Raman tweezers and Raman microscopy
for single call analysis

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Outline

I.   Introduction: single cell analysis

II. Technology: Raman tweezers & Raman microscopy

- Microfluidic LTRS
- Combination of phase contrast & fluorescence microscopy
- Multi-trap LTRS
- Rapid confocal Raman imaging
- Multifocus confocal Raman microspectroscopy

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 microorganisms
- Biotech: probing recombinant proteins produced in transgenic cells
- Medical: cancer cells, virus-infection and thalassemia

2

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

a single cell could give rise to 2³⁶ cells (more than 3 ×
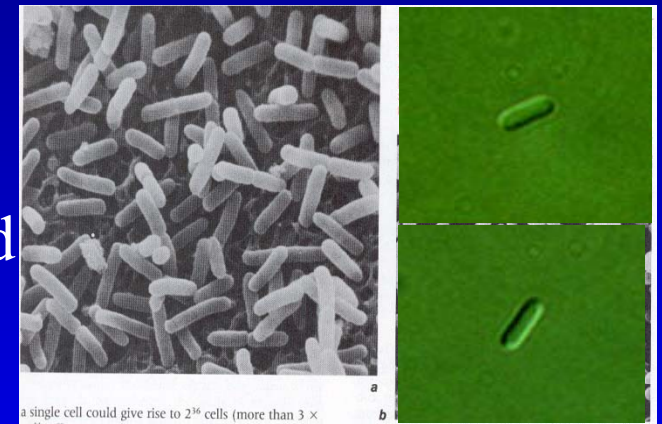
- **Needs for single cell analysis:**

  - 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 …

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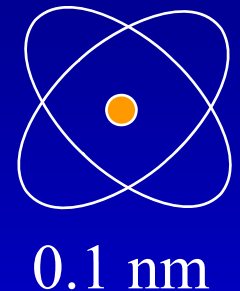
❑ Single cells:
- chemical composition, spatial distribution of different molecules (structure)
- key molecules that control the cell's function & signal transduction
- cellular heterogeneity. Is each cell the same?
- cell-cell & cell-environment interactions (light, thermal, sound, nutrient, pH value,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

1 µm

Challenges to single cell analysis

- Sensitivity: must be very high → single photon detection

- Non-invasive: keep the cell alive for further identification→ NIR

- Immobilization: locate the cell for stable observation since most may flow in liquid and air environment → optical trapping

  Brownian motion and Cell motility
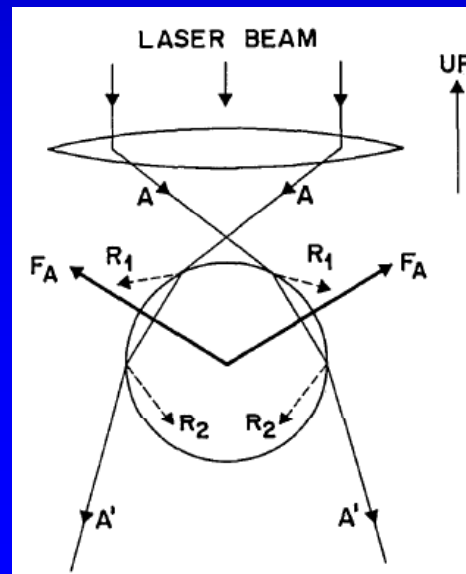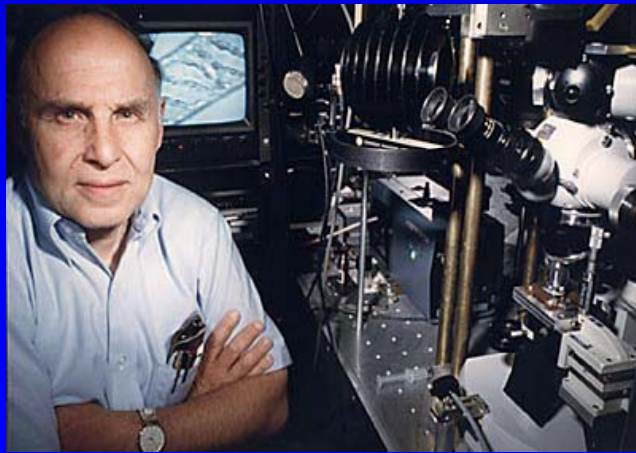
- Rapid: real-time analysis for dynamic processes→ sec/min

Random "walk"

1 μm
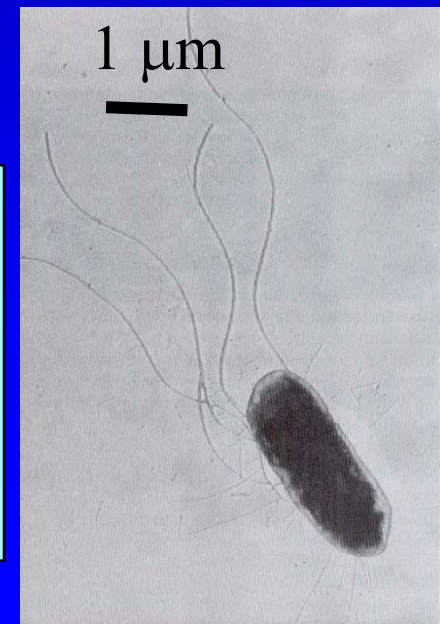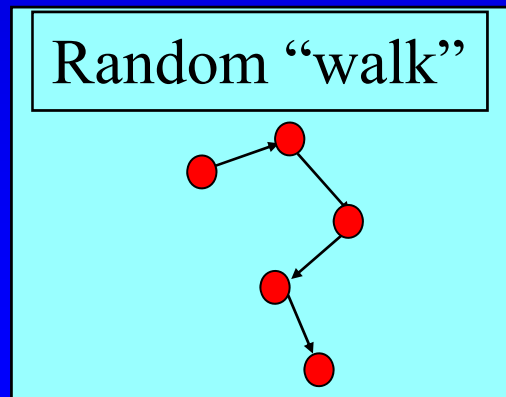
Optical Tweezers

## 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, Nature/Science, manipulation of single cells and intracellular materials (organelles and chromosomes).
- 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.

protein

Tracking bead
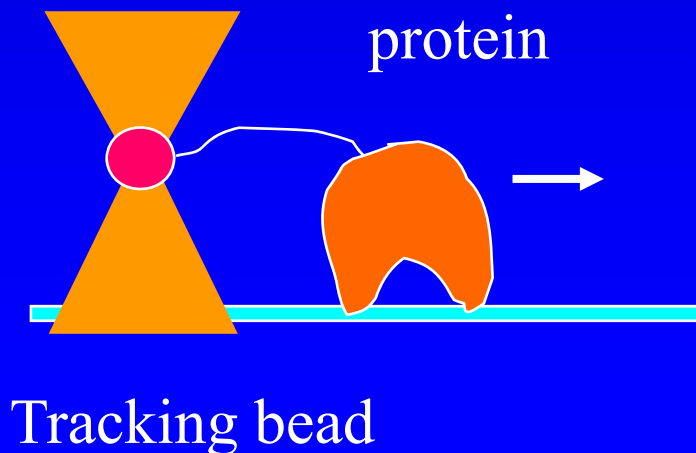
Applied Force Reveals Mechanistic and Energetic Details of Transcription Termination

Matthew H. Larson,[1,5] William J. Greenleaf,[2,5] Robert Landick,[4] and Steven M. Block[2,3,*]

Cell *132*, 971–982, March 21, 2008 ©2008 Elsevier Inc. 971

B

DNA template

RNA

RNAP

7
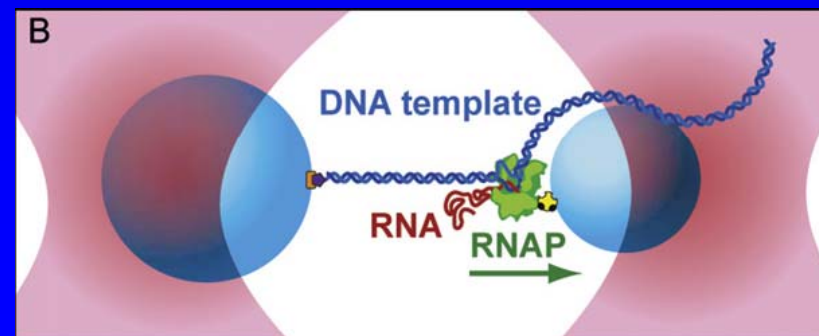
Raman spectroscopy combined with optical tweezers allows analyzing **Biochemical Properties** of single cells in aqueous media

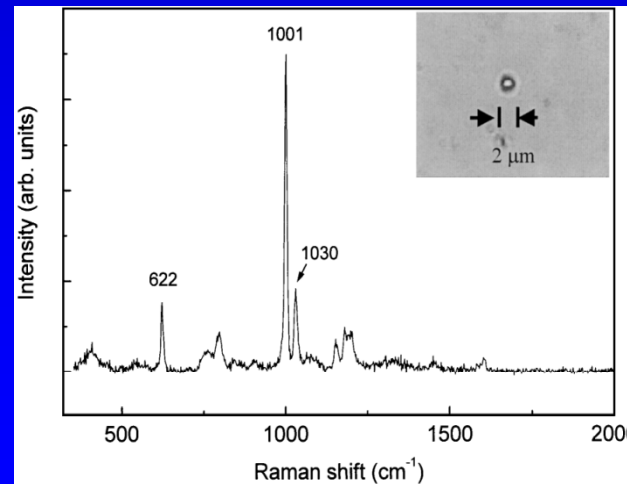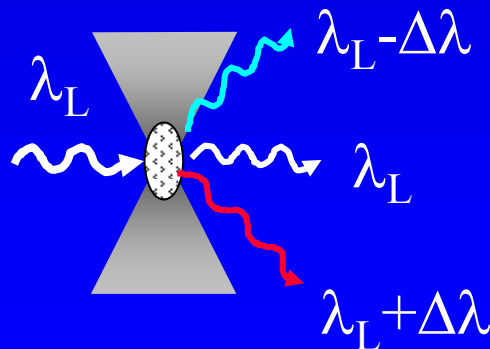## Near-infrared Raman spectroscopy of single optically trapped biological cells
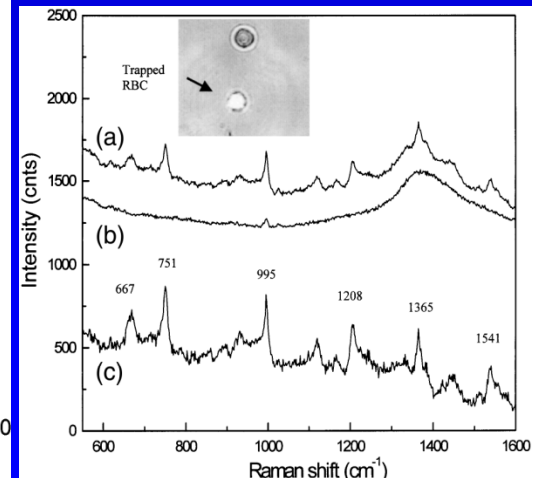
Changan Xie, Mumtaz A. Dinno, and Yong-qing Li

Department of Physics, East Carolina University, Greenville, North Carolina 27858-4353

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

λ_L

$\lambda_L - \Delta\lambda$

λ_L

$\lambda_L + \Delta\lambda$
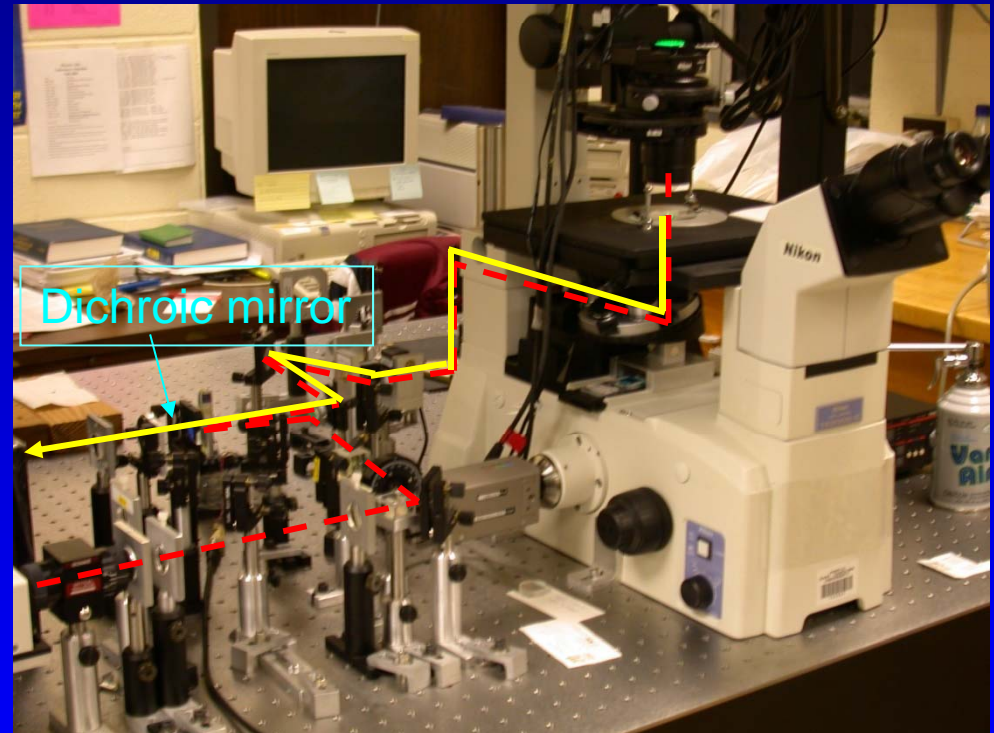
Polystyrene sphere

Human red-blood cell

Laser tweezers Raman spectroscopy (LTRS)

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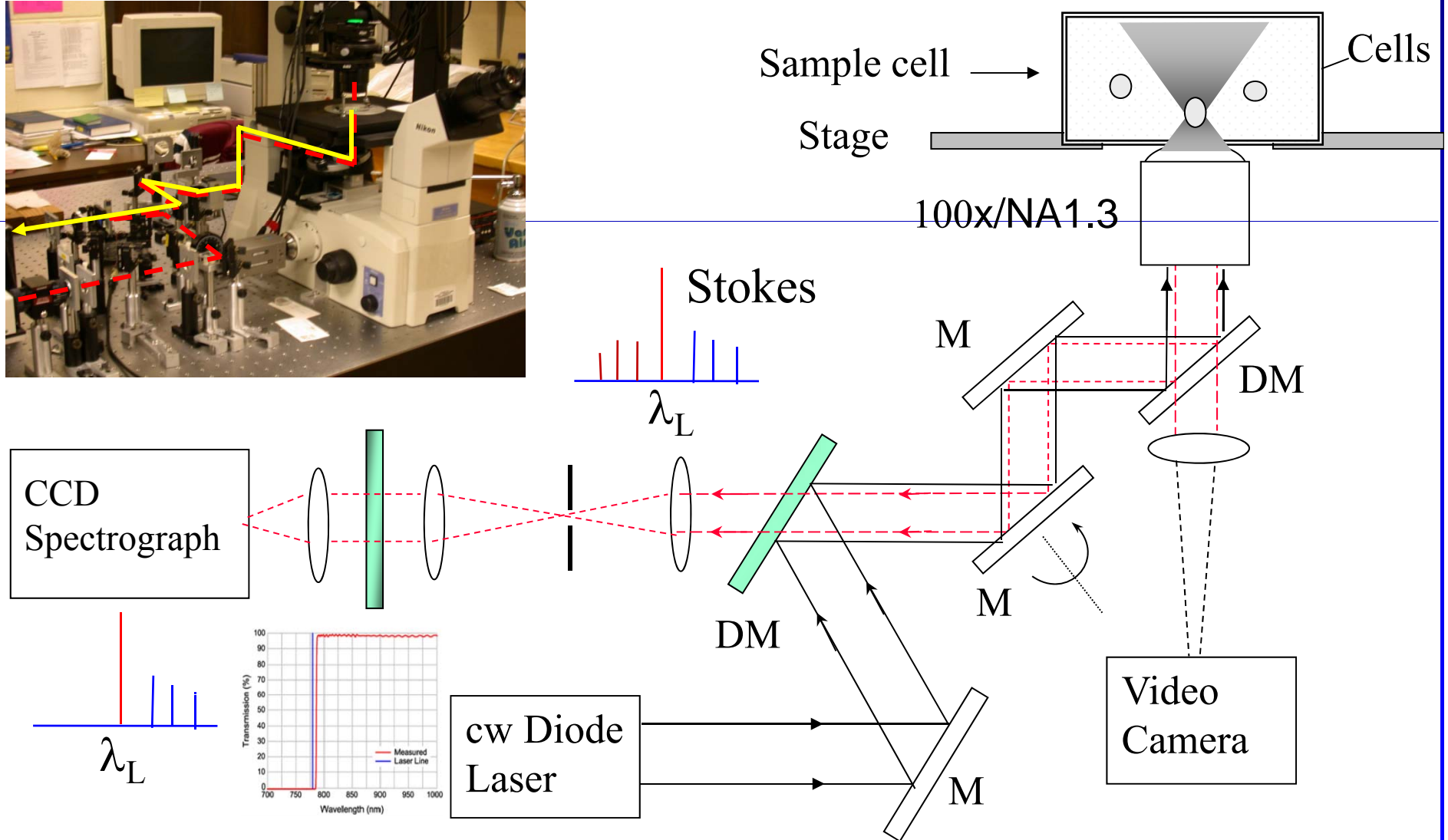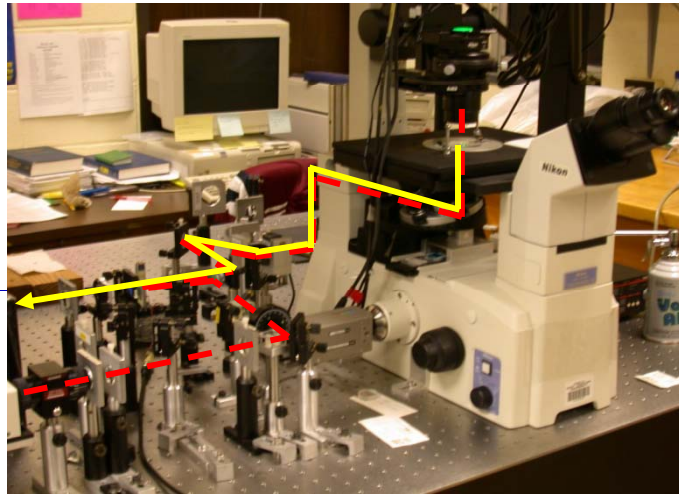- Optical tweezers
- Raman spectroscopy.

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

Dichroic mirror
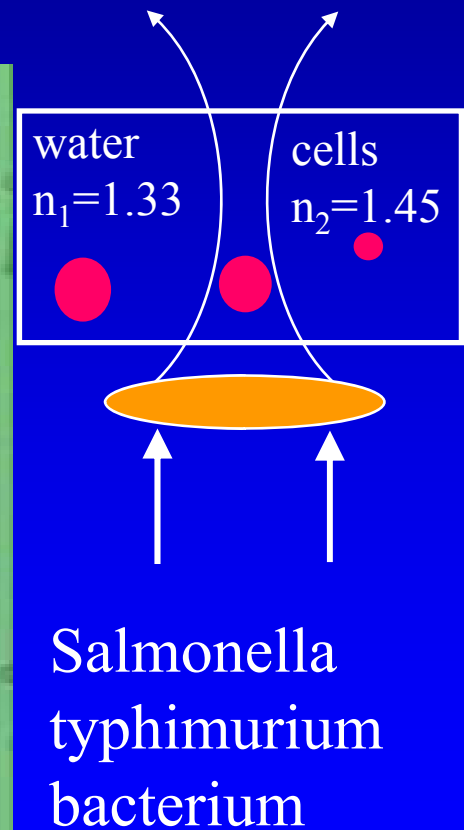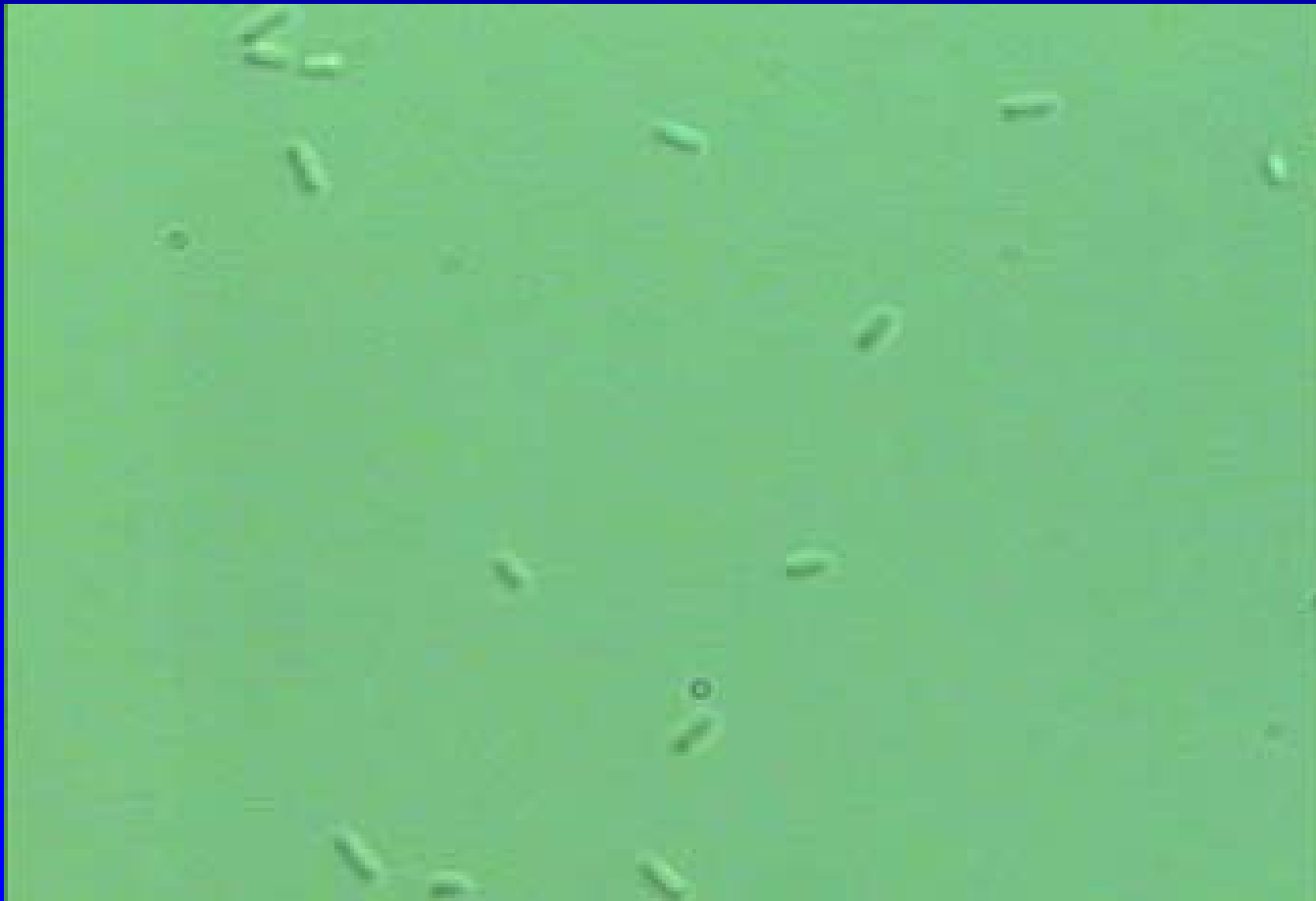
Kong et al, Nat. Prot. 6, 625-639 (2011); *Opt. Lett.* 27, 249-251 (2002); *APL.* 81, 951-953 (2002).
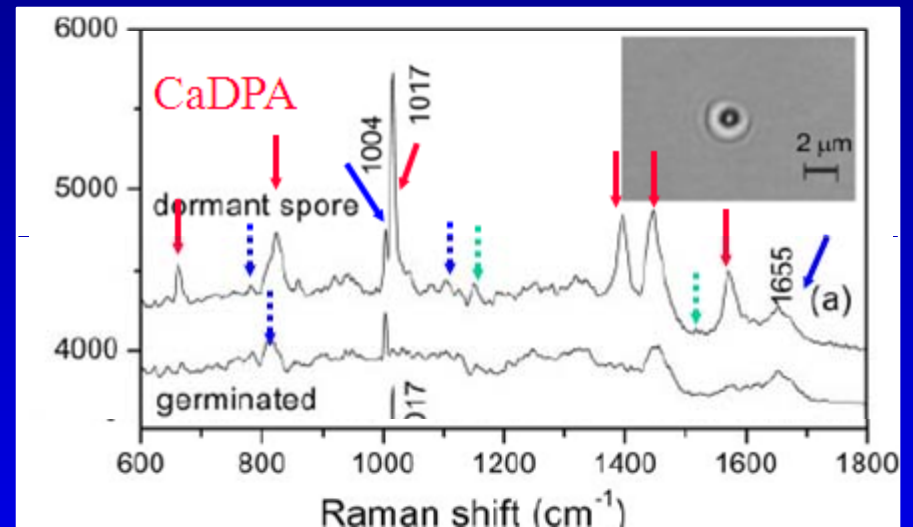
Single-trap LTRS Setup

