Label-free detection of anticancer drug paclitaxel in living cells by confocal Raman microscopy


Citation: Applied Physics Letters 102, 113701 (2013); doi: 10.1063/1.4794871
View online: http://dx.doi.org/10.1063/1.4794871
View Table of Contents: http://scitation.aip.org/content/aip/journal/apl/102/11?ver=pdfcov
Published by the AIP Publishing
Label-free detection of anticancer drug paclitaxel in living cells by confocal Raman microscopy

H. Salehi,¹ L. Derely,¹ A.-G. Vegh,¹,² J.-C. Durand,¹ C. Gergely,³,⁴ C. Larroque,⁵ M.-A. Fauroux,¹ and F. J. G. Cuisinier¹

¹Laboratoire Biologie-Santé Nanosciences, EA 4203, UFR Odontologie, Université Montpellier 1, Montpellier 34193, France
²Institute of Biophysics, Biological Research Centre of the Hungarian Academy of Sciences, 6726 Szeged, Hungary
³Université Montpellier 2, Laboratoire Charles Coulomb UMR 5221, F-34095 Montpellier, France
⁴CNRS, Laboratoire Charles Coulomb UMR 5221, F-34095 Montpellier, France
⁵IRCM/INSERM896, Centre Régional de Lutte contre le Cancer Val d’Aurelle - Paul Lamarque, Université Montpellier 1, 34298 Montpellier, France

(Received 18 June 2012; accepted 18 December 2012; published online 18 March 2013)

Confocal Raman microscopy, a non-invasive, label-free, and high spatial resolution imaging technique is employed to trace the anticancer drug paclitaxel in living Michigan Cancer Foundation-7 (MCF-7) cells. The Raman images were treated by K-mean cluster analysis to detect the drug in cells. Distribution of paclitaxel in cells is verified by calculating the correlation coefficient between the reference spectrum of the drug and the whole Raman image spectra. A time dependent gradual diffusion of paclitaxel all over the cell is observed suggesting a complementary picture of the pharmacological action of this drug based on rapid binding of free tubulin to crystallized paclitaxel. © 2013 American Institute of Physics. [http://dx.doi.org/10.1063/1.4794871]
37°C and 5% CO₂. Cells were cultivated onto polished CaF₂ substrates (Crystran Ltd, Dorset, UK). CaF₂ reveals a Raman peak at 320 cm⁻¹ and hence has no interference with major cell’s Raman lines. After 24 h the cells adhered on CaF₂ substrate. Paclitaxel (Taxol, Teva Pharmaceutical Ind., Tel Aviv, Israel) was then added to the culture medium: cells were incubated for three, six, and nine hours in DMEM containing paclitaxel and then washed three times with PBS (Phosphate Buffer Saline) (Invitrogen, Grand Island, USA), enabling the time tracing of paclitaxel in the cell. The CaF₂ substrates with adhered cells were transferred into Petri dishes containing 5 ml PBS. PBS was selected because it does not create fluorescence as DMEM. Clinical amount of paclitaxel in each 5 ml PBS. PBS was selected because it does not create fluorescence as DMEM. Clinical amount of paclitaxel (22500 spectra for one image, each spectrum corresponding to one spectrum region) in specific spectrum regions (2800–3000 cm⁻¹) corresponding to the CH band. The Witec Project software provides a Sum Filter. By choosing the specific spectra region in Sum Filter, we could extract an image based on integrated Raman intensities of a specific region. Bright yellow hues indicate highest intensities and dark hues the lowest integrated intensities of the chosen region, respectively. In (c), bright yellow hues corresponding to high intensities of CH stretching band, belong inside the cell and dark hues the lowest integrated intensities of CH stretching band belong to PBS with no CH band. (b) Shows the results of K-mean cluster analysis (KMCA) of the data set for six clusters. K-means clustering partitions data into k mutually exclusive clusters. K-means treats each observation in the data set as an object having a location in space. It finds a partition in which objects within each cluster are as close to each other as possible, and as far from objects in other clusters as possible. K-means uses an iterative algorithm that minimizes the sum of distances from each object to its cluster centroid, over all clusters. This algorithm moves objects between clusters until the sum cannot be decreased further. The result is a set of clusters that are as compact and well-separated as possible.

The distance between each spectral vectors $S^a_i$ and cluster centroids $m_k$ has to be minimized for each group of clusters. KMCA was realized using the Witec Project Plus (Ulm, Germany) software. K-mean clustering was performed in the 500–1800 cm⁻¹ and 2600–3200 cm⁻¹ spectral domains. These regions were chosen because they contain sufficient spectral information of cell components and provide the best clustering results. Figure 1(c) depicts a Raman image constructed via KMCA revealing clusters matching nucleus (blue), nucleolus (pink), cytoplasm (orange), membrane (light green), endoplasmic reticulum (dark green), as well as different components in cytoplasm.

