Raman tweezers and Raman microscopy for single call analysis

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Outline

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   - Biotech: probing recombinant proteins produced in transgenic cells
   - Medical: cancer cells, virus-infection and thalassemia
I) Introduction: single cell analysis in real-time

- **Population analysis:** most of cellular and molecular analyses are based on an ensemble of cells.
  - Need large number of cells
  - Slow response due to culture
  - But 99% microbes cannot be cultured
  - Report average results

- **Needs for single cell analysis:**
  - Monitor dynamic processes of individual cell in real-time.
  - Explore heterogeneity among individual cells.
  - Measure features of individual cell that are masked by population measurement.
What do we concern about single cells?

- Single atoms: energy levels (structure), velocity (temperature), atom-atom interaction, atom-environment (light, EM, cavity) interaction …

- Single cells:
  - chemical composition, spatial distribution of different molecules (structure)
  - key molecules that control the cell’s function & signal transduction
  - cellular heterogeneity. Is each cell the same?
  - cell-cell & cell-environment interactions (light, thermal, sound, nutrient, pH value, drug)
  - ……
Challenges to single cell analysis

• Sensitivity: must be very high \(\rightarrow\) single photon detection
• Non-invasive: keep the cell alive for further identification \(\rightarrow\) NIR
• Immobilization: locate the cell for stable observation since most may flow in liquid and air environment \(\rightarrow\) optical trapping

Brownian motion and Cell motility

• Rapid: real-time analysis for dynamic processes \(\rightarrow\) sec/min
Optical Tweezers

Observation of a single-beam gradient force optical trap for dielectric particles

A. Ashkin, J. M. Dziedzic, J. E. Bjorkholm, and Steven Chu

AT&T Bell Laboratories, Holmdel, New Jersey 07733
Applications of optical trapping for Biomechanics

- 1987-00, measurement of mechanical properties of cells (elasticity, stiffness, rigidity and torque).
- 1995-12, single-molecule biomechanics: protein motor and elastics of DNA molecules.
Raman spectroscopy combined with optical tweezers allows analyzing **Biochemical Properties** of single cells in aqueous media

Near-infrared Raman spectroscopy of single optically trapped biological cells

Changan Xie, Mumtaz A. Dinno, and Yong-qing Li

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Laser can not only control the cell, but also analyze the cell!

![Graphs showing Raman spectroscopy results for Polystyrene sphere and Human red-blood cell](image)
Laser tweezers Raman spectroscopy (LTRS)

Combination of
- Light microscopy
- Optical tweezers
- Raman spectroscopy.

It allows simultaneous
- imaging
- manipulation
- analysis in real-time.

Optical trap allows hold & manipulate individual cells in solution

- Water: $n_1 = 1.33$
- Cells: $n_2 = 1.45$

Salmonella typhimurium bacterium
Why Raman spectroscopy

- To obtain the chemical information of trapped particles

![Diagram of Raman spectroscopy](image)

- **CaDPA specific bands**
  - 824, 1017, 1395, 1572 cm\(^{-1}\)

- **Other spore’s components**
  - Nucleic acids – 788, 812, 1085 cm\(^{-1}\)
  - Phenylalanine - 1004 cm\(^{-1}\)
  - Protein amide I - 1665 cm\(^{-1}\)
Raman spectral dependence

- **Composition analysis**: Vibration frequencies are specific for each type of small molecules and functional groups.
- **Conformation analysis**: Vibration frequencies depend on the structure of molecules.
- **Quantitative analysis**: Vibration intensity at a specific band depends on the concentration of molecules.
II-1) Microfluidic LTRS

- How to measure large number of individual cells: automatic sampling.

*Huang et al; J. Bacteriol. 189, 4681-4687 (2007).*
Diagnosis of thalassamia: Hemoglobin heterogeneity - α- and β-thalassemia

$I_{1545}/I_{1450}$ Histogram

$I_{1545}/I_{1450}, \text{Bin}=0.1$

- norm = 360
- major = 330
- HbH = 359

Raman shift (cm$^{-1}$)
Cellular heterogeneity of Ca-DPA levels in individual spores of different species

- a) *B. cereus* T;
- b) *B. megaterium* QM B1551;
- c) *B. subtilis* FB62 (gerD);
- d) *B. subtilis* PS832 (wt) grown with 2% xylose;
- e) *B. subtilis* PS3413 (P$_{xyl^-}$ spoVA) grown with 2% xylose; and
- f) *B. subtilis* PS3413 grown with 0.5% xylose.