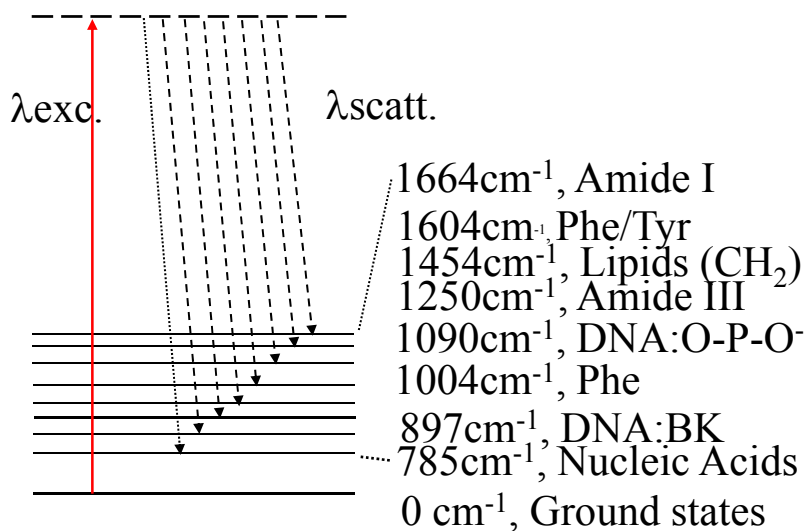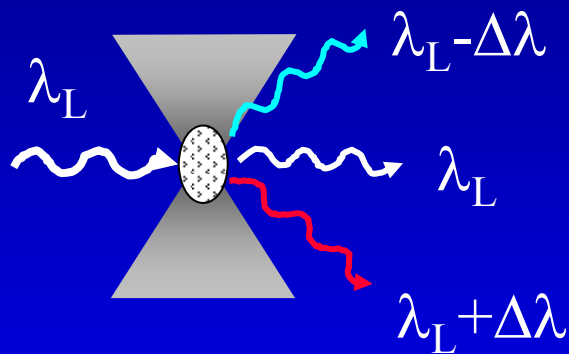# Optical trap allows hold & manipulate individual cells in solution

water
$n_1=1.33$

cells
$n_2=1.45$

Salmonella typhimurium bacterium

Why Raman spectroscopy

❑ To obtain the chemical information of trapped particles

λ_L

$\lambda_L - \Delta\lambda$

λ_L

$\lambda_L + \Delta\lambda$

λexc.

λscatt.

$1664 cm^{-1}$, Amide I
$1604 cm^{-1}$, Phe/Tyr
$1454 cm^{-1}$, Lipids (CH_2)
$1250 cm^{-1}$, Amide III
$1090 cm^{-1}$, DNA:O-P-O$^-$
$1004 cm^{-1}$, Phe
$897 cm^{-1}$, DNA:BK
$785 cm^{-1}$, Nucleic Acids
$0 cm^{-1}$, Ground states

CaDPA

dormant spore

germinated

1004

1017

1655

(a)

Raman shift (cm^{-1})

- CaDPA specific bands
  - 824, 1017, 1395, 1572 cm^{-1}
- Other spore's components
  - Nuclic acids – 788, 812, 1085 cm^{-1}
  - Phenylalanine - 1004 cm^{-1}
  - Protein amide I - 1665 cm^{-1}

12

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

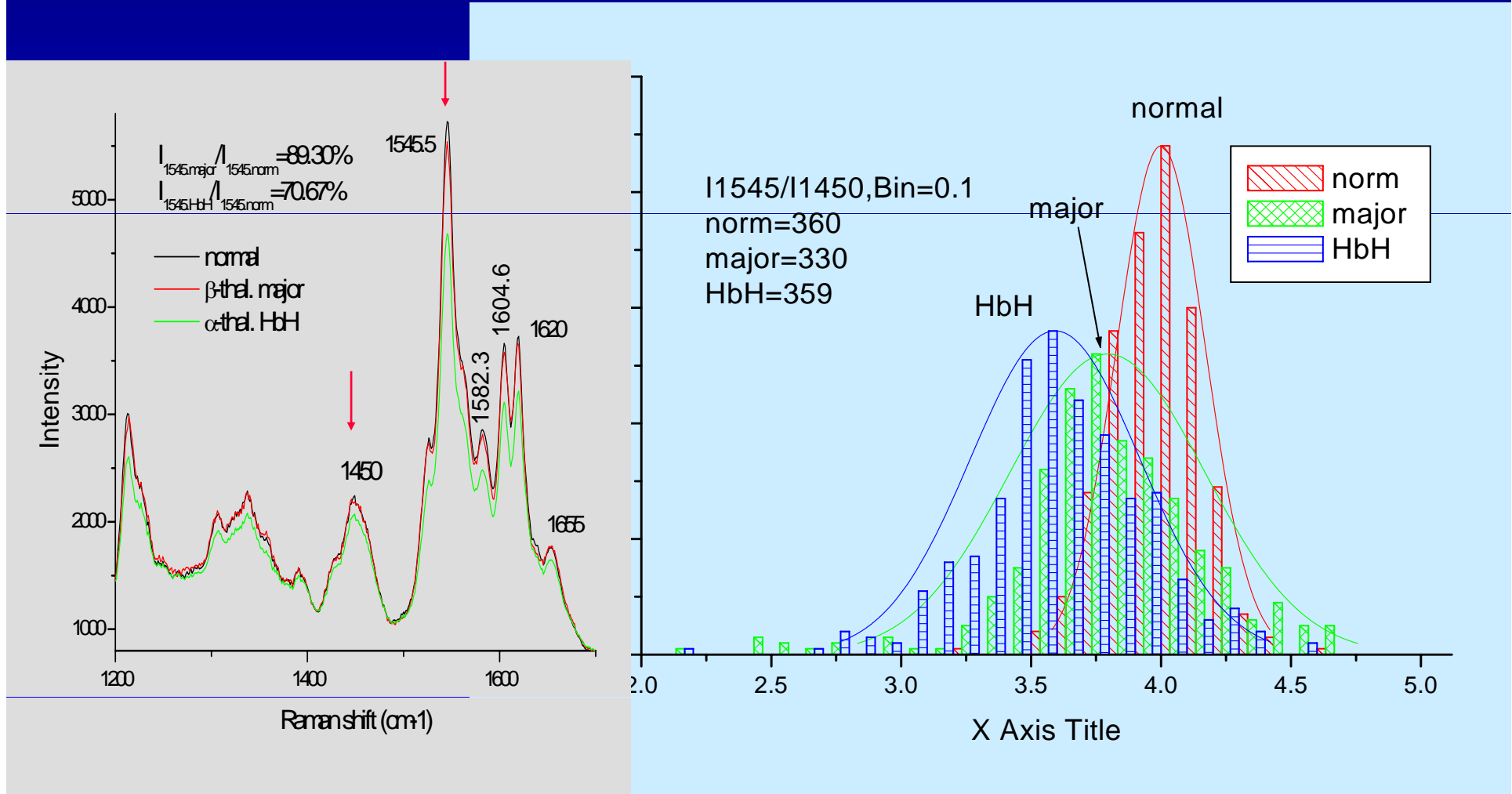- How to measure large number of individual cells: automatic sampling.

Drain   Source

Micro-
channel

0:07:12
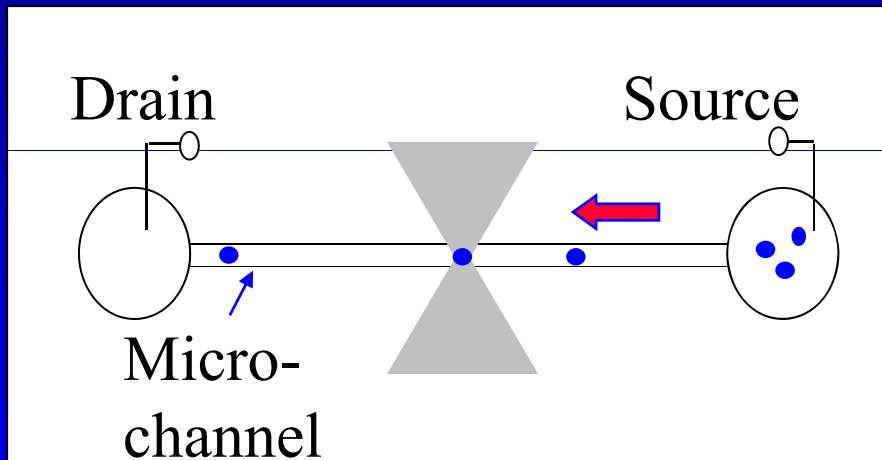
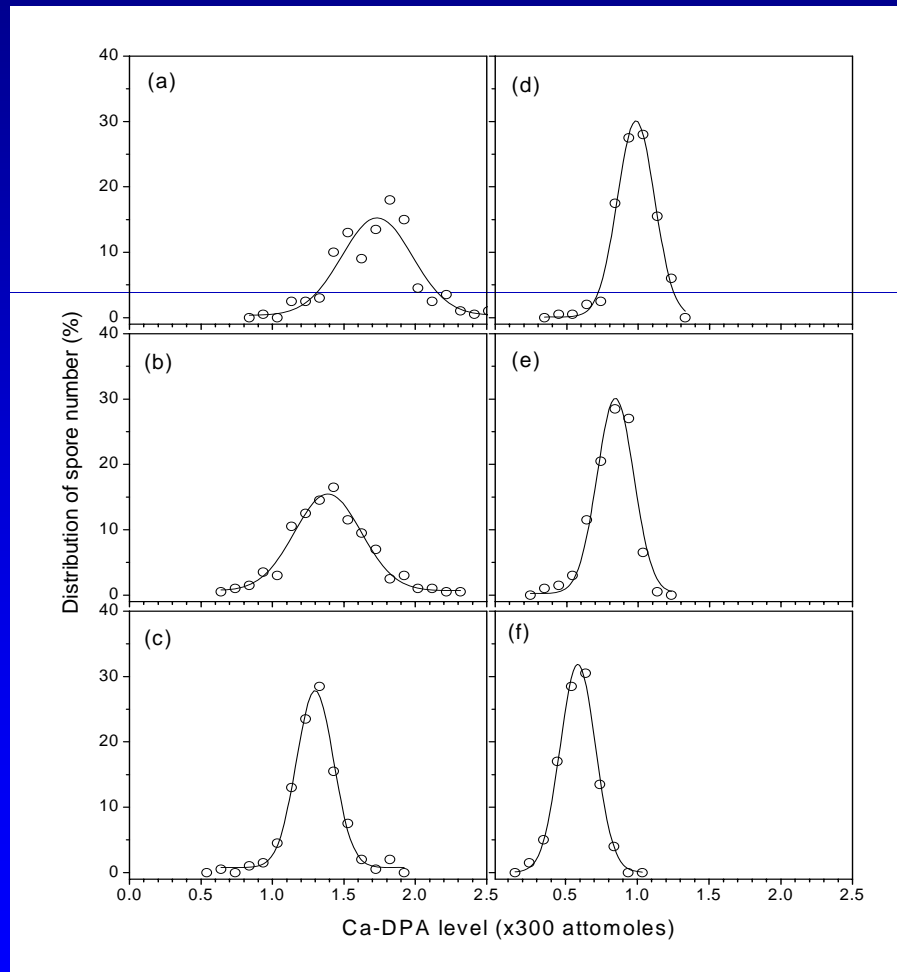*Huang et al; J. Bacteriol.* 189, 4681-4687 (2007).

Diagnosis of thalassamia: Hemoglobin heterogeneity - α- and β-thalassemia
# I_{1545}/I_{1450} Histogram

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.

Huang et al, *J. Bacteriol.* **189**, 4681-4687 (2007).

16

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

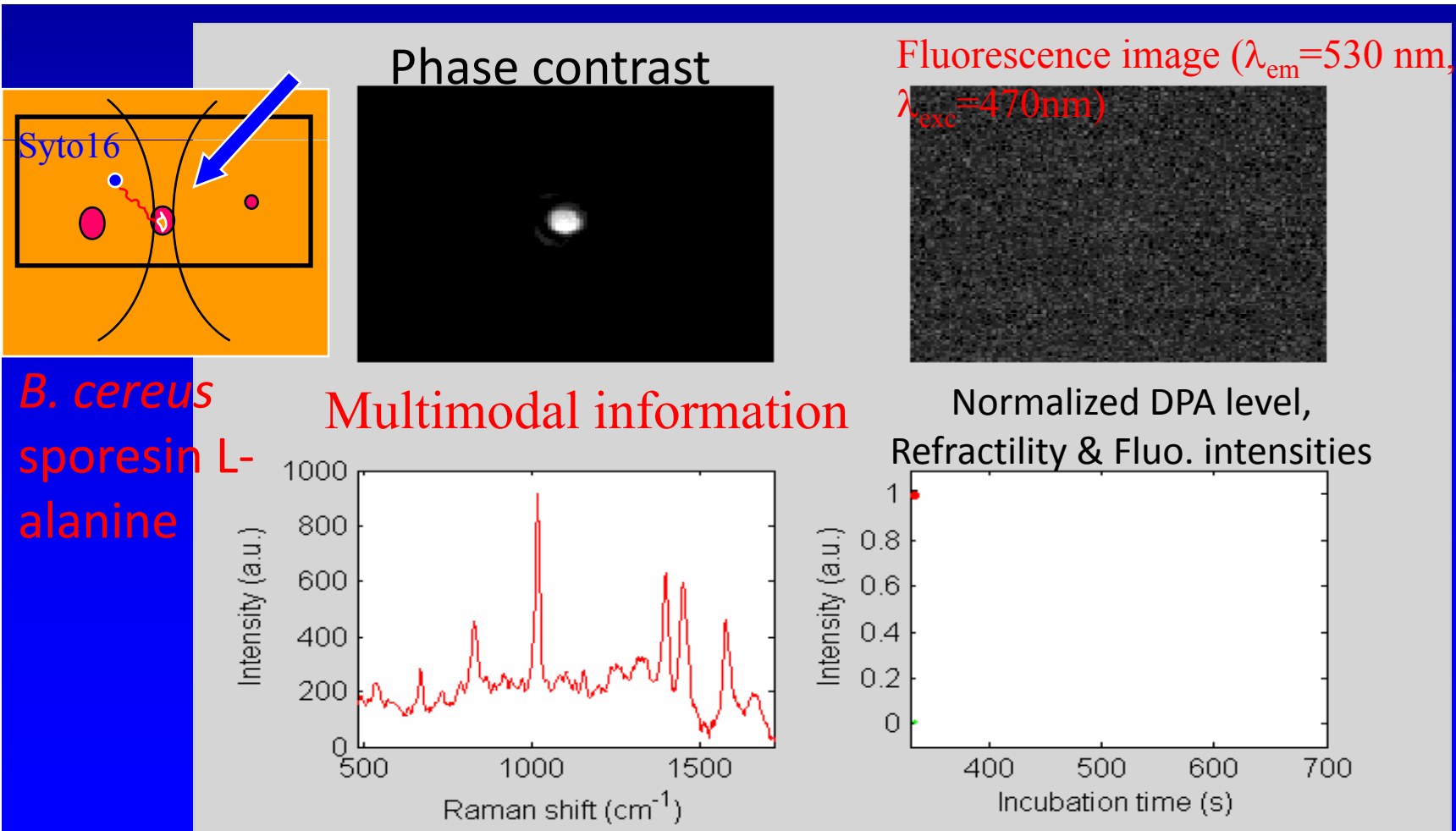Multimodal information about the trapped cells:

λ_L

- Fluorescence

λ_L – Elastic scattering

$\lambda_L + \Delta\lambda$ – Raman scattering

- Phase contrast:
index of refraction
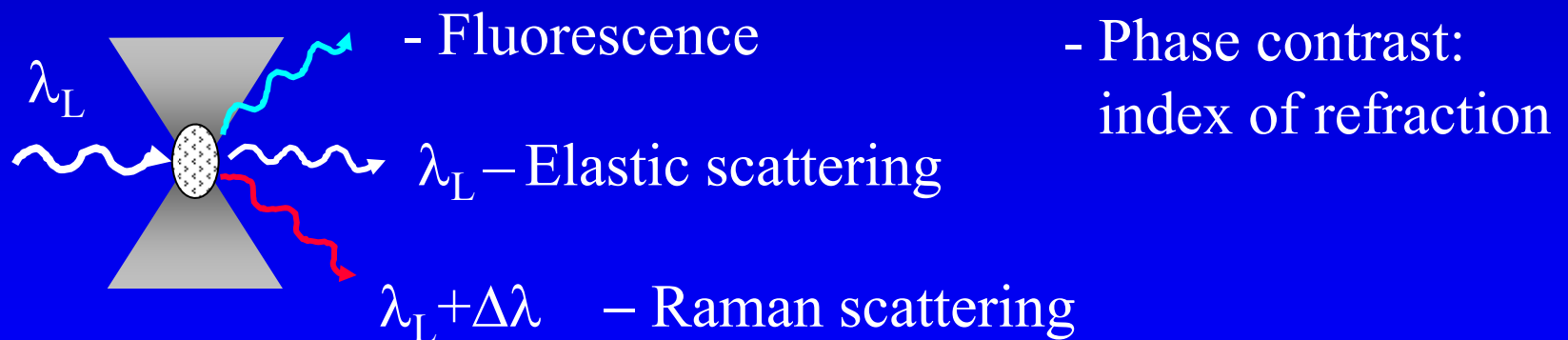
Experimental System
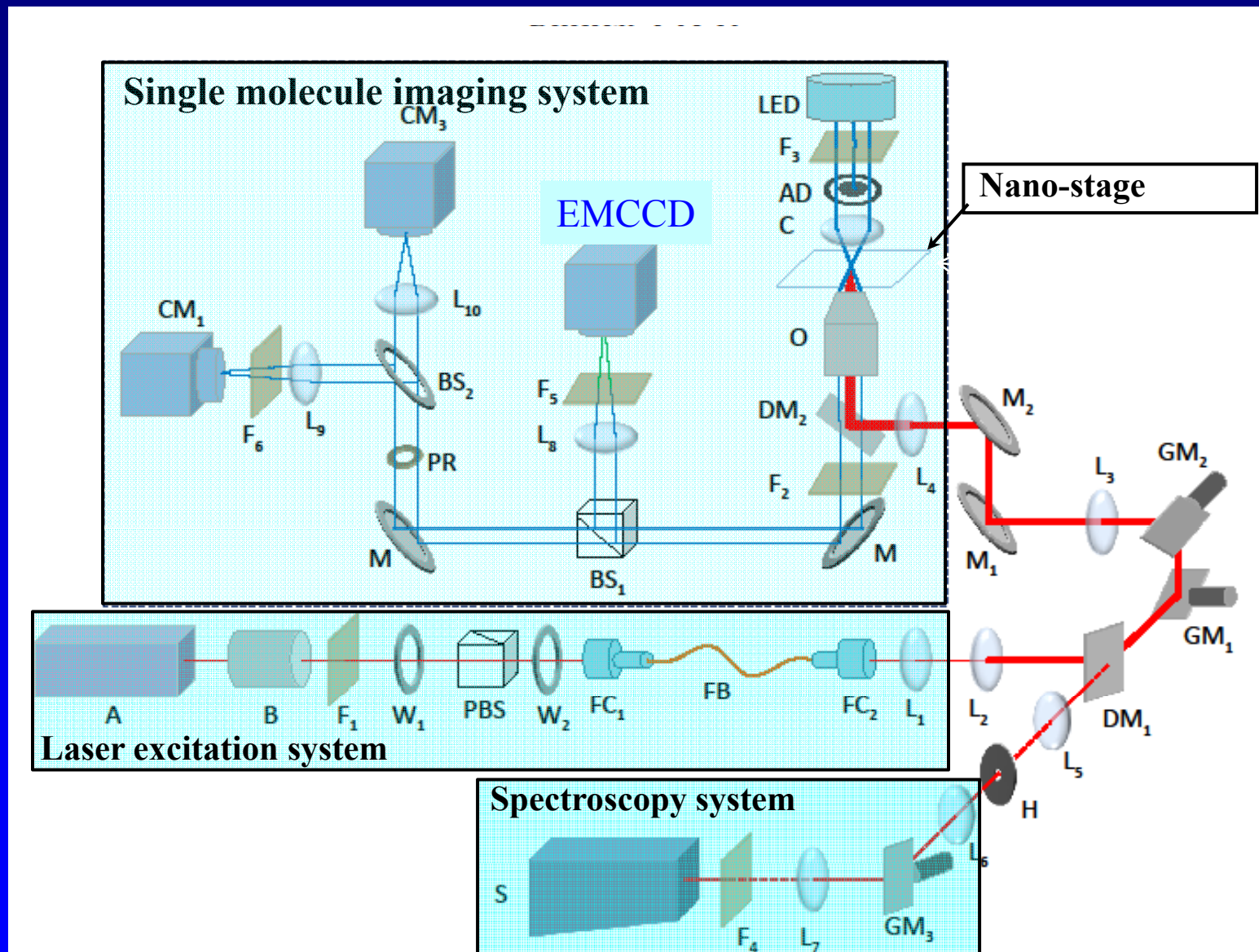
Kong et al; *Nature Protocols, 6, 625-639, (2011)*.