In Fig. 2 we present a typical Raman spectrum of a cell (a), the Raman spectrum of the paclitaxel solution prepared from paclitaxel (Teva Ind, Tel Aviv, Israel) (b) and the Raman spectrum of paclitaxel (Sigma-Aldrich, France 95% 

FIG. 1. (a) Bright-field microscopic image of MCF-7 cell in buffer solution, 60× objective. (b) Integrated Raman intensities in the 2800–3000 cm⁻¹ region of the cell shown in (a) collected at a dwell time of 0.5 s/point and a point spacing of around 300 nm. Bright yellow hues indicate the highest and dark hues the lowest integrated C-H stretching intensities, respectively. (c) Raman image of various cell components obtained via KMCA clusters based on data shown in (b) and corresponding to the Raman spectra region of 500–1800 cm⁻¹ and 2600–3200 cm⁻¹.

FIG. 2. Predominant bands in the Raman spectra of (a) cytoplasm and endoplasmic reticulum; (b) clinical paclitaxel solution from Teva Ind.; (c) pure paclitaxel powder from Sigma-Aldrich.
purity) in powder (c), respectively. The Raman vibrations obtained for the paclitaxel used in our research are very similar to the spectra previously reported for functional derivatives of the paclitaxel.\(^6\) Comparison between Figs. 2(a) and 2(b) shows that most of Raman peaks appearing in the spectra of cells overlap with the vibrational peaks of the paclitaxel. Moreover, the previously used peak by Ling et al.\(^\text{22}\) at 1002 \(\text{cm}^{-1}\) which is due to \(\text{sp}^3\) hybridized carbon-carbon (C-C) vibration in the paclitaxel occurs also in the cell’s spectra. Therefore in our work the peak at 1740 \(\text{cm}^{-1}\) (the \(\text{C} = \text{O}\) stretching band marked in the red spectrum of Fig. 2(b)) was used to monitor paclitaxel in cells.

Cells cultured for 24 h in DMEM and then incubated for 3 h in a solution of DMEM containing paclitaxel (9.7 \(\mu\text{M}\)) were rinsed with PBS and transferred under the confocal Raman microscope. Fig. 3(a) shows the results of KMCA analysis of MCF-7 cell’s images, when the cells were incubated with paclitaxel for three hours. The images reveal seven clusters, corresponding to different parts of the cell and the background signal of the PBS buffer and the CaF2 substrate. The spectral contrast between cellular components is relatively small as they are very similar in terms of Raman vibrations. But precisely it is possible to reveal very small differences between the various parts of the cell.\(^\text{15,21}\) The average spectra of nucleus (orange) nucleolus (dark green), cytoplasm (pink), membrane (brown), endoplasmic reticulum (light blue), and paclitaxel (red) calculated by KMCA are plotted in Fig. 3(b). The peak positions, band shapes, and intensities are very alike, considering that cell basic components are quite similar; however, there are noticeable differences in specific spectral regions. For example the symmetric uracil or thymine ring breathing mode with a frequency at 785 \(\text{cm}^{-1}\) is observed quite strongly for the nuclear and cytoplasmic regions but much less for the mitochondria.\(^\text{9}\) The number of spectra contributing to the average spectra of each cluster is in order of thousands. Consequently the observed signal to noise ratio for an average spectrum is very good. Fig. 3(b) evidences that the (red) spectrum of paclitaxel is very similar to the cell components’ spectra; therefore, the drug was monitored after clustering an average spectra containing the specific peak of paclitaxel at 1740 \(\text{cm}^{-1}\) (\(\text{C} = \text{O}\) stretching in Fig. 2). As this band has a very low intensity compared to the whole spectra, clustering has to be performed several times. Finally by comparing the cluster of average spectrum with the drug reference spectrum, we are able to identify the cluster corresponding to paclitaxel.

As the paclitaxel (red cluster in Fig. 3(a)) cluster contains several single spectra, each of them was compared to the reference paclitaxel spectrum (Fig. 2) and showed a very good match. Beside the K-mean cluster, a spectral correlation matrix was calculated,\(^\text{21}\) to find the most similar spectrum to the reference spectrum of paclitaxel. To quantify the similarity, as a “distance,” the Pearson’s correlation coefficient was calculated between the two spectra. The best correlation is obtained for the paclitaxel in cell (red spectrum); the region with no correlation to paclitaxel is due to the PBS buffer (blue spectrum).