II-2) Combination of phase contrast/fluorescence microscopy, elastic scattering, Raman spectroscopy and optical tweezers

Multimodal information about the trapped cells:

- Fluorescence
- Phase contrast:
  - index of refraction
- Elastic scattering
- Raman scattering
Experimental System

Single molecule imaging system

Nano-stage

Laser excitation system

Spectroscopy system

Characterization of bacterial spore germination using phase-contrast and fluorescence microscopy, Raman spectroscopy and optical tweezers

Lingbo Kong¹, Pengfei Zhang¹, Guiwen Wang¹, Jing Yu¹, Peter Setlow² & Yong-qing Li³

Phase contrast image

Fluorescence image ($\lambda_{em}=530$ nm, $\lambda_{exc}=470$ nm)

Normalized DPA level, Refractility & Fluo. intensities

B. cereus spores in L-alanine

Multimodal information
II-3) Multiple-trap LTRS array

Single-trap LTRS can only analyze one spore at a time. How to increase the system efficiency and monitor multiple spores in one-run experiment (1-2 hours)?
Multiple-trap laser tweezers Raman spectroscopy for simultaneous monitoring of the biological dynamics of multiple individual cells

Pengfei Zhang,1 Lingbo Kong,1 Peter Setlow,2 and Yong-qing Li1,*
Phase-contrast and Raman spectral images of multiple trapped dormant *B. cereus* spores
Monitoring four trapped B. cereus spores germinating in 10 mM L-alanine, 25 mM Tris buffer and 0.5 μM SYTO 16 using LTRS array
Advantages of LTRS

- **Allow capture and analysis** of single or multiple individual living in aqueous environment.
- **Identify by molecular vibration**. No fluorescent label is needed generally.
- **High sensitivity** (confocal excitation and collection).
- **Rapid response** (no incubation, no sample preparation).
- **Non-invasive** to the cells (no adding chemicals, no breaking the cells, low absorption in NIR).
- **Multiple information**: integrated with microfluidics, phase contrast & fluorescence microscopy.
II-4) Rapid confocal Raman imaging with multifoci-scan

- How to obtain spatial distribution of specific cellular molecules (such as CaDPA, carotenoid, proteins, or nuclei acids) of single cells?
- Spontaneous Raman imaging allows label-free molecular imaging of different components simultaneously.
- The point-scan Raman mapping affords the ultimate sensitivity, spatial resolution, image quality and large spectral range capability, but too slow (40-60 min per frame) for monitoring living cells.

*Kong et al; Appl. Phys. Lett. 98, 213703 (2011).*
Multifoci-scan confocal Raman microscopy

Synchro multifoci-scan allows acquiring 40-80 spectra simultaneously while retaining point-scan resolution. The image acquisition time is 40-80 times faster.
• Measured lateral and axial resolution using a 100-nm diameter polystyrene bead Raman band at 1001 cm$^{-1}$. 

Spatial Resolution

(b) lateral 0.4 µm

(c) axial 1.6 µm
The distance between two 1-µm polystyrene beads:

- Point-scan (40 min): 1.919 ± 0.008 µm
- Multifoci-scan (1 min): 1.921 ± 0.008 µm
Raman images of human red blood cells and *B. megaterium* spores
Imaging speed: 33 s/frame
II-5) Image-guided multifocus confocal Raman microspectroscopy

• Conventional Raman microspectroscopy has single-focus excitation and can only analyze one particle. It becomes time-consuming when the analysis of large numbers of single particles is desired.

• How can monitor multiple (80-100) individual cells in random positions on a cover slip for 10-24 hours or longer (i.e. for slow biological process)?

There are two challenges

1) Microscope stabilization in z-direction (focusing) and x-y direction (stage horizontal movement) over >60 min is required. 100-200 nm drift will cause Raman signal significantly reduction.