PROTOCOL

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

Lingbo Kong[1], Pengfei Zhang[1], Guiwen Wang[1], Jing Yu[1], Peter Setlow[2] & Yong-qing Li[1]

Phase contrast

Fluorescence image (λ_{em}=530 nm, λ_{exc}=470nm)

Syto16

B. cereus spores in L-alanine

Multimodal information

Normalized DPA level, Refractility & Fluo. intensities

19

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)?
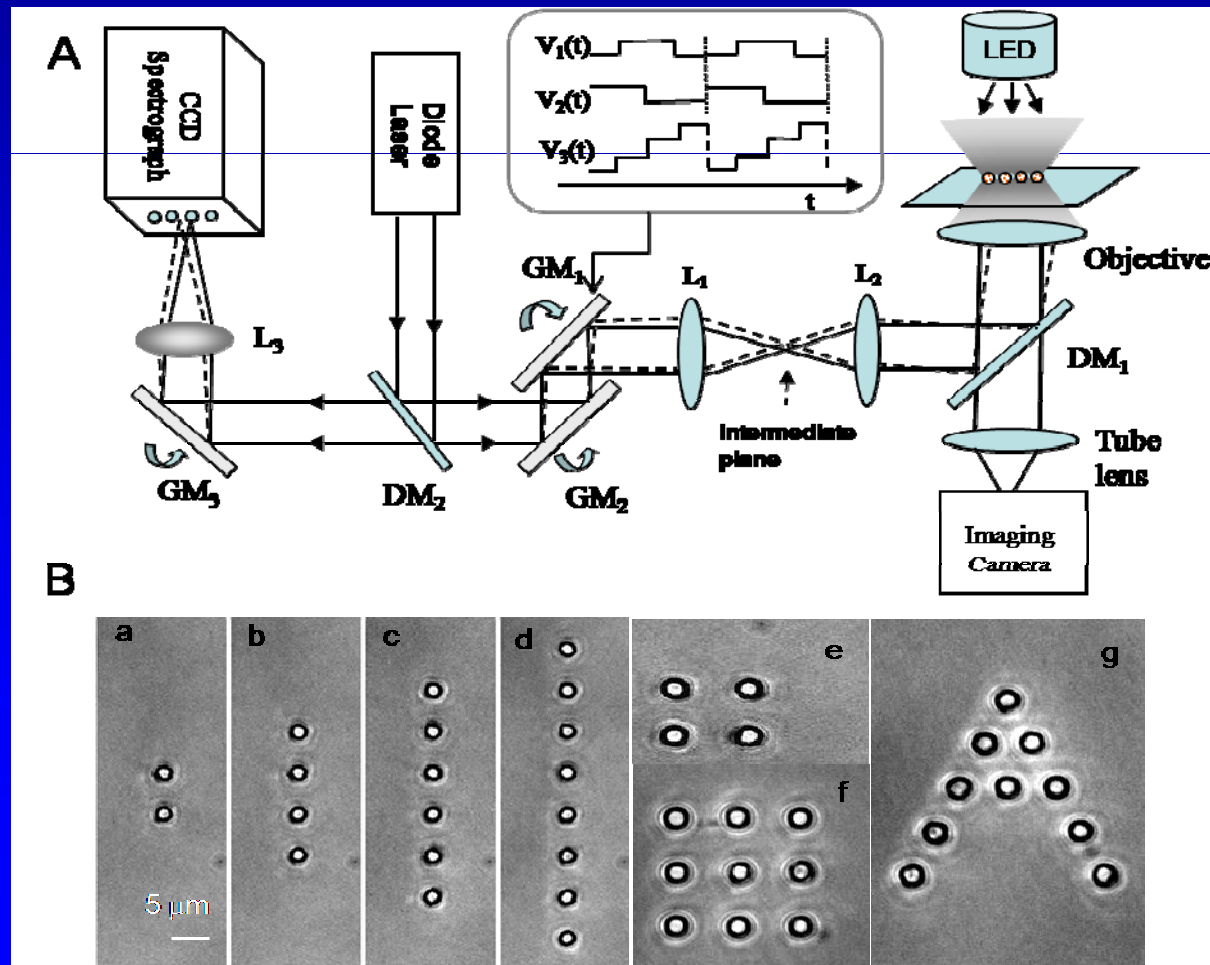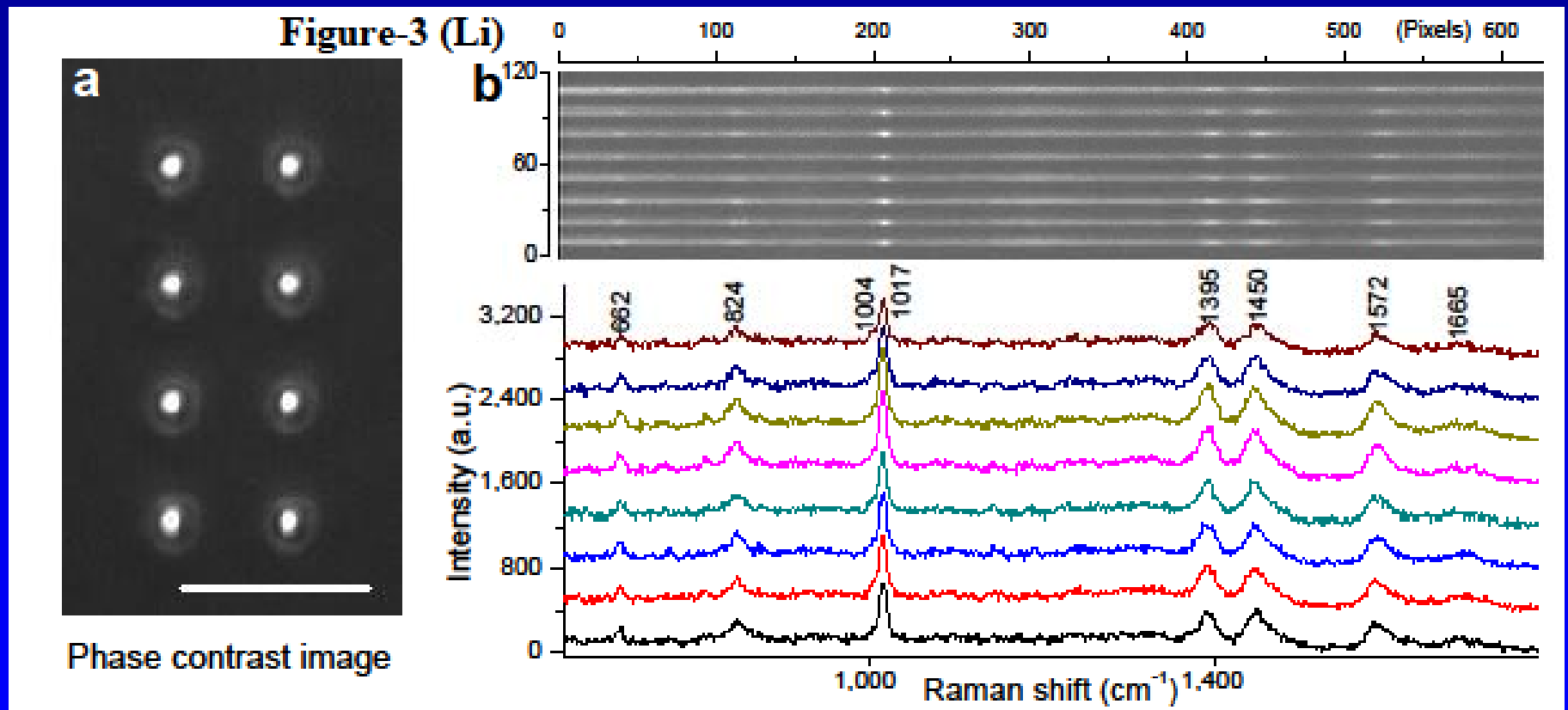
Multiple-trap laser tweezers Raman spectroscopy for simultaneous monitoring of the biological dynamics of multiple individual cells
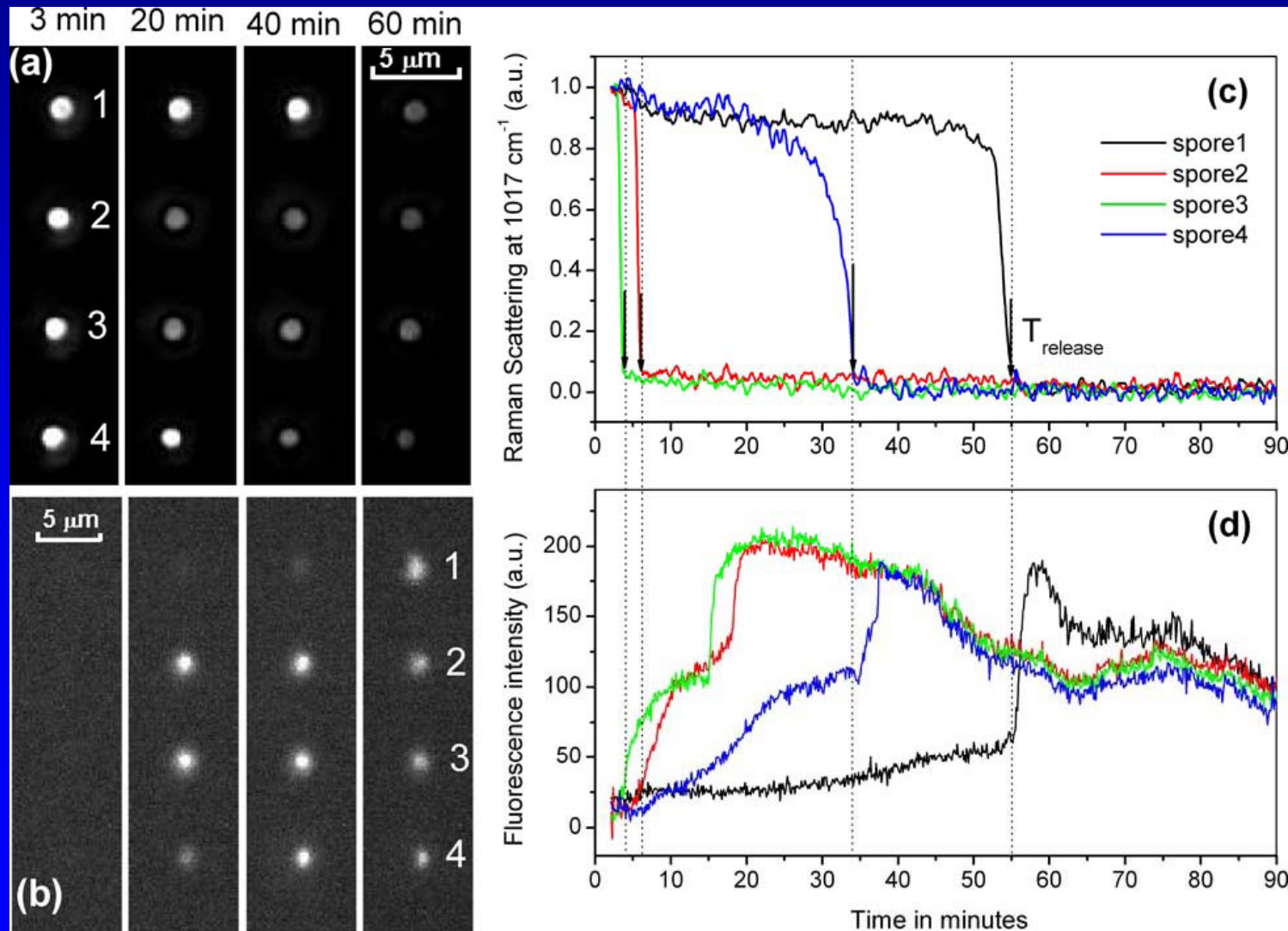
Pengfei Zhang,[1] Lingbo Kong,[1] Peter Setlow,[2] and Yong-qing Li[1,*]
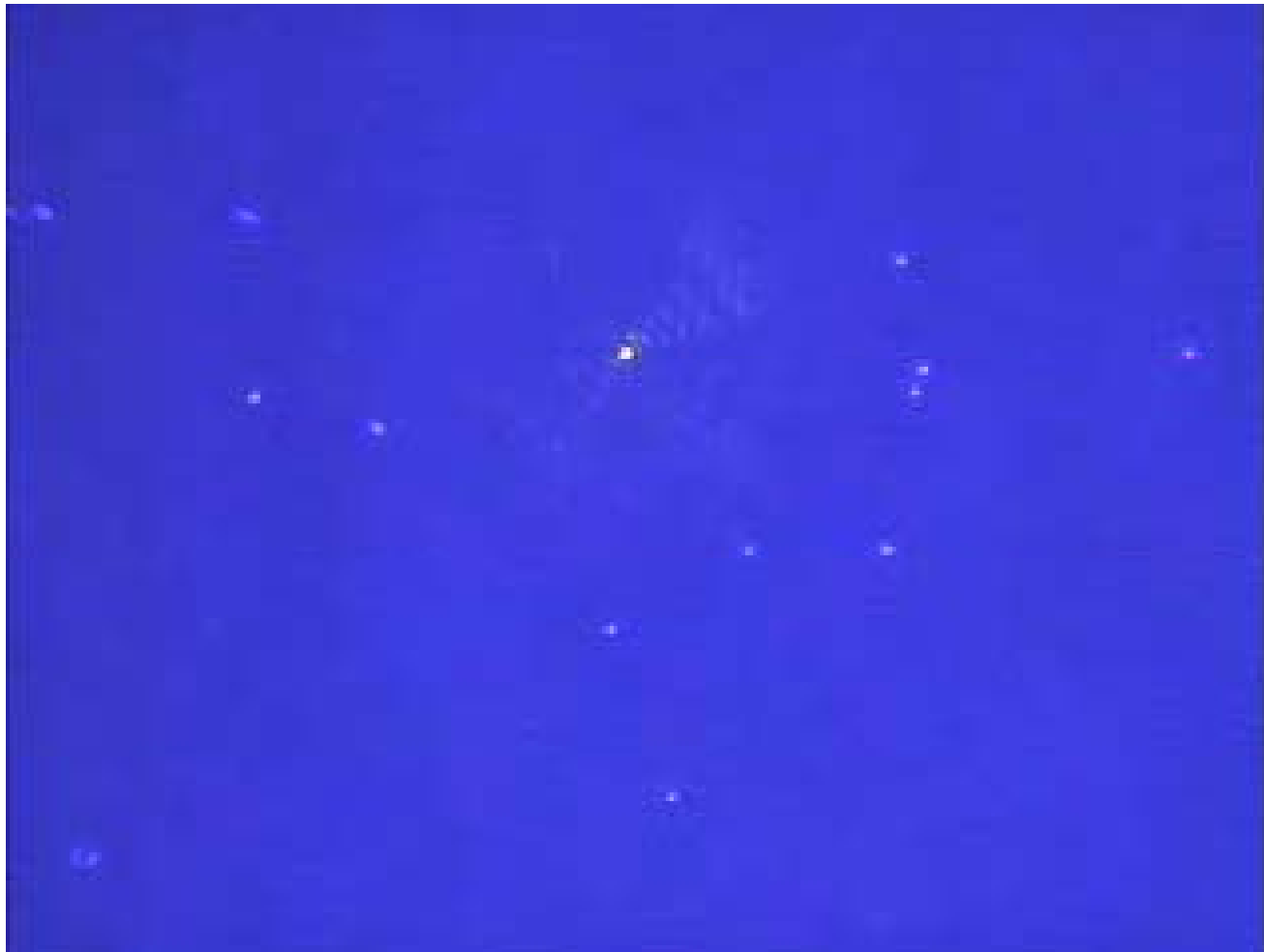
# Phase-contrast and Raman spectral images of multiple trapped dormant *B. cereus* spores

Figure-3 (Li)

a Phase contrast image

b

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

23

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.

24

II-4) Rapid confocal Raman imaging with multifoci-scan

1 μm $1017\ cm^{-1}$
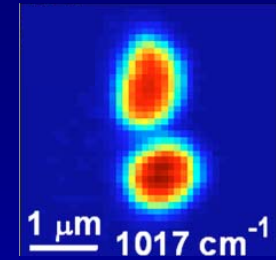
- How to obtain spatial distribution of specific cellular molecules (such as CaDPA, carotenoid, proteins, or nuclei acids) of single cells?

- 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).*

25

Multifoci-scan confocal Raman microscopy
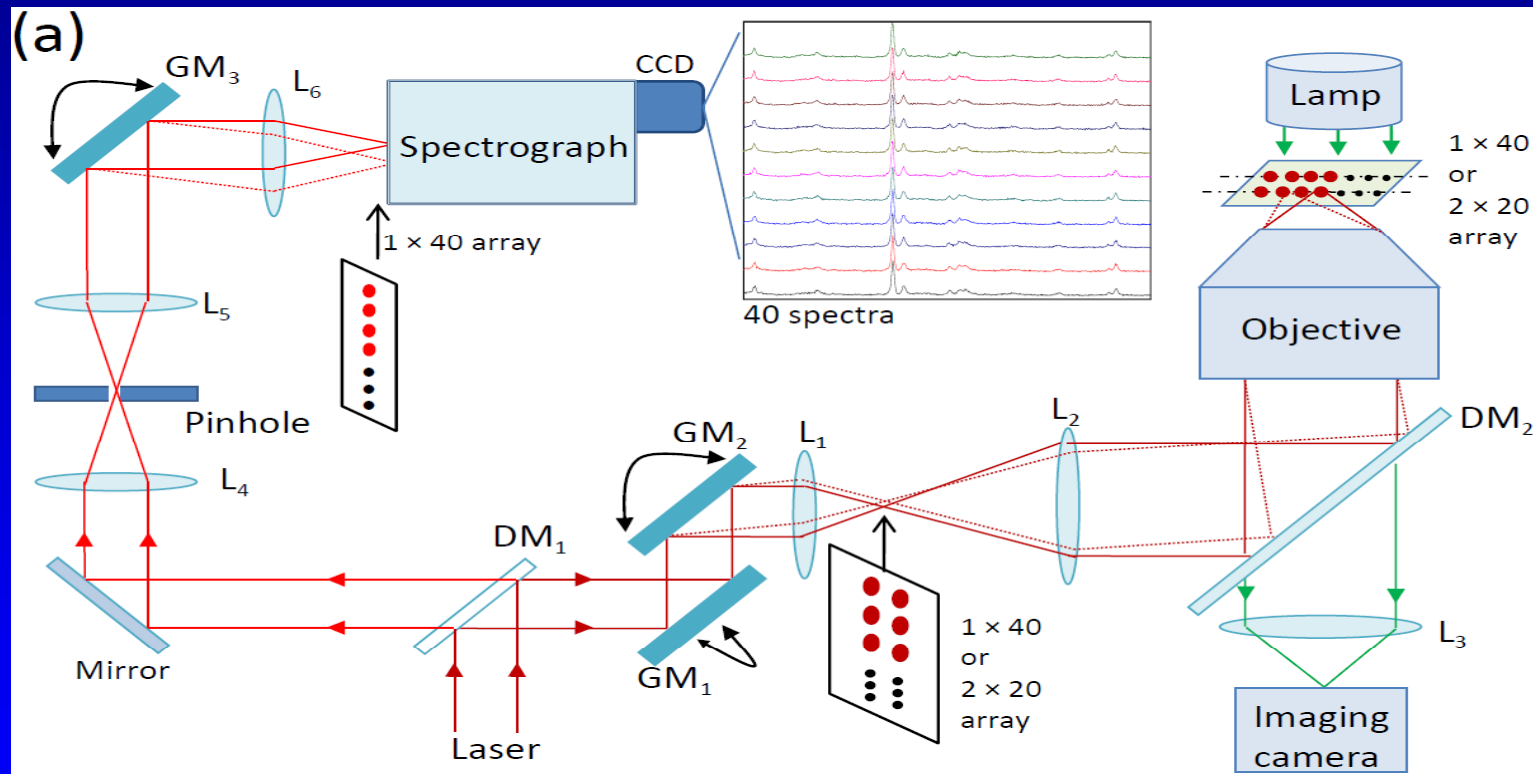
Synchro multifoci-scan allows acquiring 40-80 spectra simultaneously while retaining point-scan resolution. The image acquisition time is 40-80 times faster.

Spatial Resolution
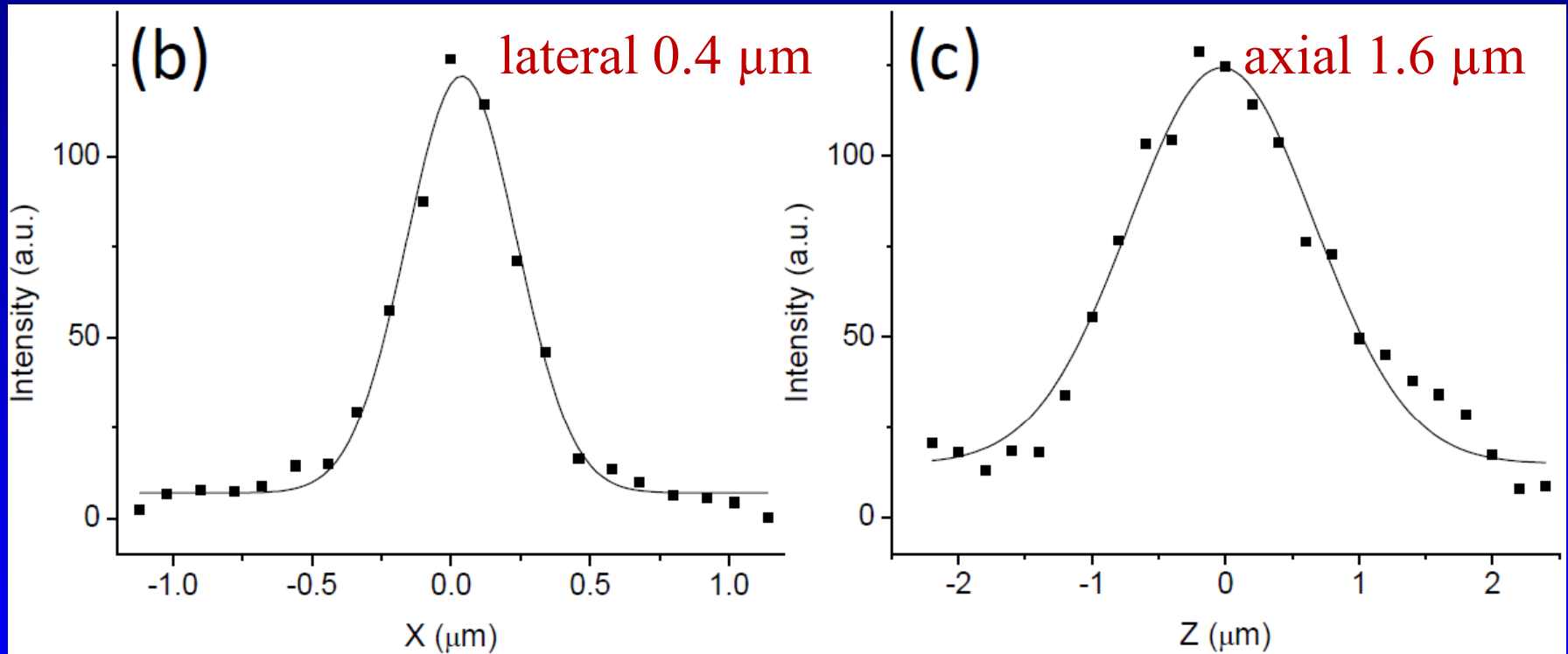
- Measured lateral and axial resolution using a 100-nm diameter polystyrene bead Raman band at 1001 cm^{-1}.