FIG. 3. (a) Seven cluster Raman map of MCF7 cells. (b) Average spectra corresponding to clusters in (a) (same colors as in (a)): the average spectra of nucleus (orange) nucleolus (dark green), cytoplasm (pink), membrane (brown), endoplasmic reticulum (light blue), PBS buffer (light green) and paclitaxel (red). (c) Correlation map of the same image. (d) The correlation coefficient between the whole spectra and the one of paclitaxel taken as a reference (black spectrum). The best correlation is obtained for the paclitaxel in cell (red spectrum); the region with no correlation to paclitaxel is due to the PBS buffer (blue spectrum).

FIG. 4. Raman images of MCF7 cells incubated in paclitaxel. Integrated Raman intensities in the 2800–3000 \(\text{cm}^{-1}\) region of the cell, marking paclitaxel as red spot. (a) 3 h; (b) 6 h; (c) 9 h incubation of cells in culture medium containing paclitaxel.
coefficient was calculated for each pair of spectra, given by the following formula:

\[ r = \frac{\sum_{i=1}^{N}(x_i - \bar{X})(y_i - \bar{Y})}{\sqrt{\sum_{i=1}^{N}(x_i - \bar{X})^2 \sum_{i=1}^{N}(y_i - \bar{Y})^2}}, \]

where \( N \) is the number of points within the spectrum, \( x_i \) and \( y_i \) are the individual points, and \( X \) and \( Y \) are the mean value of each spectrum. The value of \( r \) can vary between \(-1\) and \(1\); thus, it can be expressed as a percentage ranging from \(-100\%\), no correlation, to \(100\\%\), the perfect match. From these values, a pseudo color map can be constructed, reflecting the quantified similarities. All calculations were performed with a homemade code written in MatLab (Math Works, Inc, Massachusetts, USA). The correlation coefficient map of the same image is depicted in Fig. 3(c). Paclitaxel spectrum as a reference was compared to all spectra of the same image, resulting in a correlation map shown in Fig. 3(c). The numbers of 100 and \(-100\) in the color bar stand for the best and worst correlation (expressed in %) between all cell spectra and paclitaxel spectrum. We observe that the lowest correlation (\(-14.5\%\), turquoise color) corresponds to PBS and the best correlation indicated by the red color corresponds to paclitaxel. In the correlation coefficient map the same position of paclitaxel as in the cluster analysis was obtained, confirming our KMCA results. In Fig. 3(d) we present the (black) spectrum of paclitaxel and the corresponding, best correlation (47.2\%, red spectrum) as well as the worst correlation (\(-14.5\%\), blue spectrum) obtained for the phosphate buffer region, respectively. Our analyses demonstrate clearly the capability of KMCA to distinguish paclitaxel based on the molecule’s Raman vibrations. Relying on the results of KMCA the next step was tracing paclitaxel versus different incubation times to monitor uptake pathways of the drug in cells. Figure 4 presents the Raman images of MCF-7 cells after different incubation times in paclitaxel solution. The paclitaxel cluster (red) obtained by KMCA was superposed on the Raman image of the cell (integrated Raman intensities in the 2800–3000 cm\(^{-1}\) region, using sum filter) using Image J software (National Institutes of Health, Maryland, USA). Fig. 4(a) shows cells after three hours of incubation in paclitaxel: the drug had passed the cell membrane and entered the cytoplasm.

The same protocol was used to obtain Figs. 4(b) and 4(c), presenting MCF7 cells incubated six and nine hours in paclitaxel containing culture medium, respectively. Images clearly reveal an increase of paclitaxel uptake by cells versus time and its distribution in numerous parts of the cells. The drug is gradually diffusing into the cytoplasm, but it does not enter cell nucleus. The ratio of the number of pixels belonging to paclitaxel cluster over the number of pixels corresponding to the cell was calculated and represented by bars in Fig. 5. This ratio representing the amount of paclitaxel in the cells increases with the incubation time of cells with the drug. The number of cells containing paclitaxel is also increasing with time (Table I). After three hours of culture with paclitaxel 85\% of cells show an uptake of the drug, while after nine hours 90\% were involved. Para-nitrophenyl phosphate (pNPP) and Trypan Blue viability tests show that after incubation for three and six hours in paclitaxel, 80\% of cells were alive and at nine hours incubation, 60\% survived. In other words interpretation of Raman images has to be done considering that for three and six hours of culture with paclitaxel, 80\% of cells were alive, thus still on the CaF\(_2\) substrate among these 85\% and 80\% uptake paclitaxel, respectively. After nine hours of culture with paclitaxel only 60\% of cells were still adhered on the CaF\(_2\) substrate and among them 90\% uptake paclitaxel.