2) Precisely targeting laser focus to each of multiple particles at random positions and collecting their Raman spectra simultaneously are required.
Image-guided multifocus confocal Raman microscopy
Active focus locking - bacterial spores

Drift = ~500 nm / 20 min

Image-guided multifocus confocal Raman microscopy

Mixed polystyrene beads (numbered in black) and *B. megaterium* spores
Monitor slow germination of individual superdormant spores for >12 hours

Dormant spores with L-valine

Superdormant spores with L-valine
III) Biological applications

- Monitoring of single cell dynamics: bacterial spore germination, wet-heat inactivation, and response to high vacuum.
- Raman sorting and flow cytometry: cells and chromosomes
- Biosensing: identification and detection of environmental microorganisms
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- Medical: cancer cells, virus-infection and thalasemia
Can we sort different types of cells in a mixed sample?

III-1) Raman Sorting

- Sample chamber
- Raman scattering
- Laser tweezers
- Micro-channel
- Collection chamber

Sorting living yeast cells

Staining

Intensity (Counts)

Raman shift (cm⁻¹)

Living

Dead

Intensities for different Raman shifts:
- 600 to 1600 cm⁻¹
- Amide I, Amide II, and Amide III peaks
- Specific peaks for DNA, Phe, Tyr, and Lipids

Counts for different intensity ranges:
- 0 to 300

Amide I:
- 1604 cm⁻¹
- 1660 cm⁻¹

Amide II:
- 1443 cm⁻¹

DNA backbone:
- 1202 cm⁻¹

Phe:
- 1085 cm⁻¹

Tyr:
- 1035 cm⁻¹

Lipids:
- 892 cm⁻¹
- 858 cm⁻¹

Staining:
- Differential staining for living and dead cells
Manipulation and discrimination of single unstained human chromosomes

- Capture an unknown chromosome
- Raman acquisition
- Manipulation
- Fixation & G-banding verification

Sample reservoir  Buffer reservoir  Fixed slide  Unstained chromosomes of leukemia cells

III-2) Detect single-cell dynamics

- Physical agents: heat, UV, ultrasonic, microwaves …
- Chemical agents: drug, PH, toxin, bleach…
- Biological: nutrient triggered germination, growth, reproduction, protein expression, virus-infected …

Examples include spore germination, heat-inactivation, production of biofuel molecules, yeast fermentation……
Biological dynamics of single bacterial spores

• Bacterial spores are metabolically dormant and can survive in this dormant state for many years. But they can sense the environment and return to life via the process of spore germination when nutrients present.

• During dynamic germination process, germination receptors (GRs) will recognize the specific germinant molecules, trigger the release of core’s CaDPA molecules, and trigger the lysis of cortex layer.

• Why spores? *Bacillus* spore is a model system in microbiology and may cause human diseases and food spoilage. Understanding of spore dynamics allows better treatment of spore-relevant diseases.
Germination components

- Permeation proteins that facilitate movement of nutrients through spore outer layers.
- Germinant receptors (GRs) that recognize nutrient germinants.
- GerD protein essential for nutrient germination.
- Channel proteins that allow release of Ca-DPA and other small molecules.
- Cortex Lytic Enzymes (CLEs) that degrade the spore’s peptidoglycan cortex.

- The CLEs in *B. subtilis* spores are CwlJ and SleB.
Real-time detection of kinetic germination and heterogeneity of single Bacillus spores


Add L-alanine
Dormant spores appear as bright and germinated spores as dark.

Based on the change in image intensity, the germination of single spores was separated into two stages.

Stage I is thought as primarily the release of the spores’ CaDPA, but not been proven.

What is the precise correlation between phase contrast image change and CaDPA release is unclear.
Simultaneous monitoring of L-alanine germination of a single optically trapped *B. cereus* spore by Raman spectroscopy and PC microscopy

*Kong et al. Anal. Chem. 82, 3840–3847 (2010).*
1. When repeat the experiments with other cells, we found their responses varied.
2. Some cells response earlier, some later. But correspondence always exists.
Monitoring the Kinetics of Uptake of a Nucleic Acid Dye during the Germination of Single Spores of *Bacillus* Species

Lingbo Kong,† Pengfei Zhang,† Jing Yu,† Peter Setlow,† and Yong-qing Li*†

- How and when external small molecules cross the inner spore membrane and enter the spore core during germination?
- SYTO 16 is a membrane-permeant nucleic acid dye that exhibits a large fluorescence upon binding to nucleic acids. How this dye gets into germinating spores and binds to nucleic acid is unclear.
- What molecules regulate this process?
Simultaneous recording of Raman spectra, phase contrast images and fluorescence images of a single trapped *B. cereus* spore germinating at 24 °C with 1 mM L-alanine plus 500 nM Syto 16 in 25 mM Tris-HCl buffer (pH 7.4)
Results obtained