Point-scan verse multifoci-scan

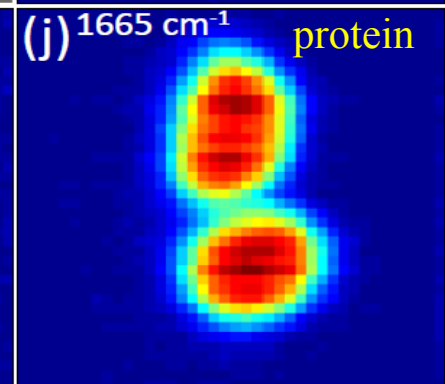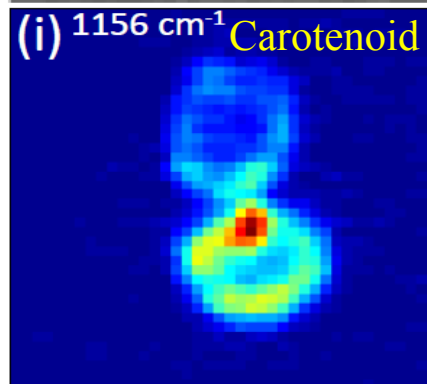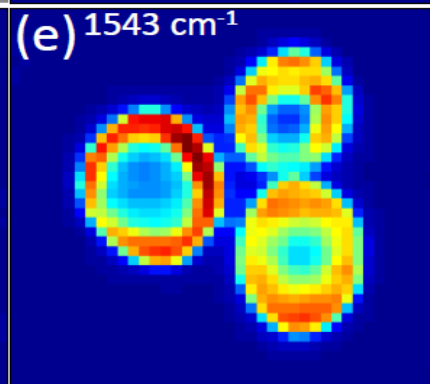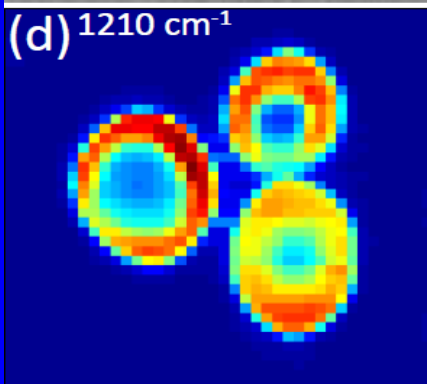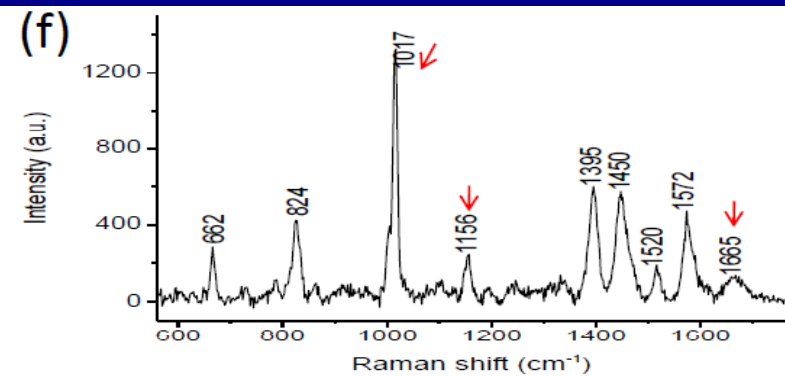Bright-field image   Point-scan (40 min)   Multifoci-scan (1min)
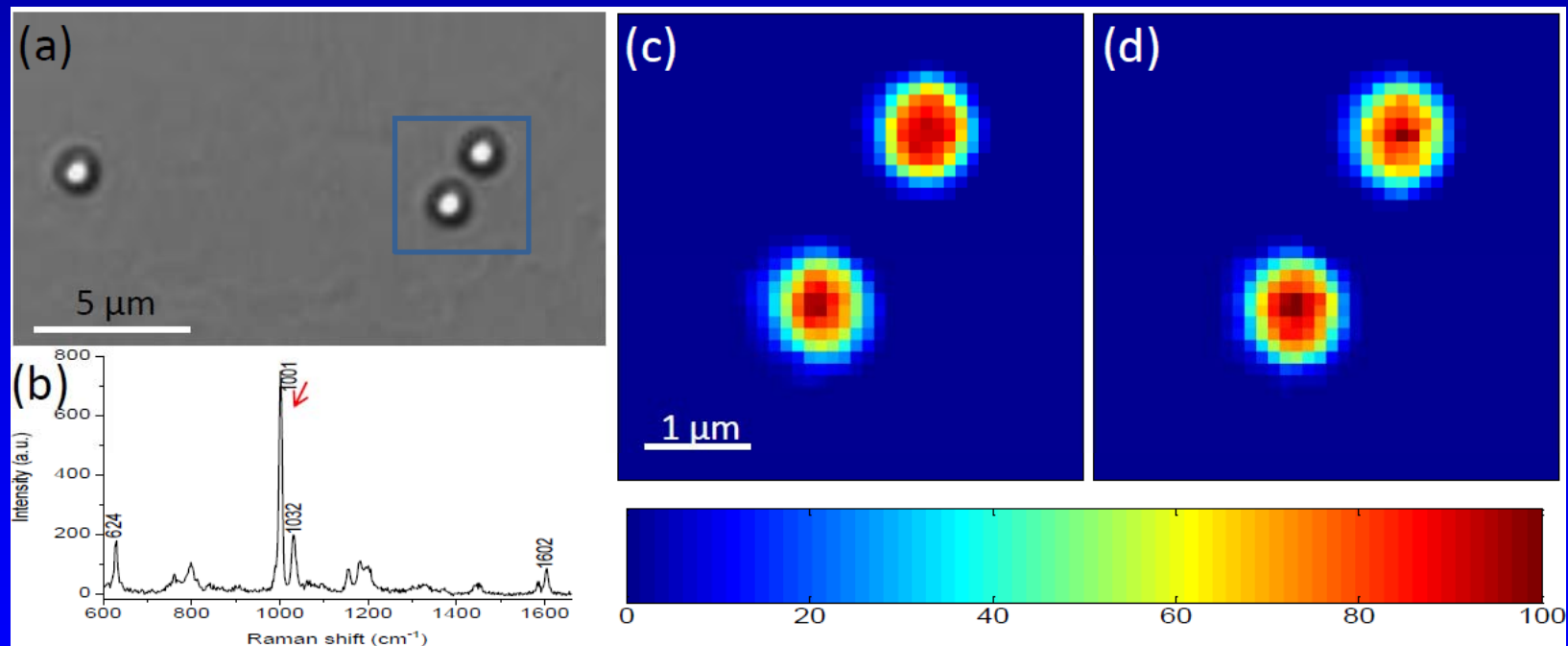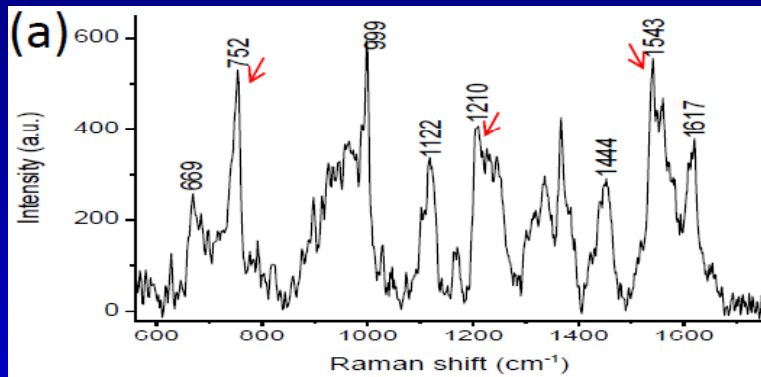

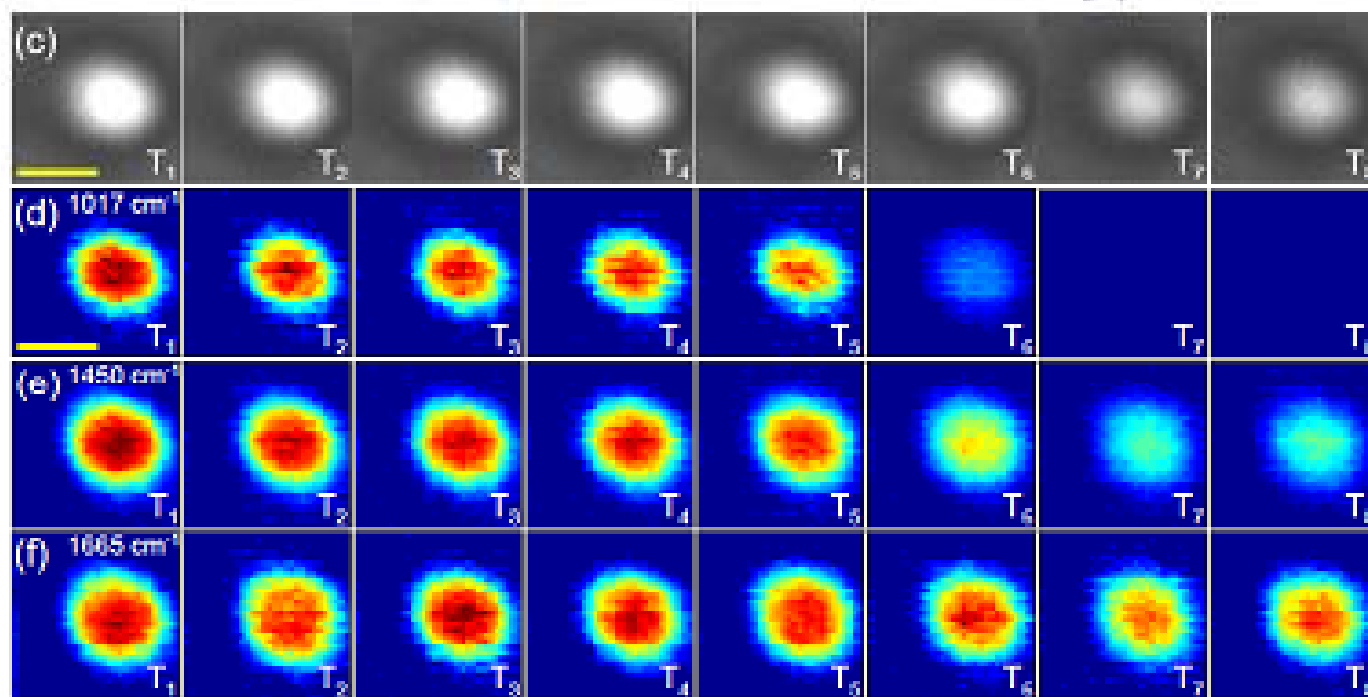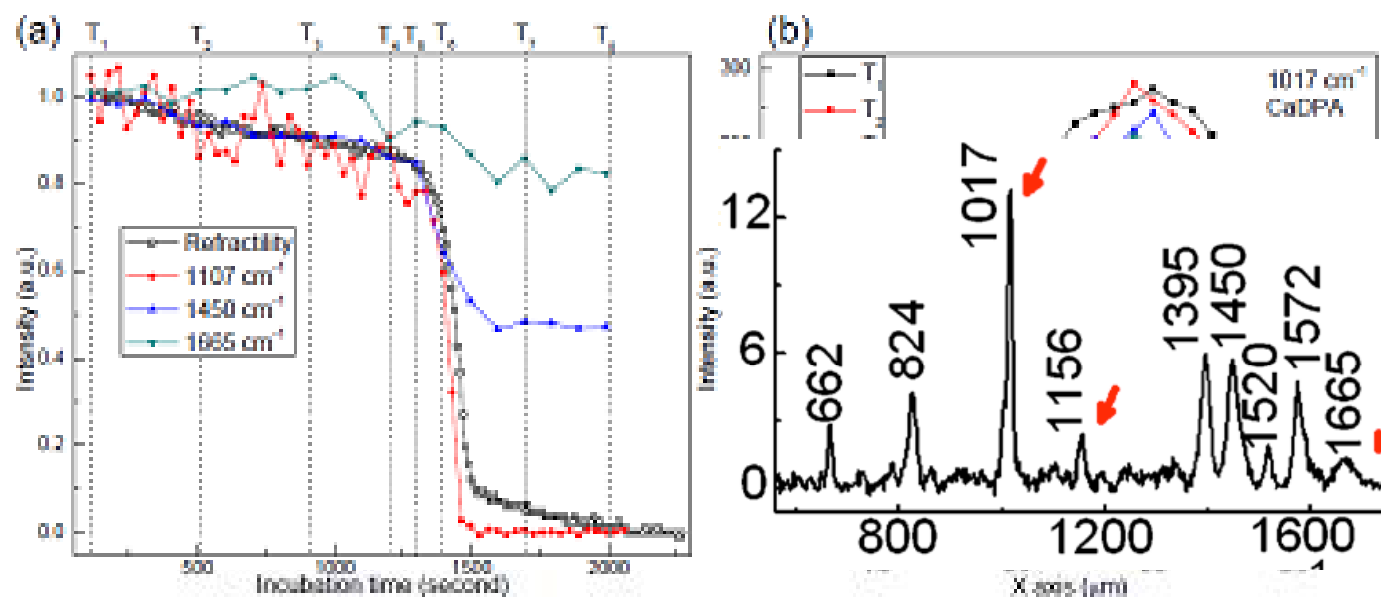The distance between two 1-µm polystyrene beads: 1.919 ± 0.008 µm   1.921 ± 0.008 µm

Raman images of human red blood cells and *B. megaterium* spores
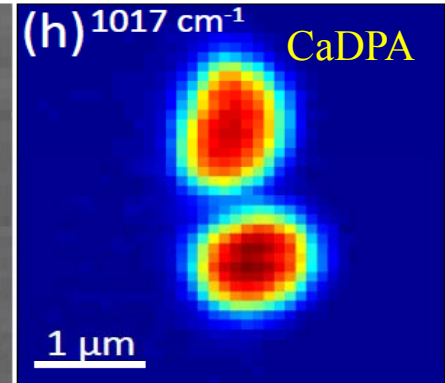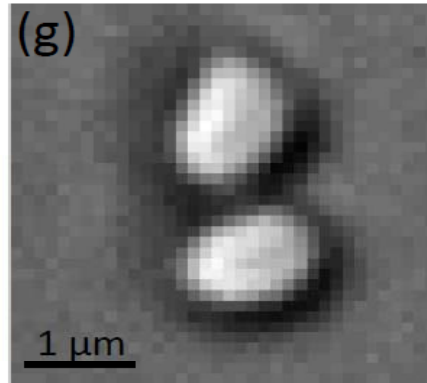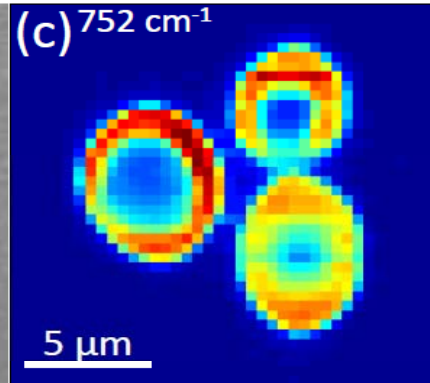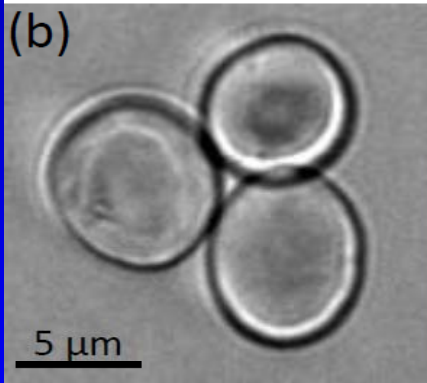
Imaging speed: 33 s /frame

30

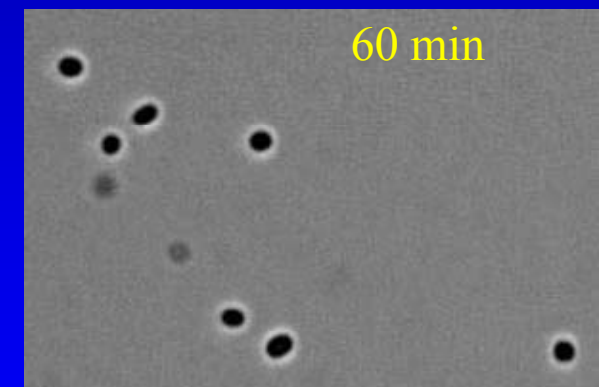# II-5) Image-guided multifocus confocal Raman microspectroscopy

- 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)?

  L.B. Kong et al, *JBO* 16, 120503 (2011).
  P.F. Zhang et al, JAM (2012).

31

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.

0 min

60 min
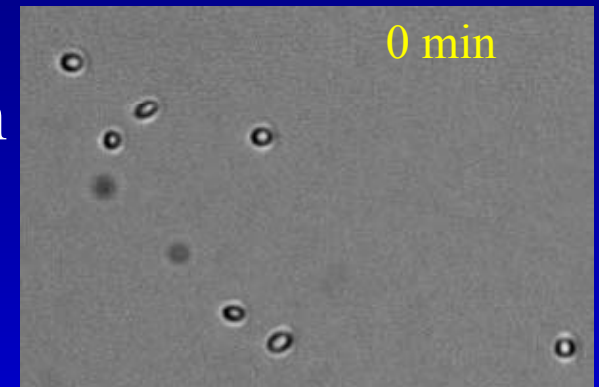
Drift ~500 nm / 20 min

Image-guided multifocus confocal Raman microscopy
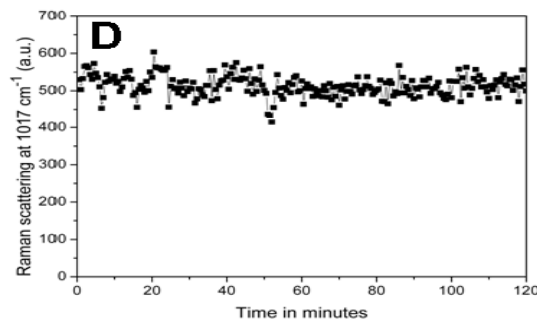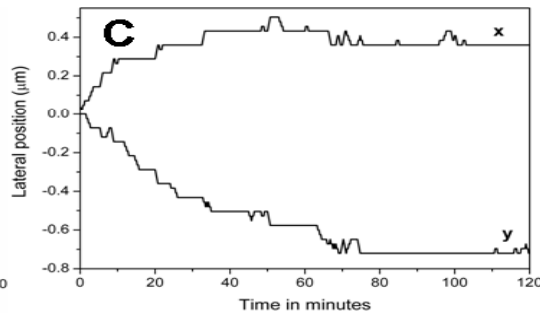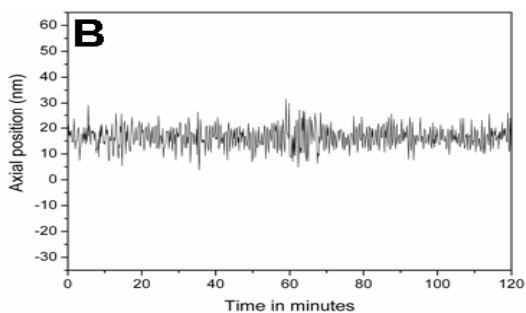
Active focus locking - bacterial spores