Intracellular pharmacokinetics of Paclitaxel was developed taking into account saturable drug binding to extracellular proteins, saturable and nonsaturable drug binding to intracellular components, time- and concentration-dependent drug depletion from culture medium, and cell density-dependent drug accumulation.\(^{24}\) Differential equations were solved to calculate the paclitaxel concentration in MCF7 cells (Python Software Foundation, Nederland). Initial cell number, initial drug concentration, average cell volume, and medium volume taken from our measurement, Michaelis–Menten constants, and some physical constants for calculation from Kuh et al.\(^{24}\) were introduced in differential equations to obtain the time dependent concentration simulation of our measurement.

\[
\frac{dC_{total,c}}{dt} = \left[ \frac{-A + \sqrt{A^2 + 4.4K_{d.m}C_{total,m}}}{2} - B + \sqrt{B^2 + 4(1+NSB)K_{d,c}C_{total,c}} \right] \frac{CL_f}{V_{one\ cell}} - k_{cell\ number\ C_{total,c}},
\]

where A, B, C, D, E, F, and G are constants, and CL\(_f\), V\(_{one\ cell}\), and k\(_{cell\ number\ C_{total,c}}\) are the random uptake, cell volume, and cell number, respectively.

### Table I. Paclitaxel uptake after 3, 6, and 9 h of incubation.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>% cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>85</td>
</tr>
<tr>
<td>6</td>
<td>80</td>
</tr>
<tr>
<td>9</td>
<td>90</td>
</tr>
</tbody>
</table>

*(a)Time of paclitaxel in culture medium. (b)Percentage of cells positive for paclitaxel/total number of live cells.
Our high resolution images demonstrate that paclitaxel's diffusion process in cells versus time and also a relative quantification of the uptaken amount of paclitaxel. Therefore, it is confirmed that paclitaxel's release is not observed drug localization around bundles of stabilized microtubules that would have been expected according the classical picture of paclitaxel's pharmaceutical action. Furthermore the validity of all the classical works based on fluorescent labeling is challenged by the theory of paclitaxel crystal formation. This theory is based on in-vitro observation of aster-like paclitaxel crystal and tubulin binding on crystal allowing confusion with microtubule. Paclitaxel crystals inducing sequestration of free tubulin could induce cytotoxicity in non-dividing cells leading to cell death rather than by paclitaxel's direct interference with mitotic spindle. Furthermore, pixel analysis enabled to trace the dynamics of the drug's diffusion process in cells versus time and also a relative quantification of the uptaken amount of paclitaxel. Our work settles bases towards the reinterpretation of chemotherapeutical drug’s action on cancerous cells susceptible to promote elaboration of pharmaceutical methods to increase efficiency of anticancer drugs.

\[ A = K_{d,m} + B_{max,c} - C_{total,m}, \]
\[ B = (1 + NSB)K_{d,c} + B_{max,c}(1 + k_{Bmax,c}), - C_{total,c}, \]
\[ V_{one-cell} = \frac{4\pi}{3} \cdot L \cdot W \cdot H = 2.1 \mu l/10^8 \text{ cell}, \]
\[ L \text{ maximum}, W \text{ minimum diameter of cell}, H \text{ height of cell}, \]
\[ V_m = (5 \text{ ml}): \text{ volume of the medium}, \]
\[ B_{max,m}(3.94 \mu M), K_{d,m}(781 \text{ nM}); \text{ Michaelis–Menten constants of drug binding to cellular components}, \]
\[ B_{max,c}(59.2 \mu M), K_{d,c}(4.93 \text{ nM}); \text{ Michaelis–Menten constants of drug binding to protein in medium}, \]
\[ NSB (0.148); \text{ Proportionality constant for nonsaturable binding site in cells} \]
\[ ICN (10^6); \text{ initial cell number}, \]
\[ CL_f \text{ (3.34 } 	imes 10^{-3} \mu l/h/cell); \text{ Clearance of free drug by passive diffusion}, \]
\[ k_{cell-number} (-0.0053h^{-1}); \text{ Rate constant for change in cell number}, \]
\[ k_{Bmax,c} (31.3 \times 10^{-3} h^{-1}); \text{ Rate constant for increase of } B_{max,c}, \]
\[ C_{total,c} - C_{total,m}; \text{ Total drug concentration in cell and medium}. \]

The mean cell volume is calculated by converting the pixels to micrometers for the ellipsoid MCF7 cell. Experimentally the minimum amount of paclitaxel detectable by Raman microscopy was determined as 100 μM. Therefore for 3 h of incubation, using the mean of the pixel of paclitaxel divided by cell pixel (Fig. 5) and multiplying the percentage of cell with paclitaxel, gives the concentration of drug inside the cell.

\[ \frac{dC_{total,m}}{dt} = \left[ -A + \sqrt{A^2 + 4 \cdot K_{d,m} \cdot C_{total,m}} \right] - \left[ \frac{B + \sqrt{B^2 + 4 \cdot (1 + NSB)K_{d,c} \cdot C_{total,c}}}{2 \cdot (1 + NSB)} \right], \]
\[ V_m^3, CL_f \cdot ICN \cdot e^{cell number} \]