the original Raman spectra to verify the vesicles' contents. We also observed aberrant nucleic acid distribution in some cells, where the DNA was scattered or pushed to one side of the nucleus.

Dysregulated DNA, as we saw in our autophagic cells, eventually leads to cell death – but how does this differ from apoptosis? To answer that question, we looked at early and late apoptotic cells. Our images revealed membrane blebbing in the early apoptotic cells, but not in the 24-hour apoptotic cells. In early apoptotic cells, we also observed the fragmentation of DNA and its localization to the cell membrane. There, we could see its being packaged in readiness for expulsion as apoptotic bodies – a neat way of disposing of unwanted cellular contents! Close to the cell membrane, we saw a ceramide lipid fraction consistent with our understanding of *de novo* ceramide formation as part of the early apoptotic process. In the late apoptotic cells, we not only saw DNA fragmentation and packaging into apoptotic bodies, but we were also able to spot those apoptotic bodies outside the cell after expulsion. The location of nucleic acids and ceramide to the cell periphery are features unique to apoptosis and aren't detected in normal or autophagic cells.

The differences between autophagic and apoptotic cells are very clear – and we were able to observe them without labeling, tissue destruction, or extensive sample preparation. That makes Raman imaging a very powerful tool for cell analysis, particularly when combined with statistical evaluation methods. It can reveal a multitude of morphological and chemical information, and in our study, it has helped us to gain a better understanding of autophagy, apoptosis and cancer – hopefully, to eventually lead us to better strategies for cancer prevention and treatment.

Katherine Lau is a Raman applications scientist specializing in the life sciences at Renishaw plc, UK. Frederick Coffman and Bhagavathi Ramamurthi are affiliated with the Center for Biophysical Pathology at Rutgers New Jersey Medical School, USA.

References
1. *JW Lee et al., PLoS One, 8, e83611 (2013). PMID: 24358301.*
2. *DA Gewirtz, Cancer Res, 74, 647–651 (2014). PMID: 24459182.*