Without locking

Drift = ~500 nm / 20 min

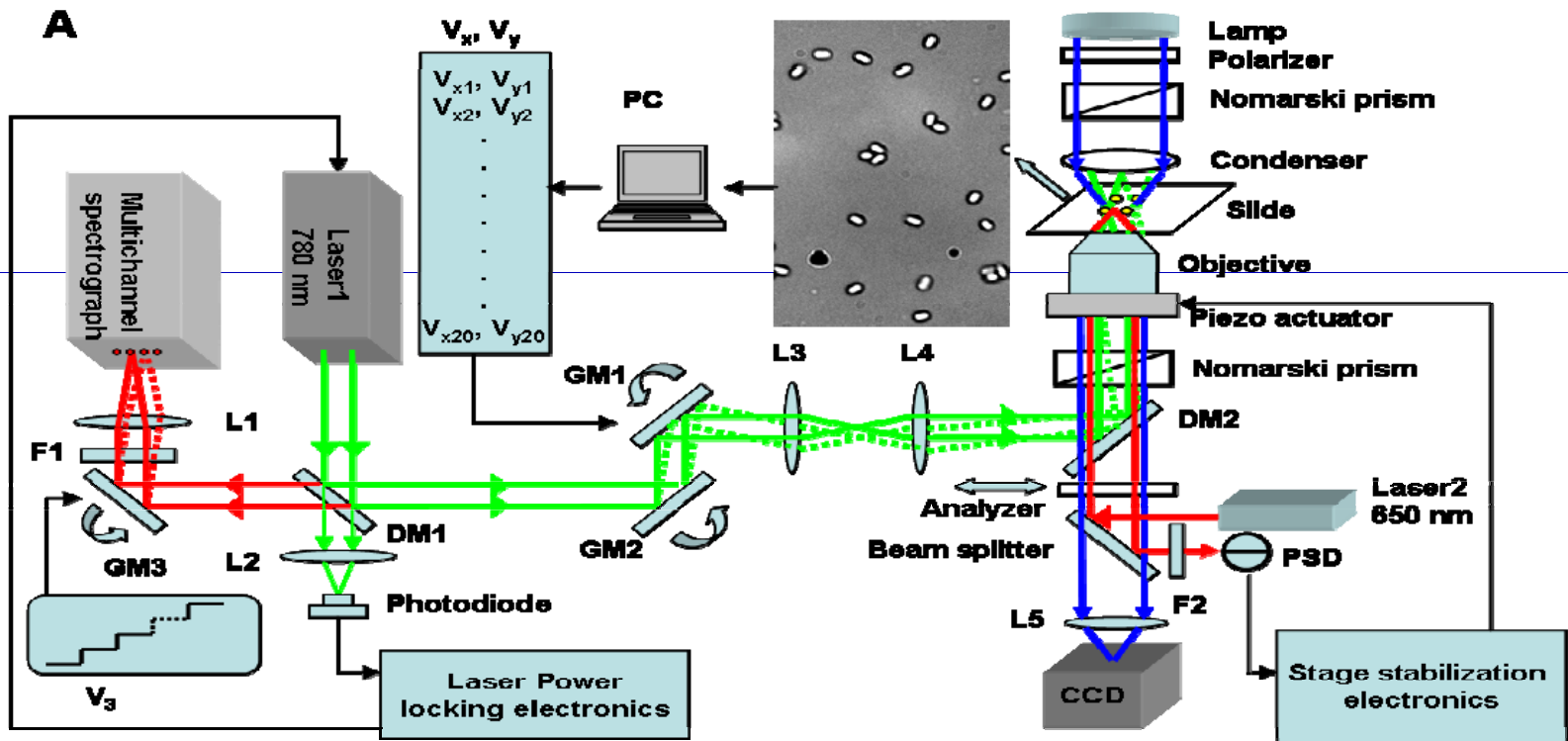After locking
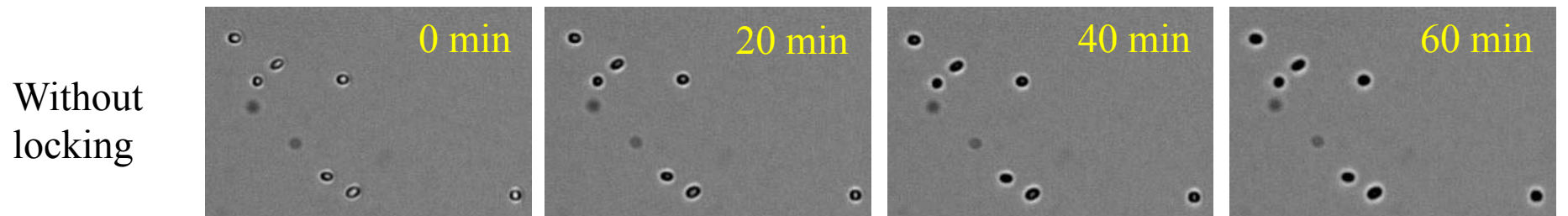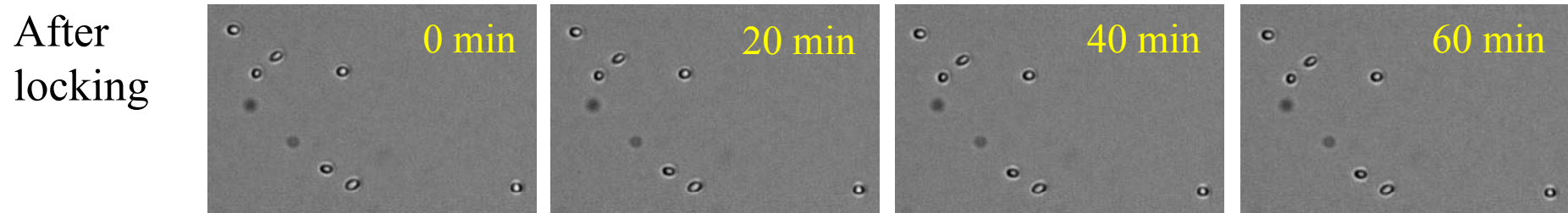
L.B. Kong et al, *JBO* 16, 120503 (2011).
P.F. Zhang et al, J. Appl. Microbiol. (2012).

Image-guided multifocus confocal Raman microscopy

1340x400 pixels

Mixed polystyrene beads (numbered in black) and *B. megaterium* spores

Monitor slow germination of individual superdormant spores for >12 hours
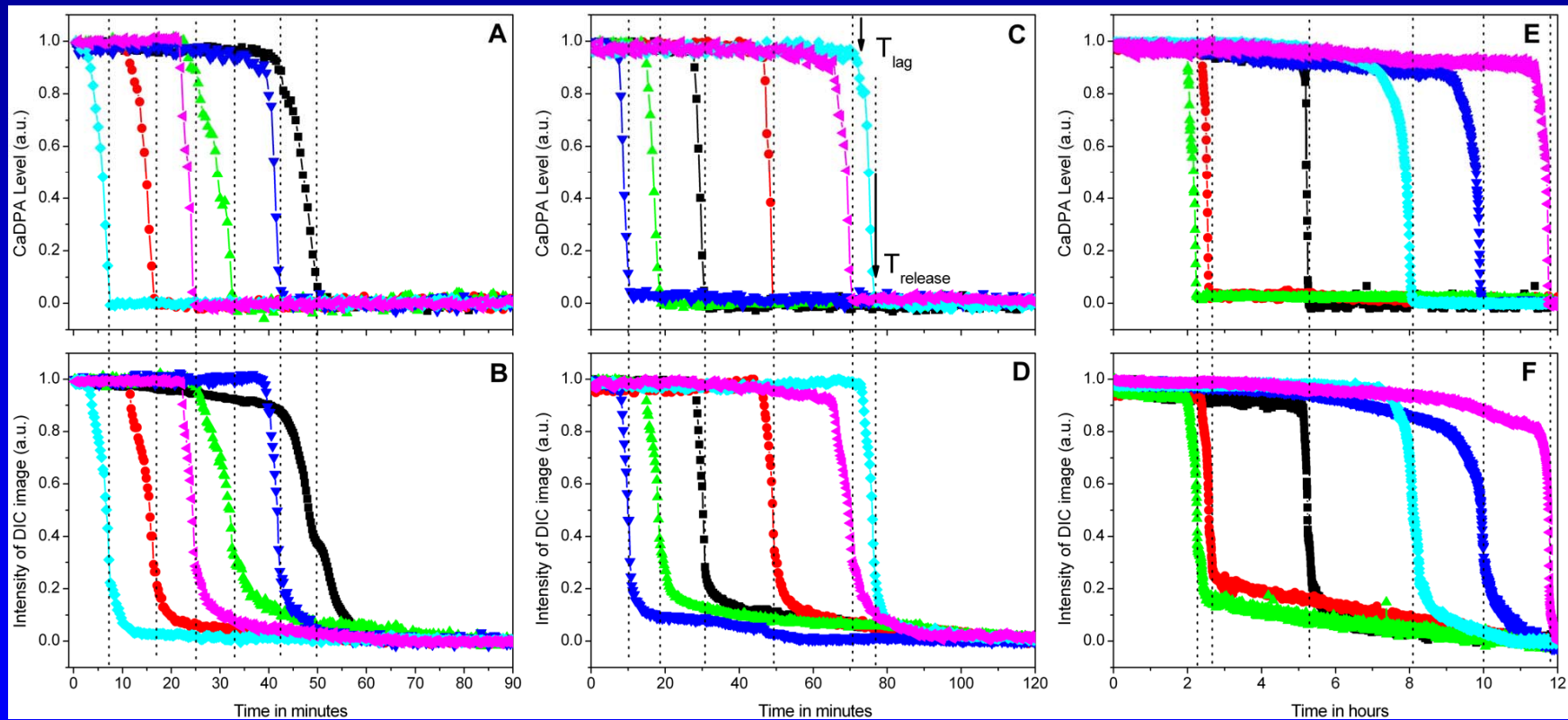
Dormant spores
with L-valine

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
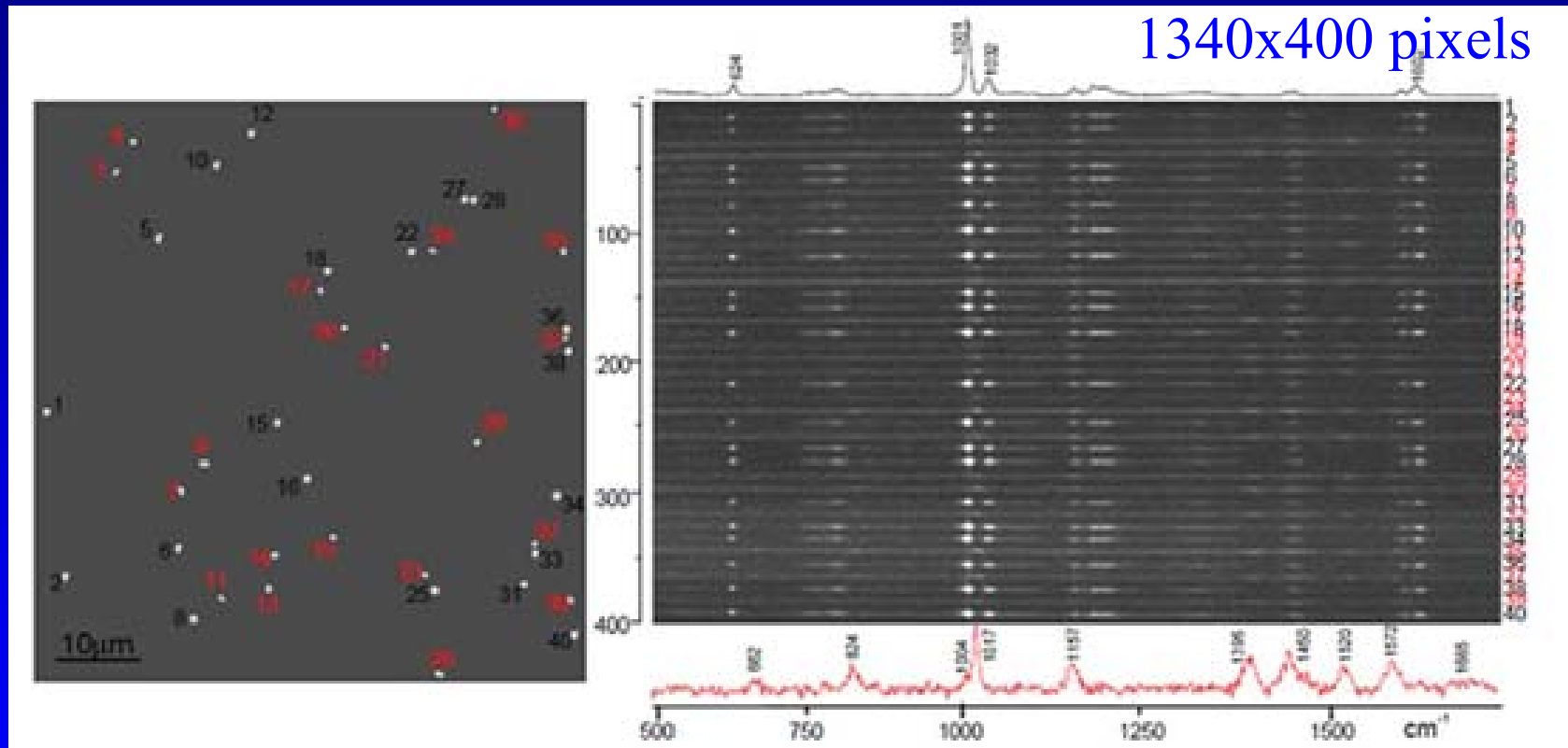- Biosensing: identification and detection of environmental microorganisms
- Biotech: probing recombinant protein production in transgenic cells
- Medical: cancer cells, virus-infection and thalasemia
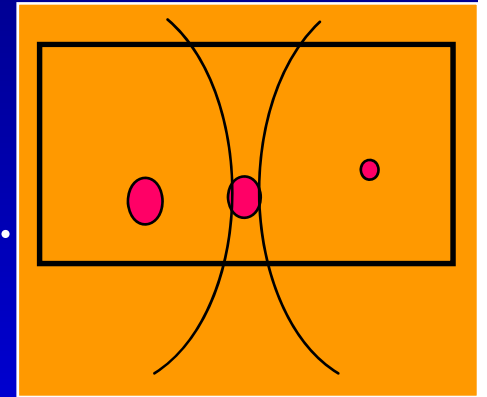
III-1) Raman Sorting

- Can we sort different types of cells in a mixed sample ?

Micro-channel

Sample chamber

Raman scattering

Laser tweezers

Collection chamber
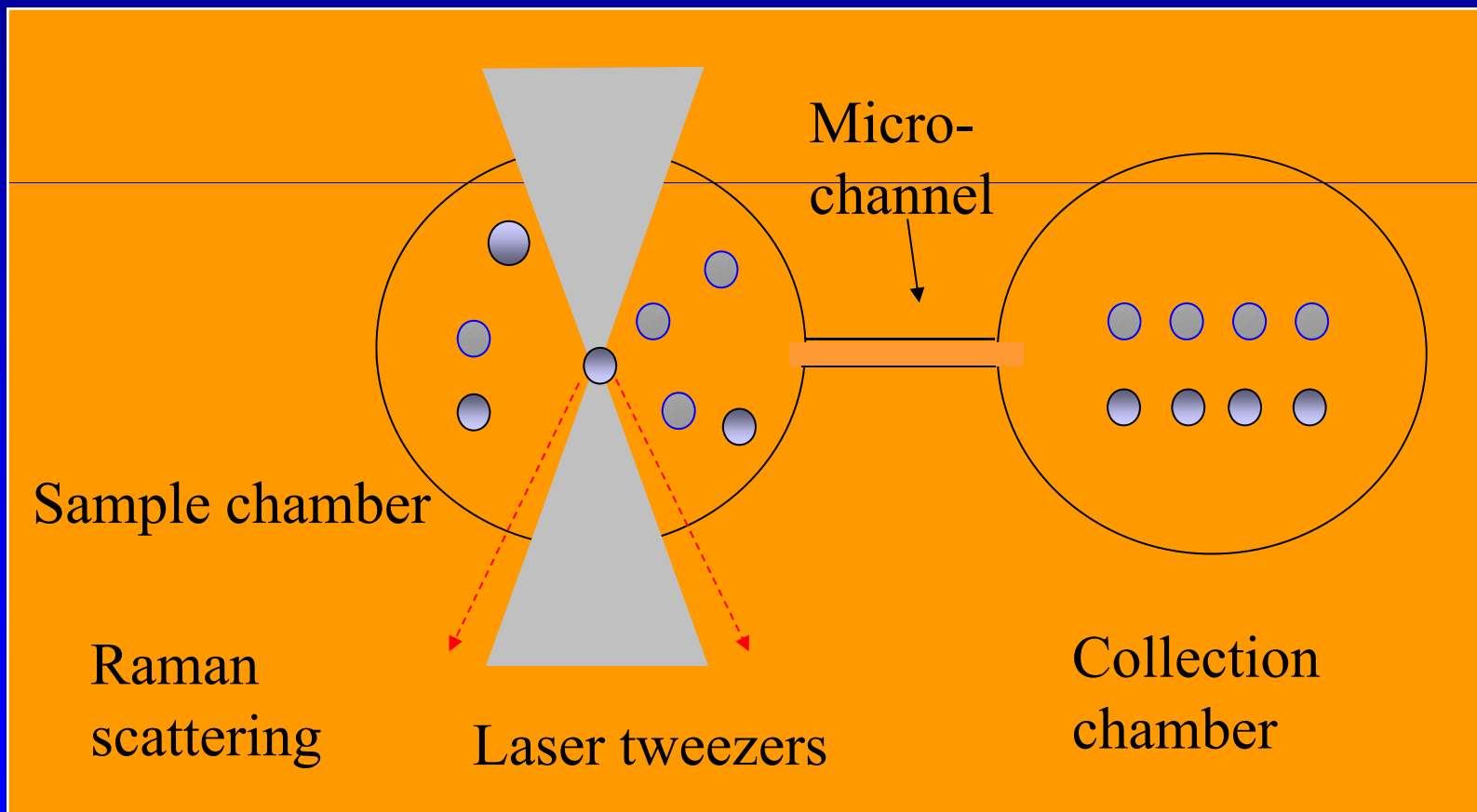
Xie et al, Opt. Lett. **30**, 1800 (2005).

38

Sorting living yeast cells
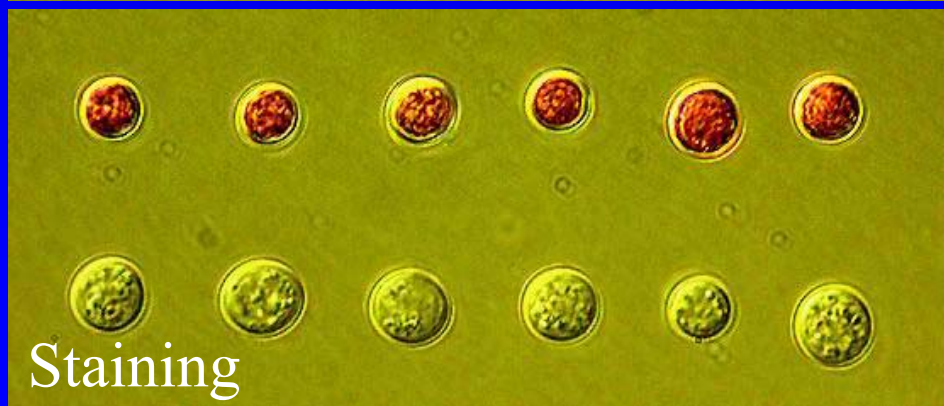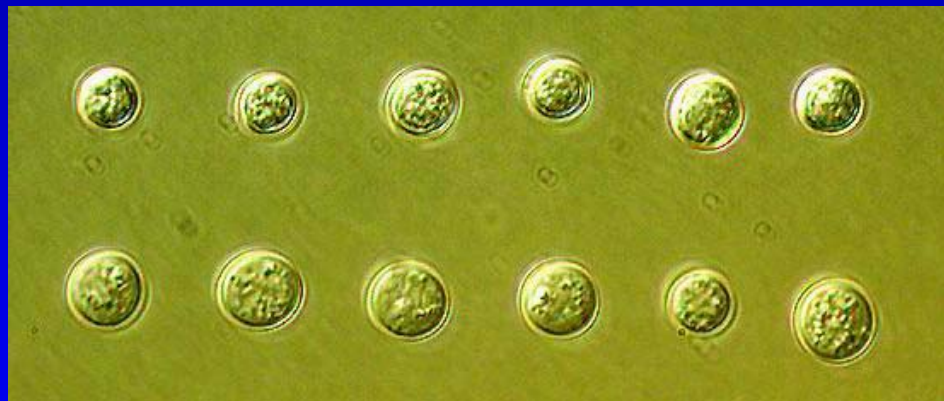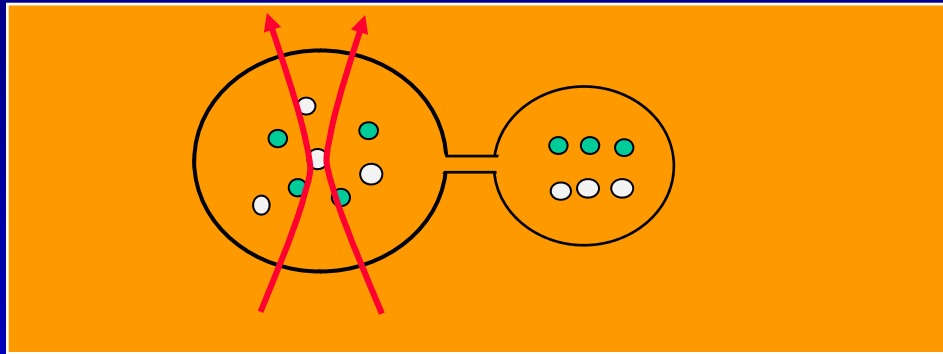
Staining
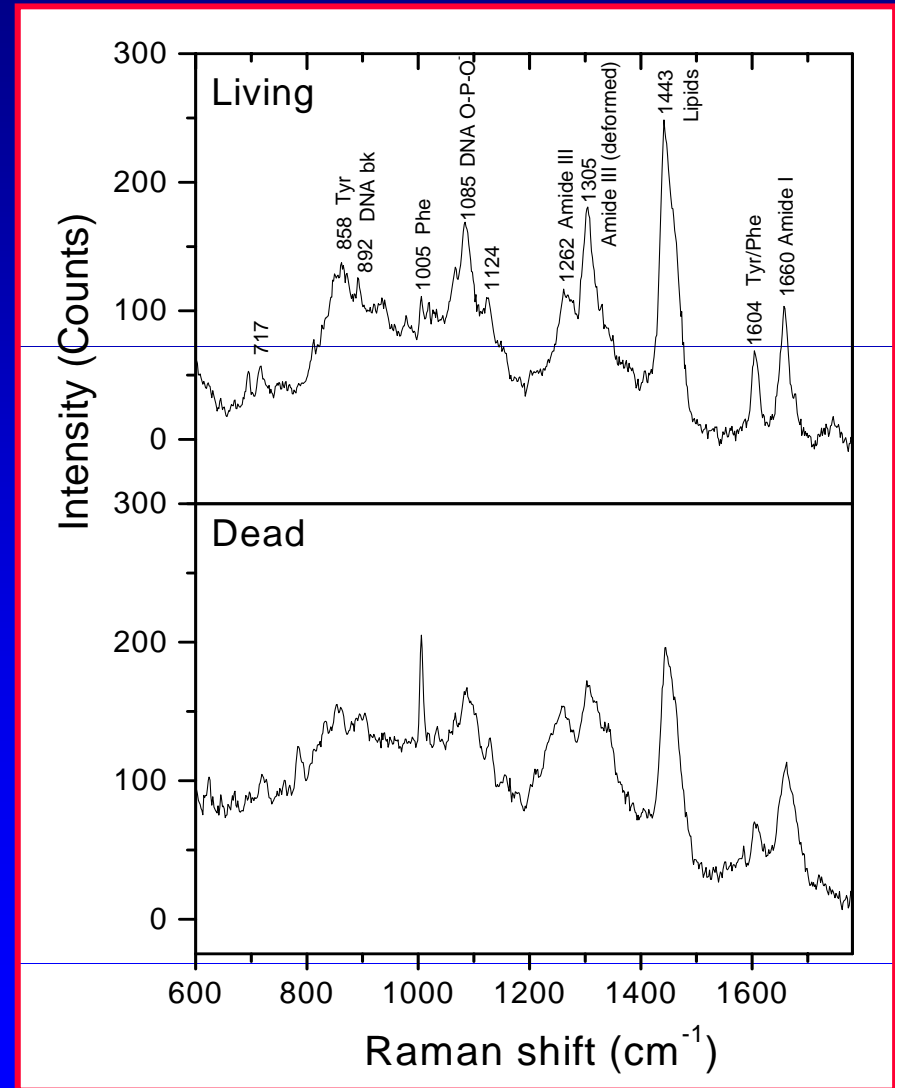
❑ Manipulation and discrimination of single unstained human chromosomes