- During nutrient germination SYTO 16 began to enter the spore core and bind to nucleic acids just when spores had released all CaDPA, and continued until hydrolysis of spores’ peptidoglycan cortex was complete.
- The time between the addition of nutrient germinants and the rapid uptake of individual spores is highly heterogeneous in a population.
C) Characterization of thermal inactivation of single spores by wet heat

- **Goal**: to study aspects of the release of Ca-dipicolinic acid (DPA), protein denaturation and cellular heterogeneity during treatment of single *Bacillus* spores with wet heat

Real-time Raman, ELS, PC or DIC imaging…
Wet heat can kill bacteria & spores but how a spore is inactivated is unclear.

Control (untreated)  Thermal treated at 90°C for 10min

Questions: 1) what is the transition between live and death states?
2) How spectra changes accordingly? What molecular events may involve?
Viability, CaDPA retention, and Raman spectra of wild-type *B. subtilis* spores with and without wet-heat treatment

90°C or 95°C

a – untreated (control)
b,c – 95°C, 30 min
Single *B. cereus* spores heat-treated at 90°C

Results: 1) DPA band dropped rapidly at a specific time $t_{\text{release}}$.

2) Protein band shifted prior to DPA released, indicating denaturation.

*APPL. ENVIRON. MICROBIOL.* 77, 4757 (2011).
Heterogeneity of individual spores inactivated by wet heat

1) Some spores release DPA earlier, some later.

2) There are always a very small percentage of spores retaining their DPA – superdormant/hard to kill.

3) Why some spores easy to kill, some difficult is unknown, but may related to DPA level contained.

How does a cell response to the exposure of high vacuum?

- The cell will be dehydrated so the water level will be reduced.
- The vibration bands of CaDPA molecules largely depend on the water content in the spore’s core such that the measurement of Raman spectra of Ca-DPA may determine the water level in spore’s core.

pressure: ~1 Pa
III-3) Identification of single cells of different bacterial species

Principal component analysis

Identification of airborne particles in the atmosphere

III-4) Detect recombinant protein production

- Transgenic cells can make recombinant proteins after induction of specific gene.
- When are new proteins made after induction?
- How many proteins are made in single cells?
- Can we detect the generated proteins within single cells without breaking the cell?

Detect SLB proteins in E. coli

III-5) Medical applications:

1. Characterize cancer cells.
2. Analyze virus-infected cells.
3. Diagnosis of thelassamia.
Raman spectroscopic analysis of apoptosis of single human gastric cancer cells

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Fig. 2. Images of gastric carcinoma cells (a) and apoptotic cells (b), observed with a DIC microscope with an objective of 100x.

Fig. 4. Raman spectra of untreated gastric carcinoma cells (curve a) and apoptotic cells (curve b). Curve c was the difference spectrum between a and b. The position of Raman bands at 782, 934, 1001, 1092, 1156, 1298, 1340, 1446, 1523, 1576, 1615 and 1655 cm\(^{-1}\) were marked.
Distinguish virus-infected B-cells

Prof. Shaw M. Akula, ECU
Microbiology & Immunology

<table>
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<th>Cell type</th>
<th>KSHV</th>
<th>EBV</th>
<th>ERK</th>
<th>VEG F</th>
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<td>-</td>
<td>++++</td>
<td>++++</td>
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<tr>
<td>BJAB (N3)</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>++</td>
</tr>
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</table>

Note: ‘+’ indicates the level of expression detected (++++ > ++); ‘-‘ indicates absence of infection.

KSHV infected

Summary

• LTRS technique in combination with optical tweezers array, microfluidics, phase contrast & fluorescence microscopy and multifocus Raman spectroscopy provides a valuable tool for the analyses of single cells.

• Applications for single-cell studies may include detection of cellular dynamics, i.e. spore germination, rapid identification of microorganisms, spectroscopic sorting of useful cells, diagnosis of cellular disorders at single cell level, and more.

• Many biological questions remain unanswered.
References