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

G-banding

Sample reservoir   Buffer reservoir   Fixed slide

-0:01:31
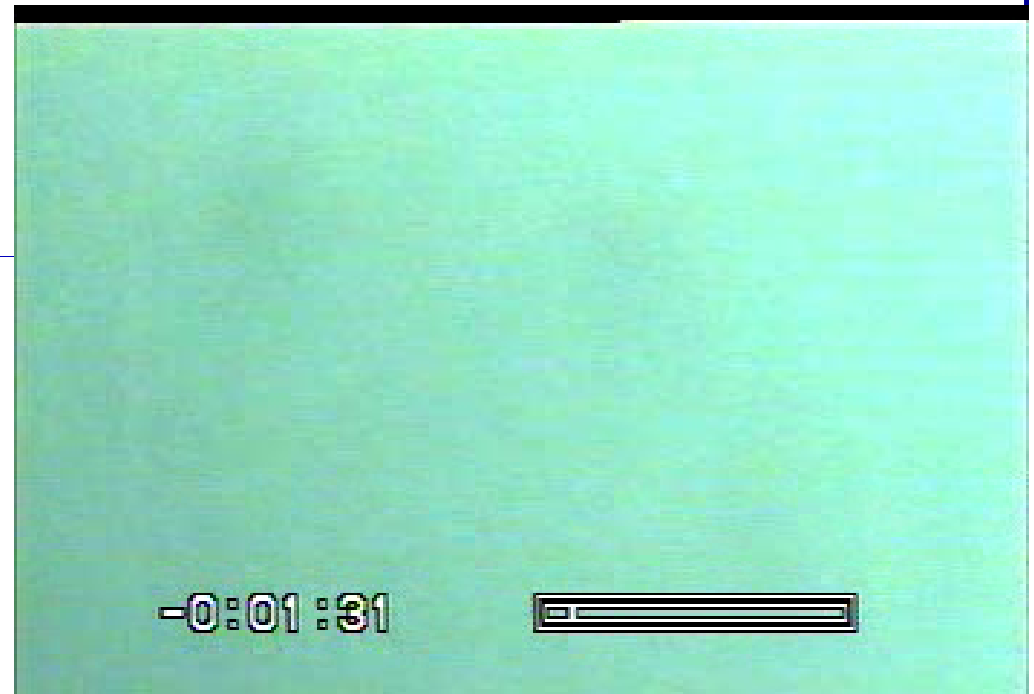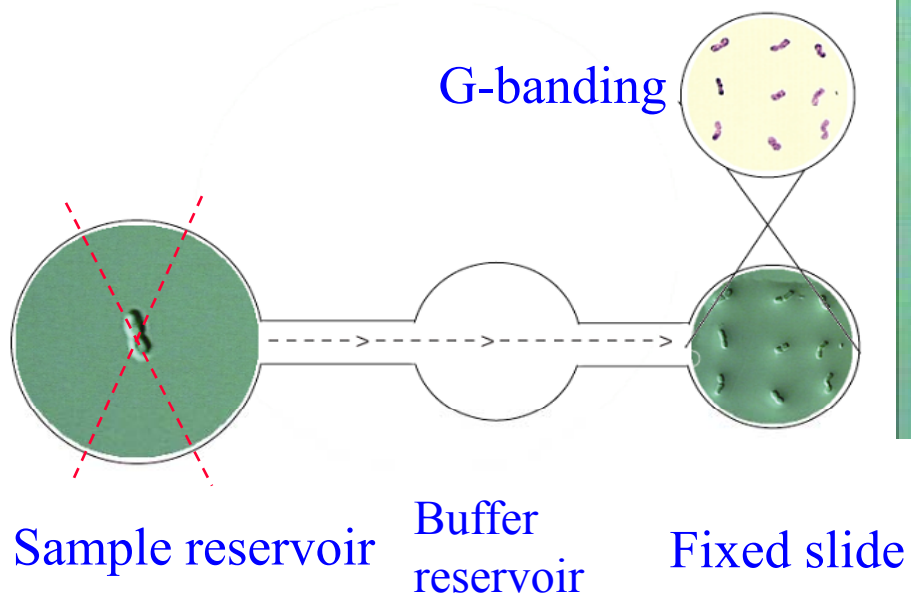
Unstained chromosomes of leukemia cells

Opt. Exp. 14, 5385-5393 (2006).

III-2) Detect single-cell dynamics
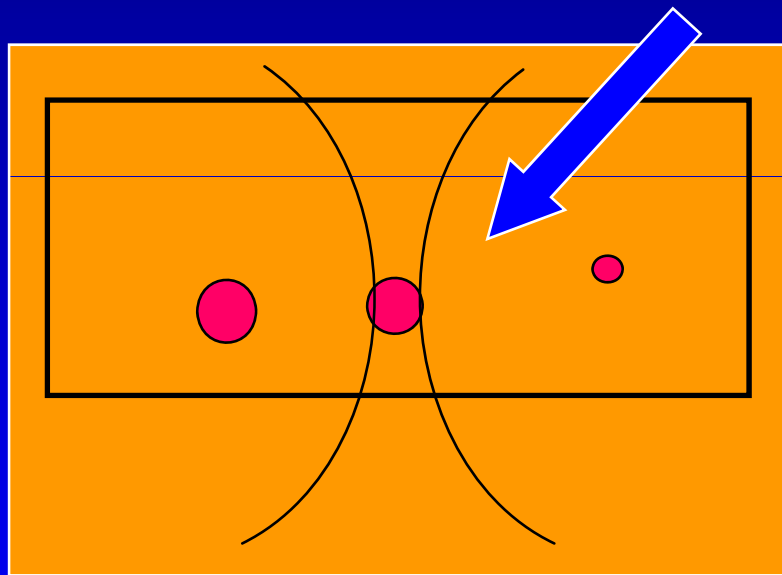
- Physical agents: heat, UV, ultrasonic, microwaves …
- Chemical agents: drug, PH, toxin, bleach…
- Biological: nutrient triggered germination, growth, reproduction, protein expression, virus-infected …

Examples include spore germination, heat-inactivation, production of biofuel molecules, yeast fermentation......

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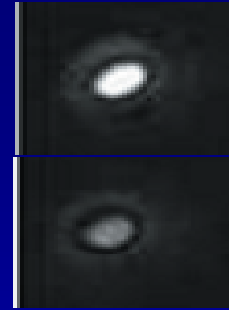
- Bacterial spores are metabolically dormant and can survive in this dormant state for many years. But they can sense the environment and return to life via the process of spore germination when nutrients present.

- 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.

dormant

germinated

EM

COATS

OUTER SPORE MEMBRANE

INNER SPORE MEMBRANE

GerP proteins

GERM CELL WALL

CORTEX
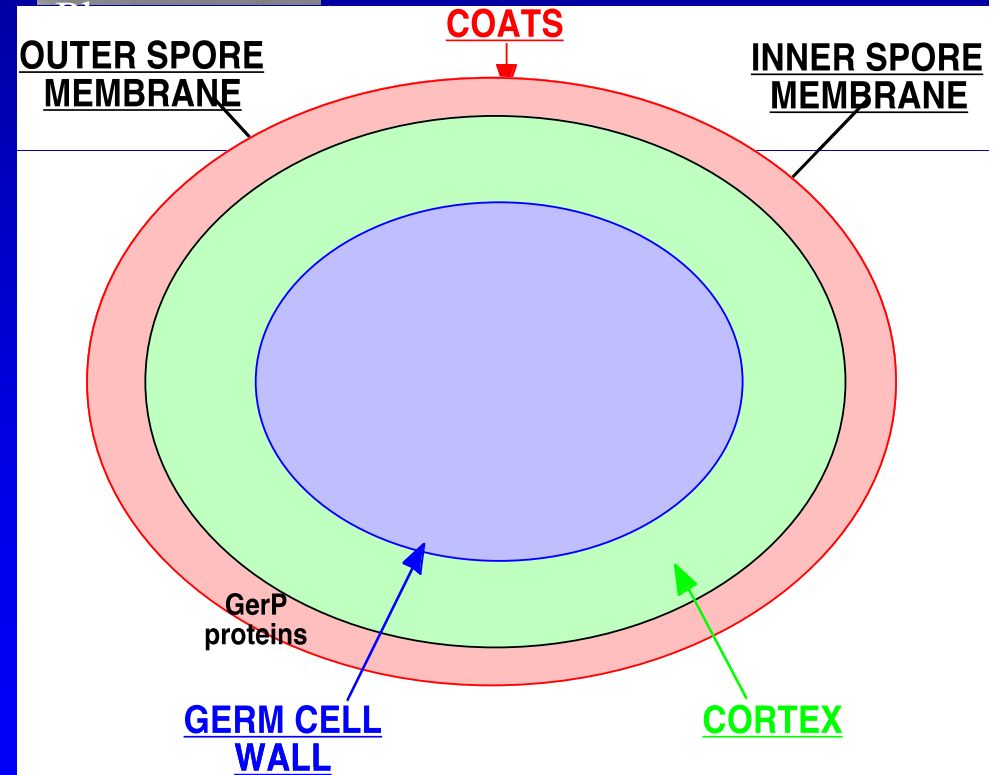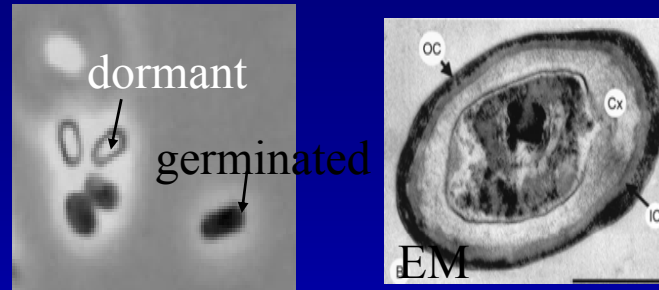
- The CLEs in *B. subtilis* spores are CwlJ and SleB.

43

Real-time detection of kinetic germination and heterogeneity of single Bacillus spores
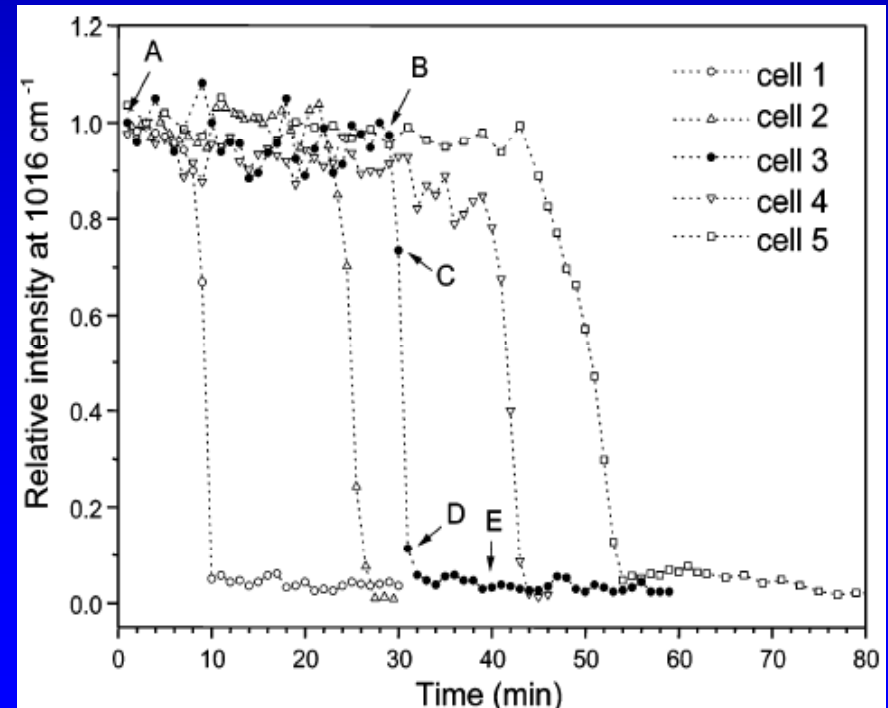
D. Chen et al, *Anal Chem*, 78:6936 (2006).
L. Peng et al; *Anal. Chem.* 81:4305 (2009).
L.B. Kong et al. *Anal. Chem. 82,* 3840–3847 (*2010).*
L.B. Kong et al. *Anal. Chem. 82,* 8717–8724 (*2010).*

Add L-alanine
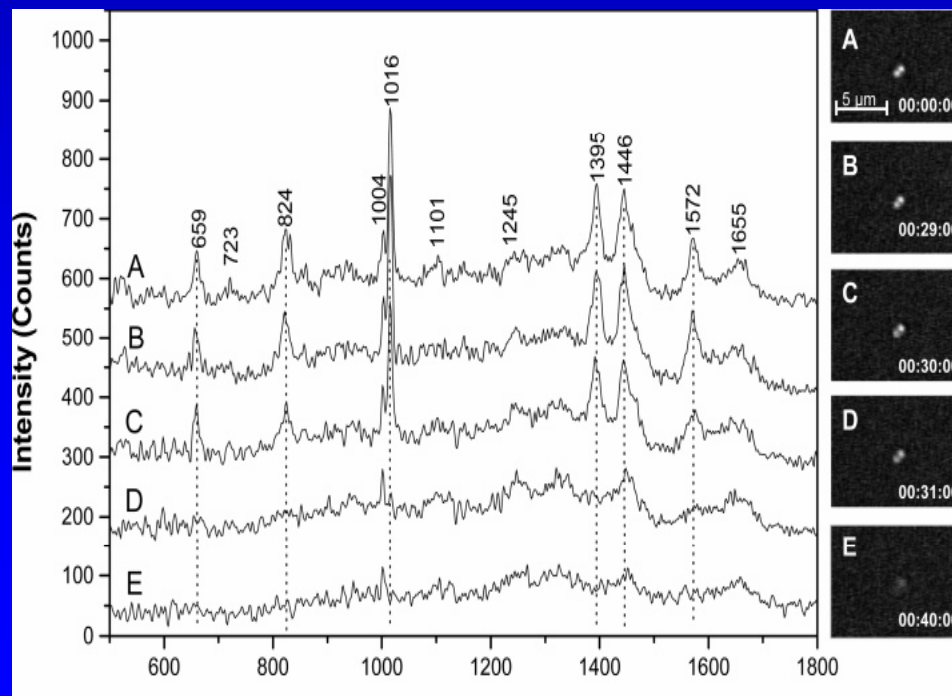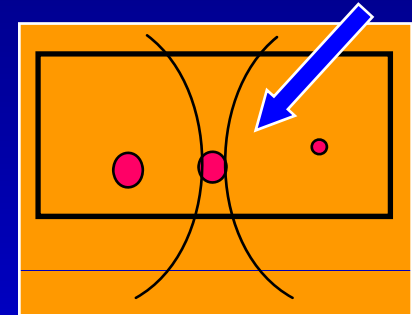
Characterization of Bacterial Spore Germination Using Integrated Phase Contrast Microscopy, Raman Spectroscopy, and Optical Tweezers

Lingbo Kong,[†] Pengfei Zhang,[†] Peter Setlow,[‡] and Yong-qing Li*,[†]

dormant
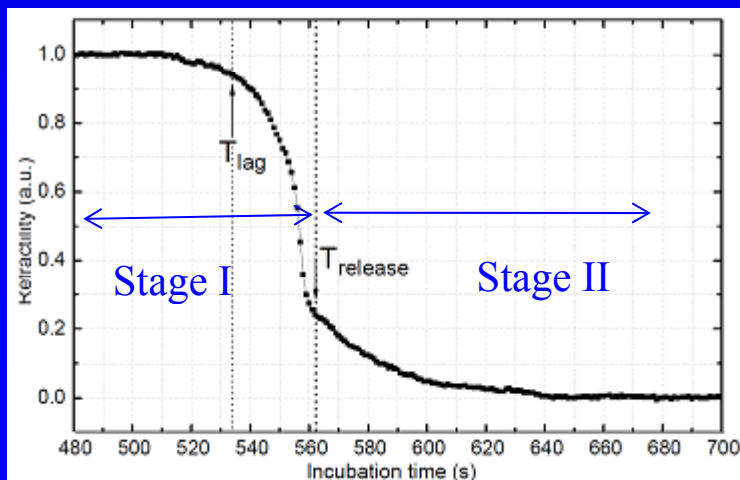
germinated

- 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.

45

Simultaneous monitoring of L-alanine germination of a single optically trapped *B. cereus* spore by Raman spectroscopy and PC microscopy
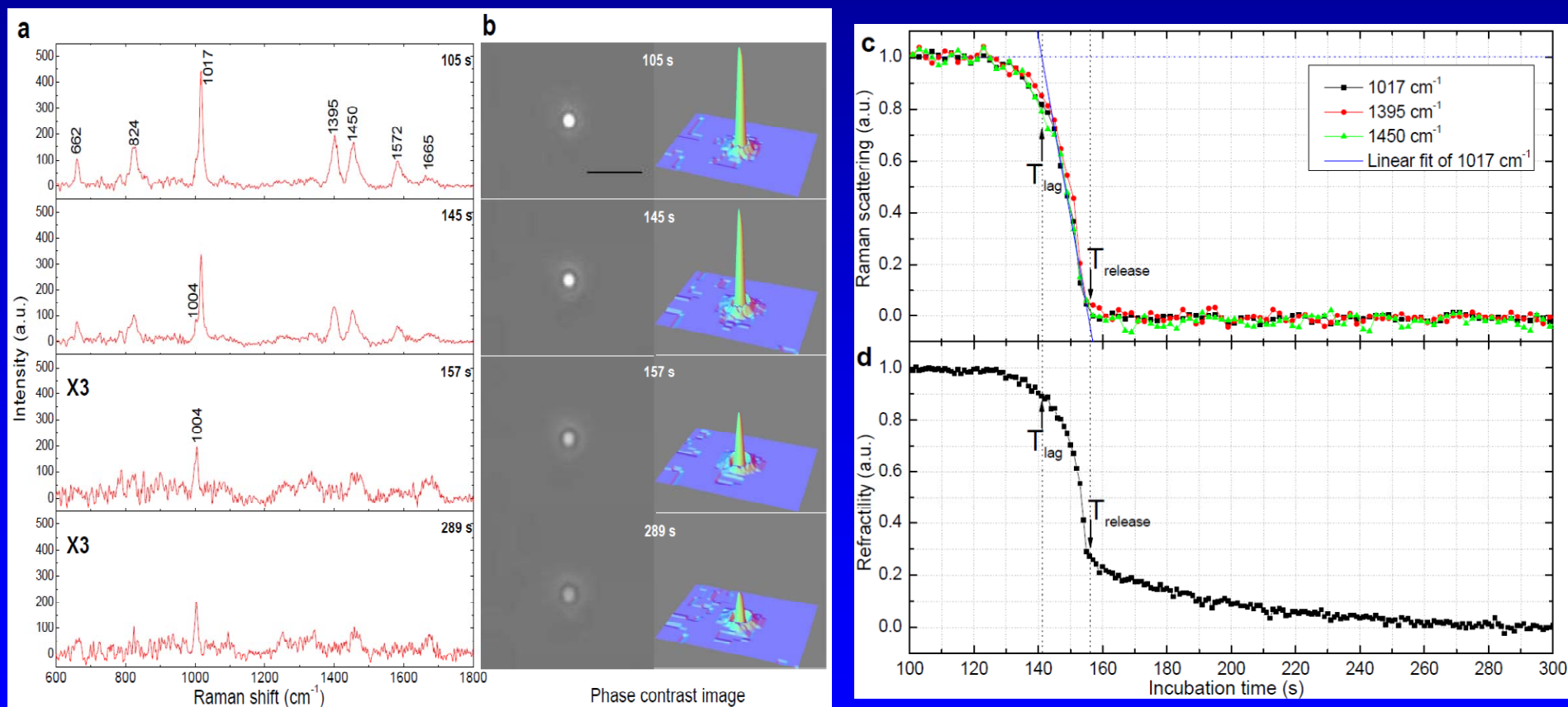
Kong et al. Anal. Chem. 82, 3840–3847 (2010).

1. When repeat the experiments with other cells, we found their responses varied.
2. Some cells response earlier, some later. But correspondence always exists.
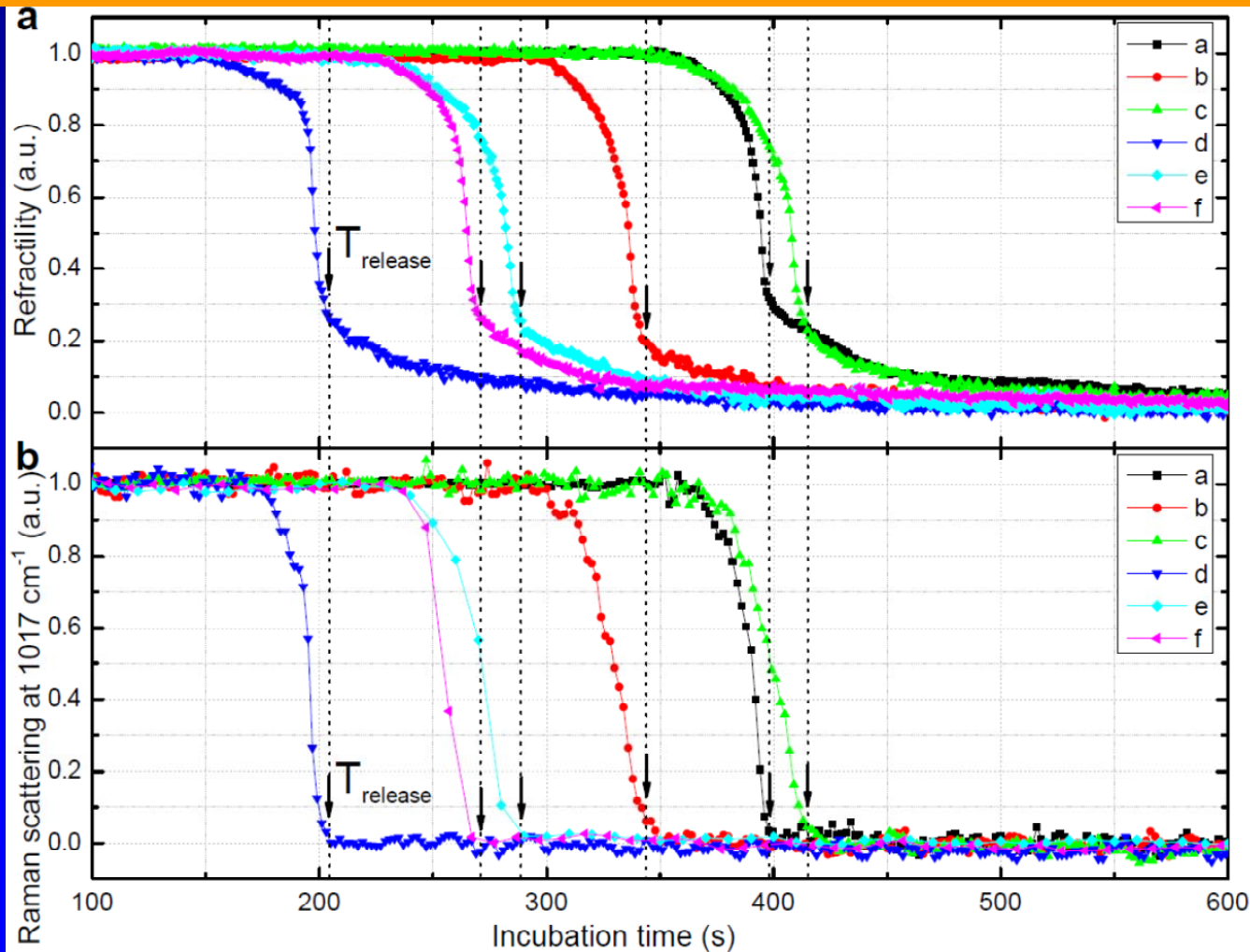
Monitoring the Kinetics of Uptake of a Nucleic Acid Dye during the Germination of Single Spores of Bacillus Species

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?

CaDPA

Water, SYTO 16 stain...

48
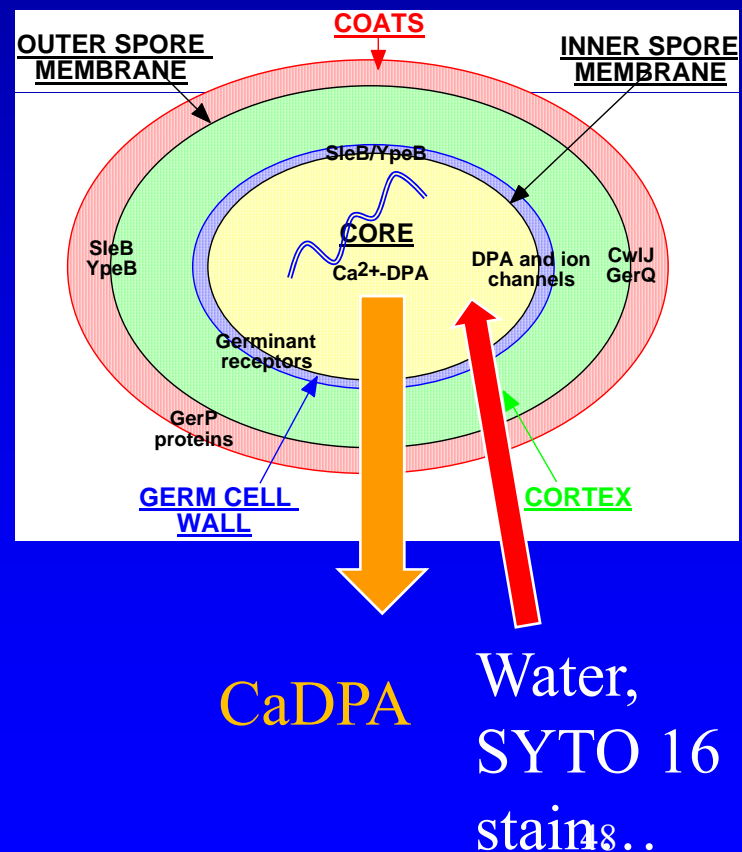
Simultaneous recording of Raman spectra, phase contrast images and fluorescence images of a single trapped *B. cereus* spore germinating at 24 °C with 1 mM L-alanine plus 500 nM Syto 16 in 25 mM Tris-HCl buffer (pH 7.4)
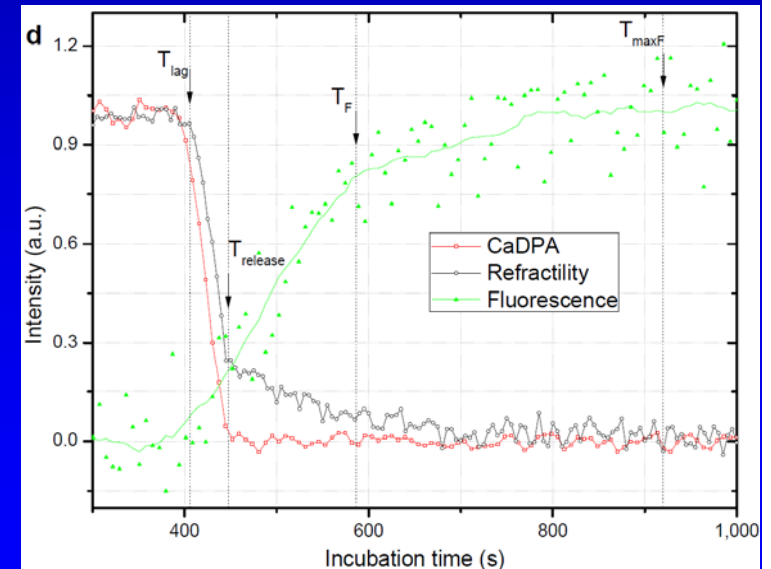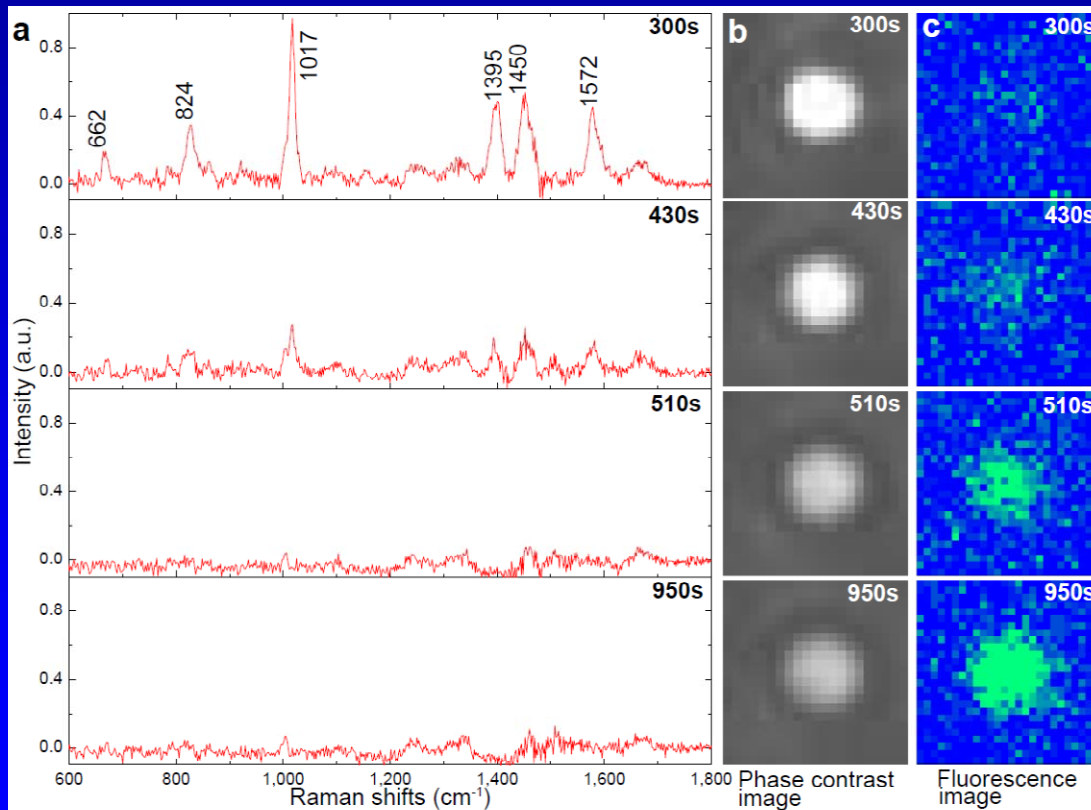
Results obtained

- 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.
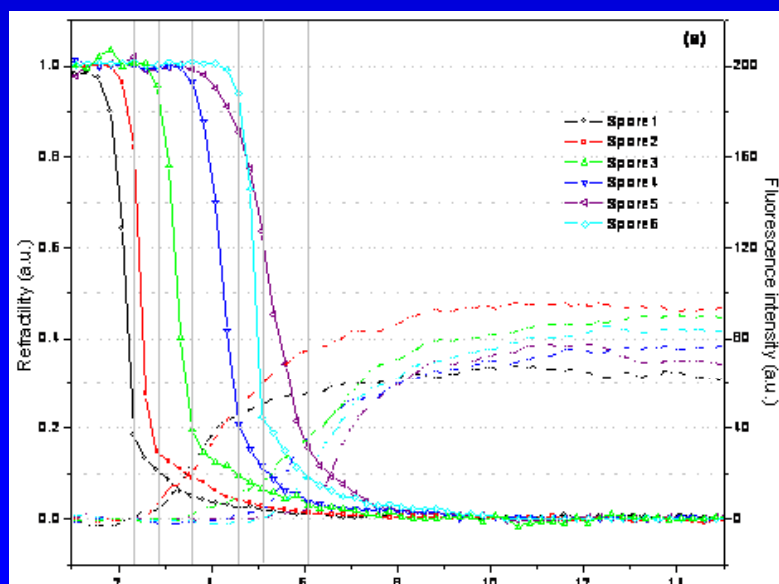
C) Characterization of thermal inactivation of single spores by wet heat

- **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

High Temp
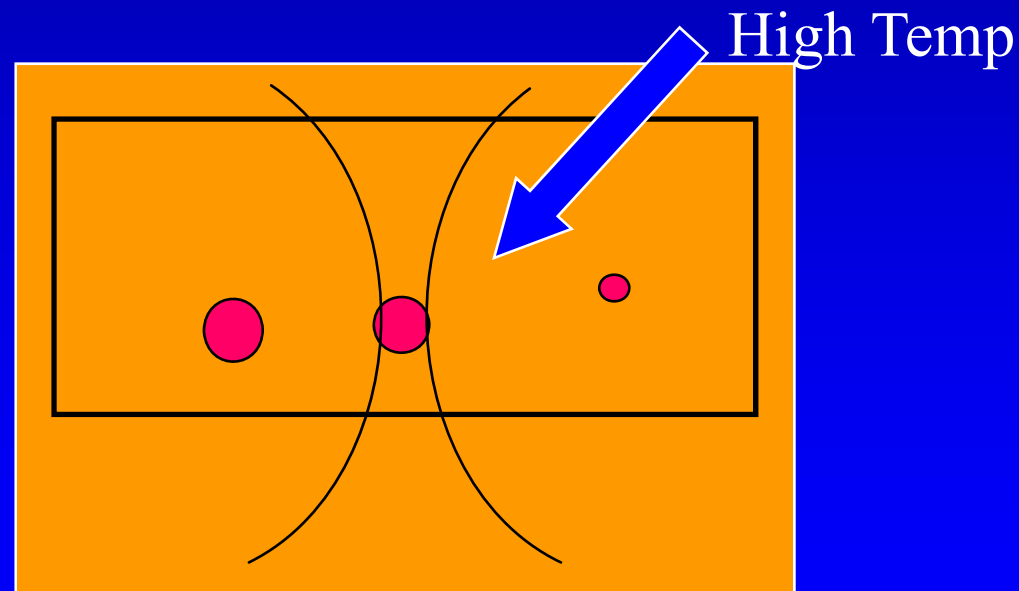
Real-time Raman, ELS, PC or DIC imaging…

Wet heat can kill bacteria & spores but how a spore is inactivated is unclear

Control (untreated)

Thermal treated at 90^0 C for 10min

388

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
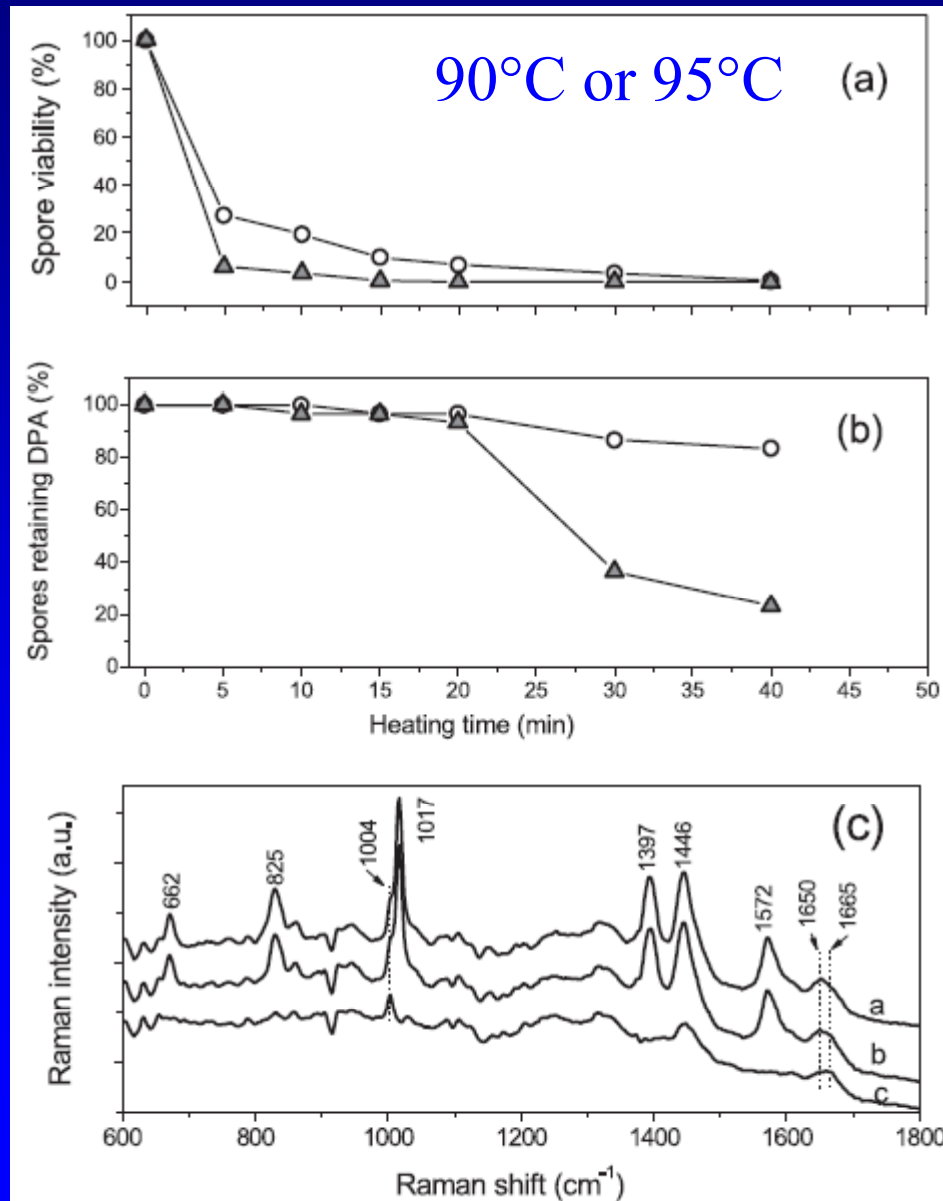
a – untreated (control)
b,c – 95ºC, 30 min

Single *B. cereus* spores heat-treated at 90°C
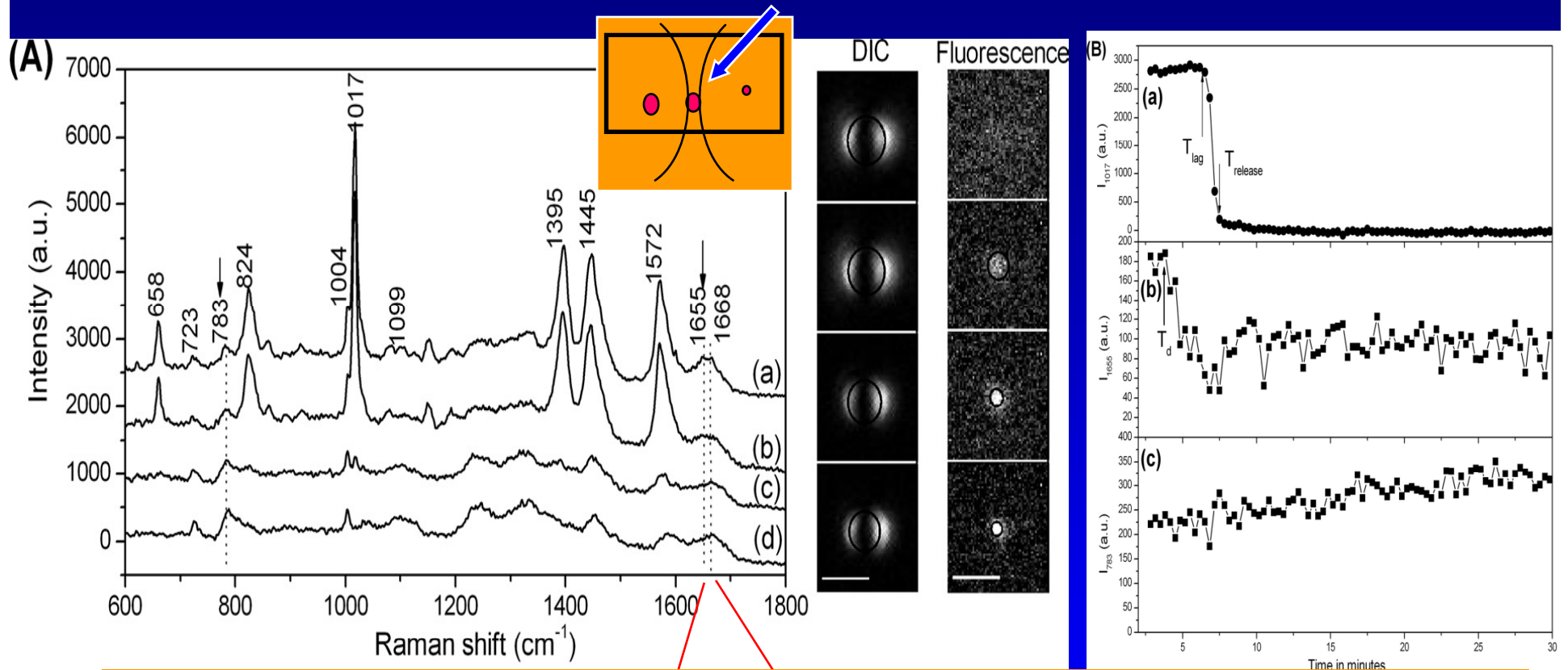
Results: 1) DPA band dropped rapidly at a specific time $t_{release}$.

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

Heterogeneity of individual spores inactivated by wet heat
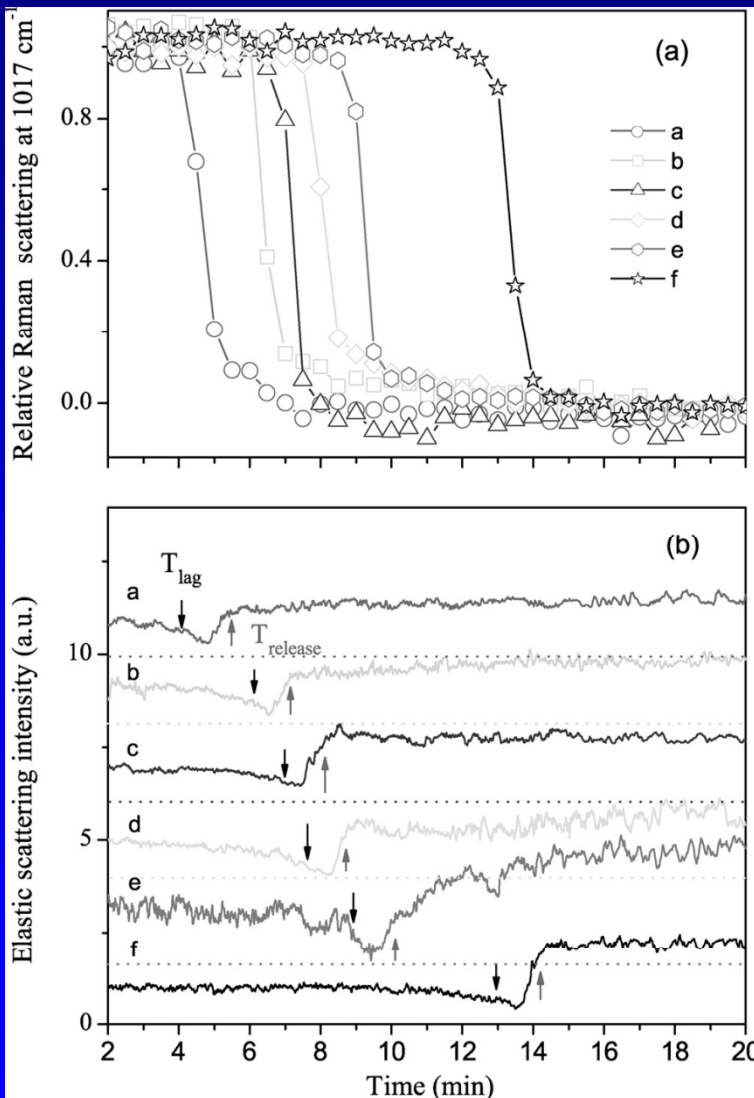
1) Some spores release DPA earlier, some later.
2) There are always a very small percentage of spores retaining their DPA – superdormant/hard to kill.
3) Why some spores easy to kill, some difficult is unknown, but may related to DPA level contained.

PAPER

Analysis of the Raman spectra of Ca²⁺-dipicolinic acid alone and in the bacterial spore core in both aqueous and dehydrated environments

Lingbo Kong,[a] Peter Setlow[b] and Yong-qing Li[*a]

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
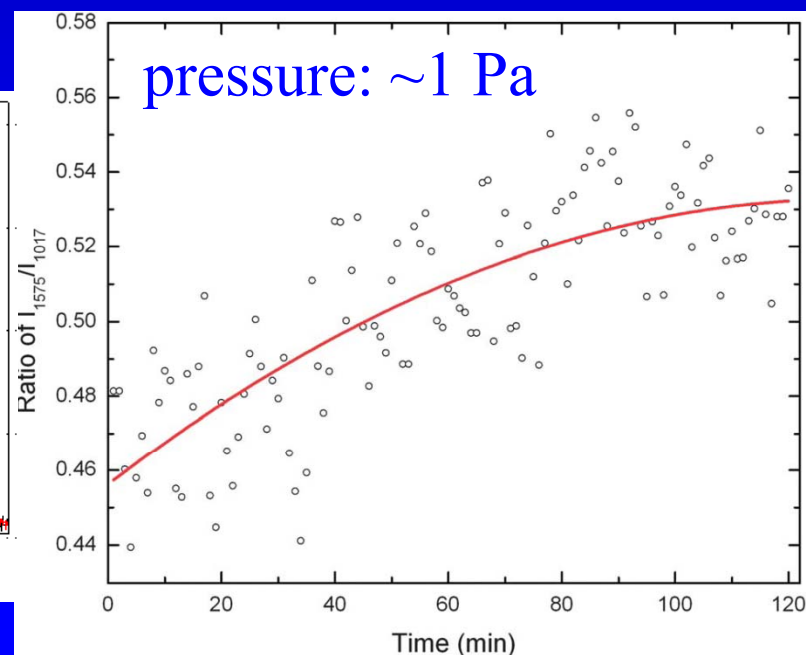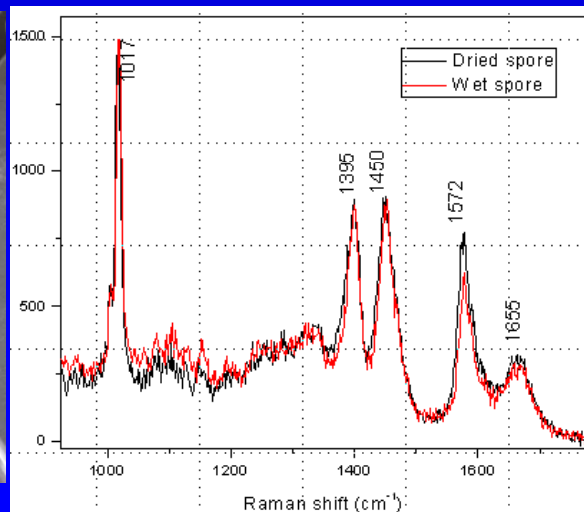
III-3) Identification of single cells of different bacterial species

a S. epidermidis

b E. faecalis

c E. coli

d E. aerogenes

e B. sporeformer
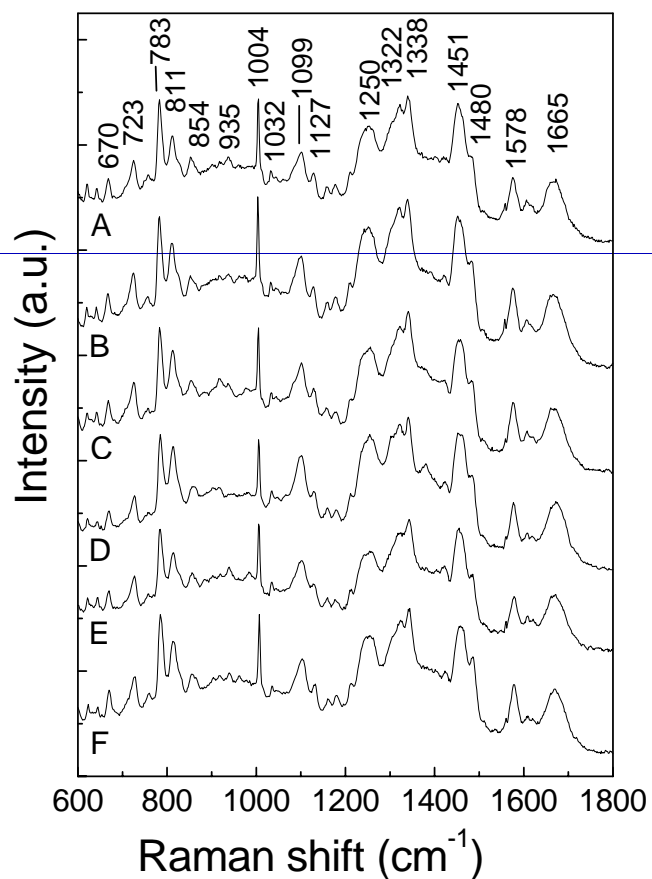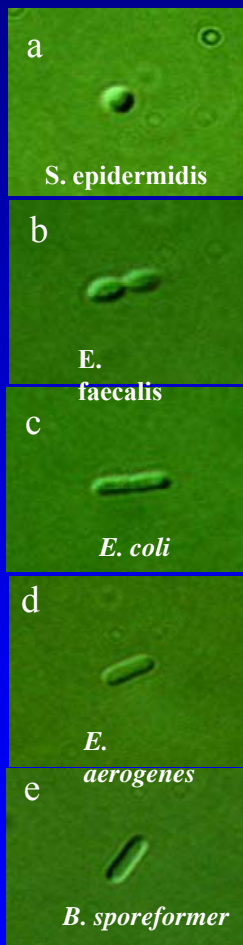
Principal component analysis
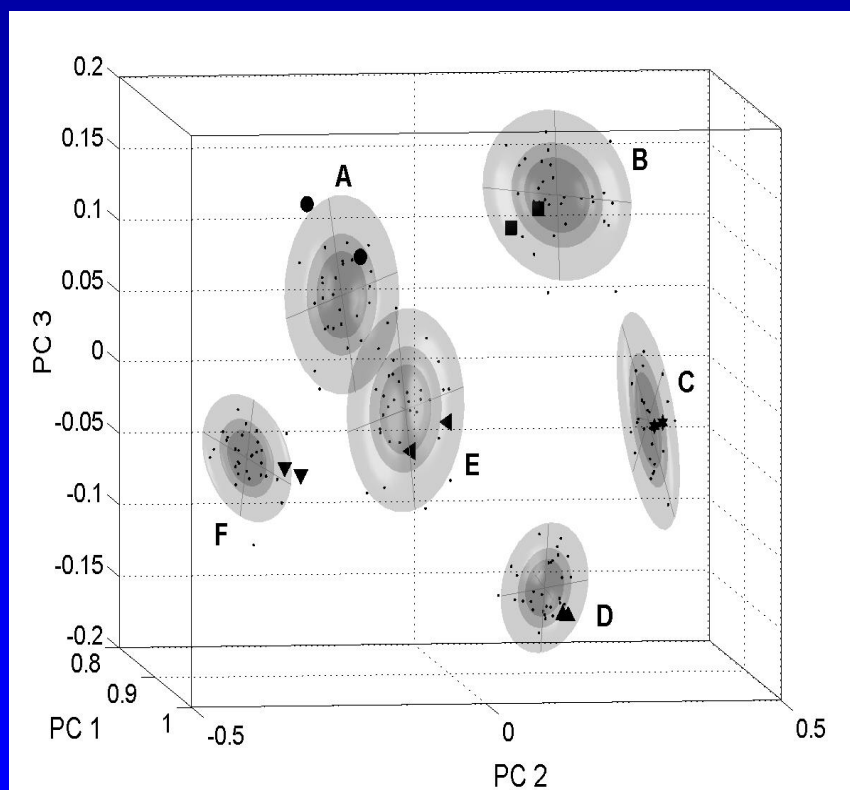
Xie et al, Anal. Chem. 77, 4390-4397 (2005).

Identification of airborne particles in the atmosphere

L.B. Kong et al, *JBO* 16, 120503 (2011).
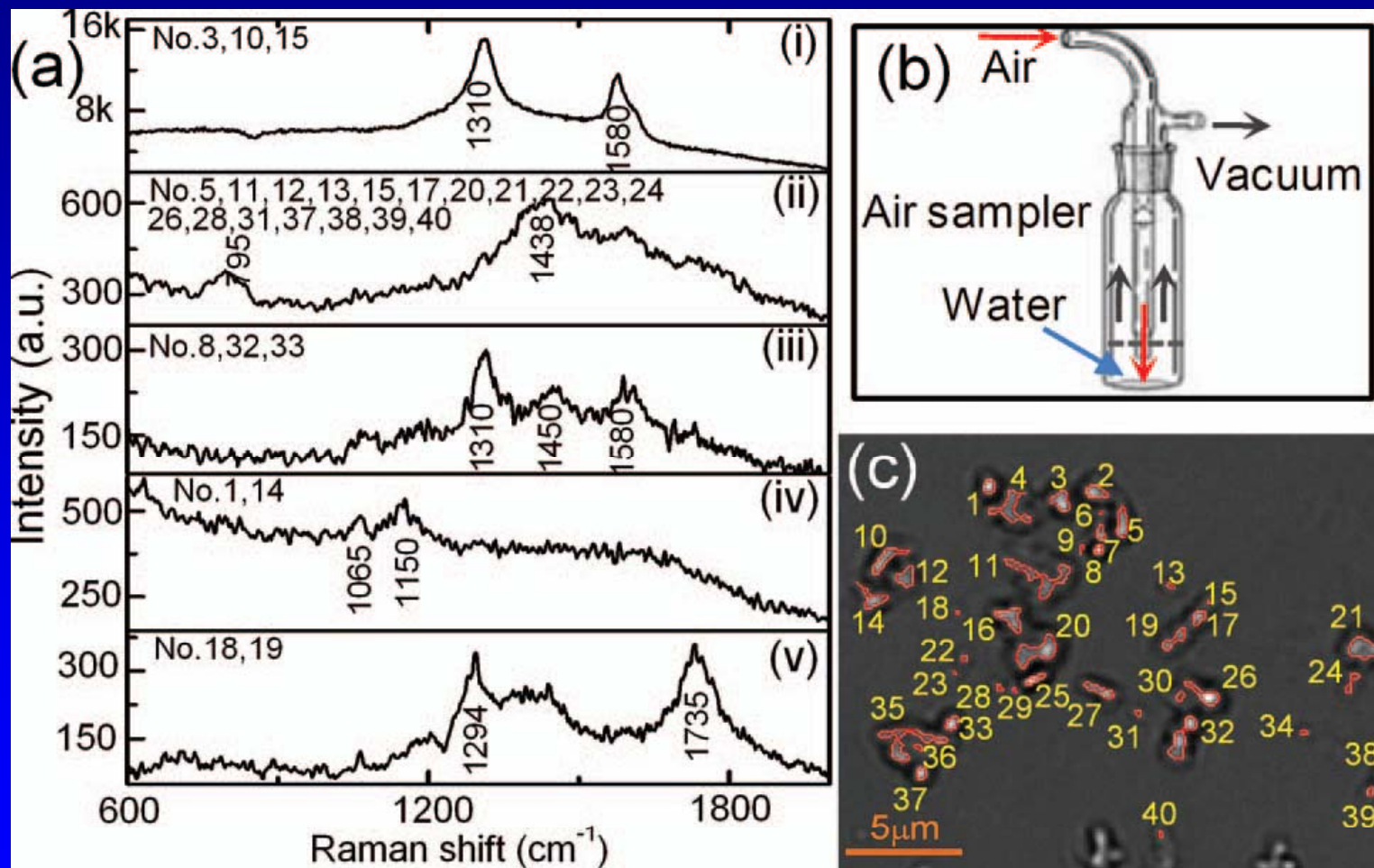
III-4) Detect recombinant protein production

(a) Raman spectra: 960, 1004, 1129, 1344, 1451, 1551, 1665; traces A (4 hr), B (12 hr), C (20 hr), D (24 hr), E (Protein); Intensity (a.u.) vs Raman shift (cm⁻¹) 900–1800.

(b) Gel: M, 0hr, 4h, 12hr, 20hr, 24hr.
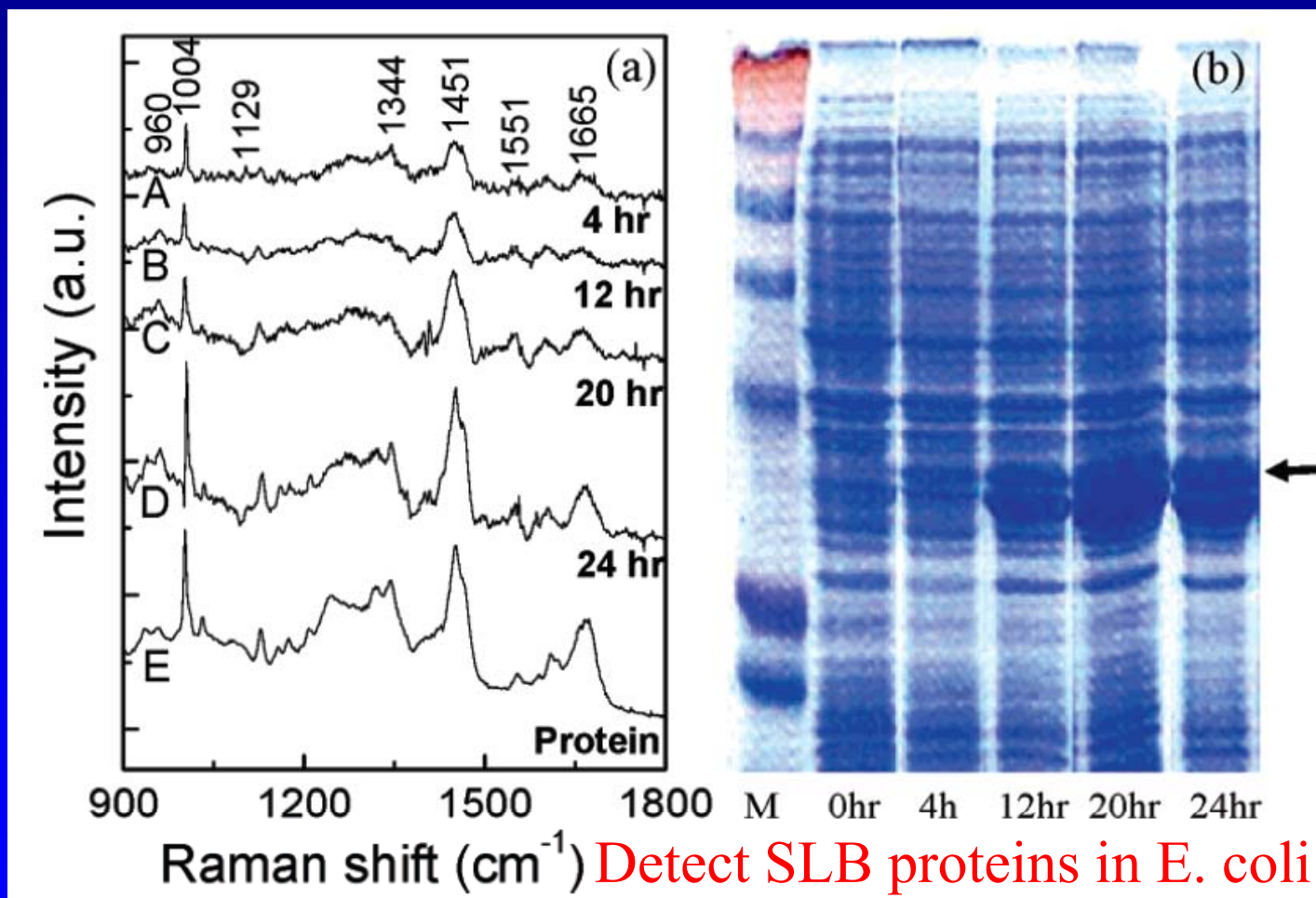
Detect SLB proteins in E. coli

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

Huilu Yao [a,*], Zhanhua Tao [a], Min Ai [a,b], Lixin Peng [a], Guiwen Wang [a], Bijuan He [c], Yong-qing Li [d]

[a] Laboratory of Biophysics, Guangxi Academy of Sciences, Nanning 530003, China
[b] College of Physics and Electronic Engineering, Guangxi Normal University, Guilin 530014, China
[c] Guangxi Academy of Sciences, Nanning 530003, China
[d] Department of Physics, East Carolina University, Greenville, NC 27858-4353, USA
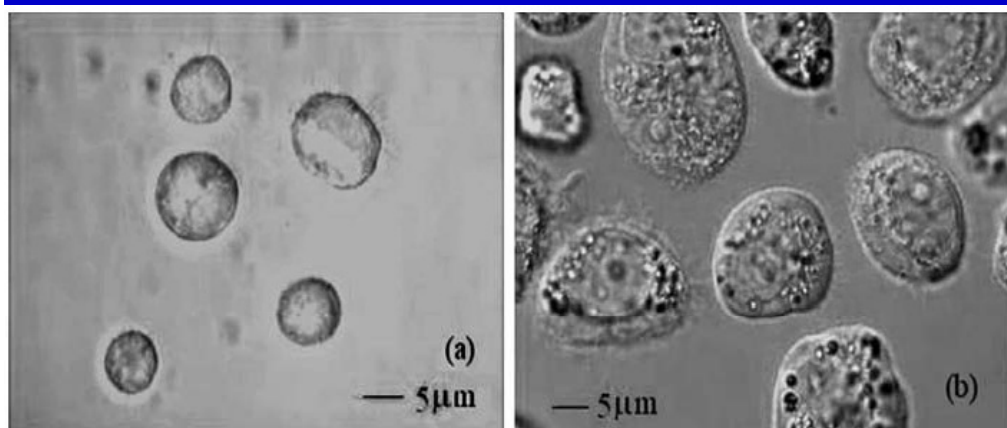
Fig. 2. Images of gastric carcinoma cells (a) and apoptotic cells (b), observed with a DIC microscope with an objective of 100×
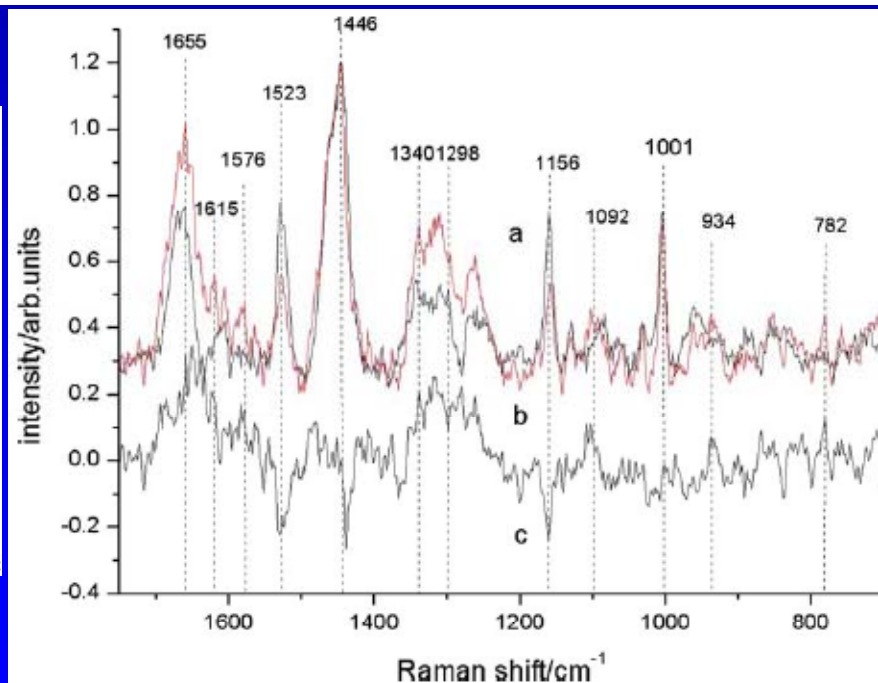
Vibration. Spectros., 50, 193-197 (2009).

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

Cell type	KSHV	EBV	ERK	VEGF
BCBL-1 (N1)	+	-	++++	++++
BC-1 (N2)	+	+	++++	++++
BC-2	+	+	++++	++++
BJAB (N3)	-	-	++	++

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

KSHV infected

J .Virol. Methods, <u>129</u>,145-51 (2005).
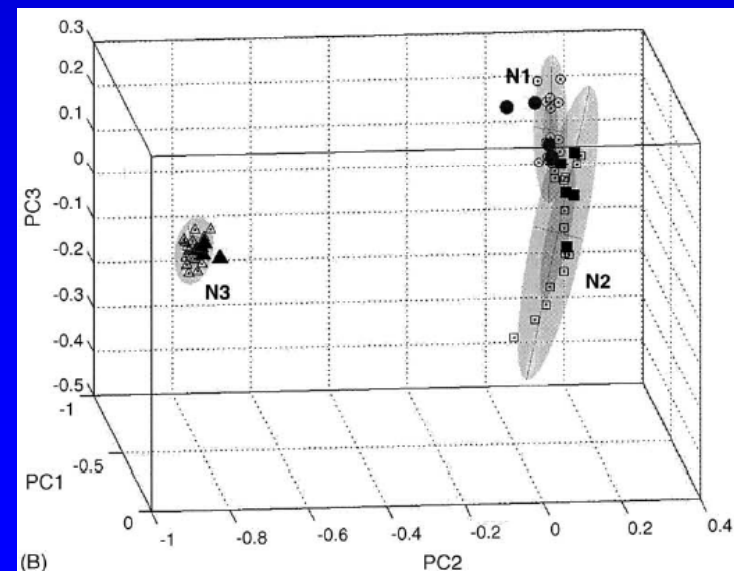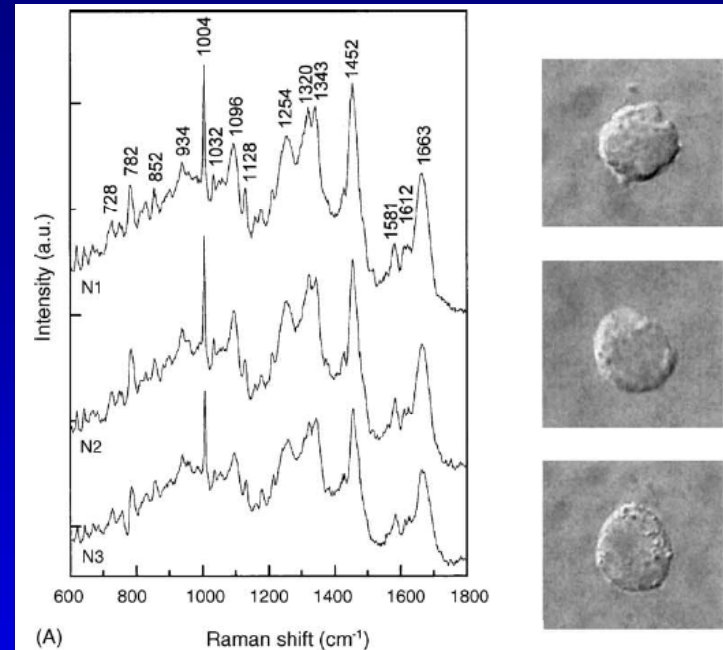
Summary

- 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

1. L.B. Kong, P.F. Zhang, J. Yu, P. Setlow, and Y.Q. Li, "Rapid confocal Raman imaging using a synchro multifoci-scan scheme for dynamic monitoring of single living cells", *Appl. Phys. Lett.* 98, 213703 (2011).
2. C.A. Xie, M. A. Dinno, and Y.Q. Li, "Near-infrared Raman spectroscopy of single optically trapped biological cells", *Opt. Lett.*, 27, 249-251 (2002).
3. P.F. Zhang, L.B. Kong, P. Setlow and Y.Q. Li, "Multiple-trap laser tweezers Raman spectroscopy for simultaneous monitoring of the biological dynamics of multiple individual cells", *Opt. Lett.* 35, 3321-3323 (2010).
4. L.B. Kong, P.F. Zhang, P. Setlow, and Y.Q. Li. "Characterization of bacterial spore germination using integrated phase contrast microscopy, Raman spectroscopy and optical tweezers", *Anal. Chem. 82,* 3840–3847 (2010).
5. D. Chen, S.S. Huang, **Y.Q. Li**. "Real-time detection of kinetic germination and heterogeneity of single *Bacillus* spores by laser tweezers Raman spectroscopy", *Anal. Chem.* <u>78</u>, 2936-6941 (2006).