24/February/2015
徳大ERATOミーティング

医学・生物学応用を中心とした線形/非線形ラマン散乱分光法

南川丈夫
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医学研究科　細胞分子機能病理学

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自己紹介

1983年 茨城県ひたちなか市（旧 勝田市） 生まれ

2005年 茨城高専 電子制御工学科 卒業
（高専 長谷川研，茨城大 伊藤研，理研 大森研）
「ELID研削加工」

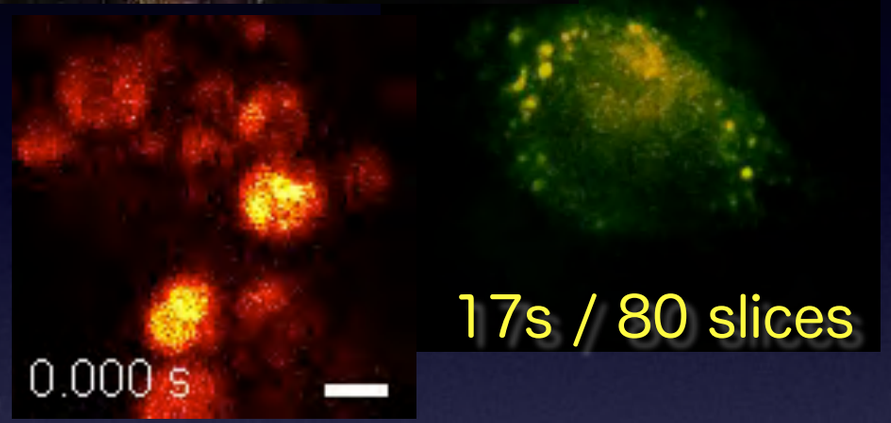
2004年
大阪大学 基礎工学部 機械科学コース
大阪大学 基礎工学研究科 機能創成専攻 生体工学領域

2010年 Ph.D.，学振DC，招聘研究員 @ 大阪大学
（荒木・橋本研究室）
「非線形ラマン散乱顕微鏡の開発と生体計測応用」

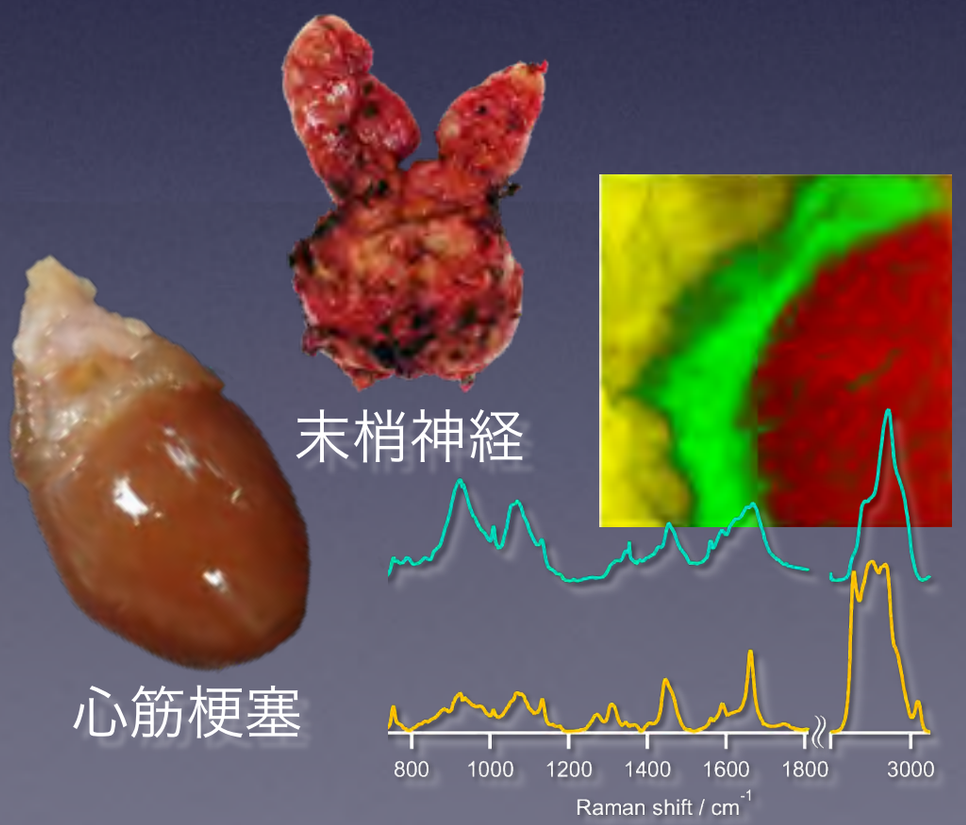
2011年 学振PD，助教 @ 京府医大
（病理，高松研究室）
「線形/非線形ラマン散乱分光法による医療応用」

2014年 客員研究員@立命館大学
「分光学的方法論による浮世絵の復元的研究」

Home-made
CARS microscope



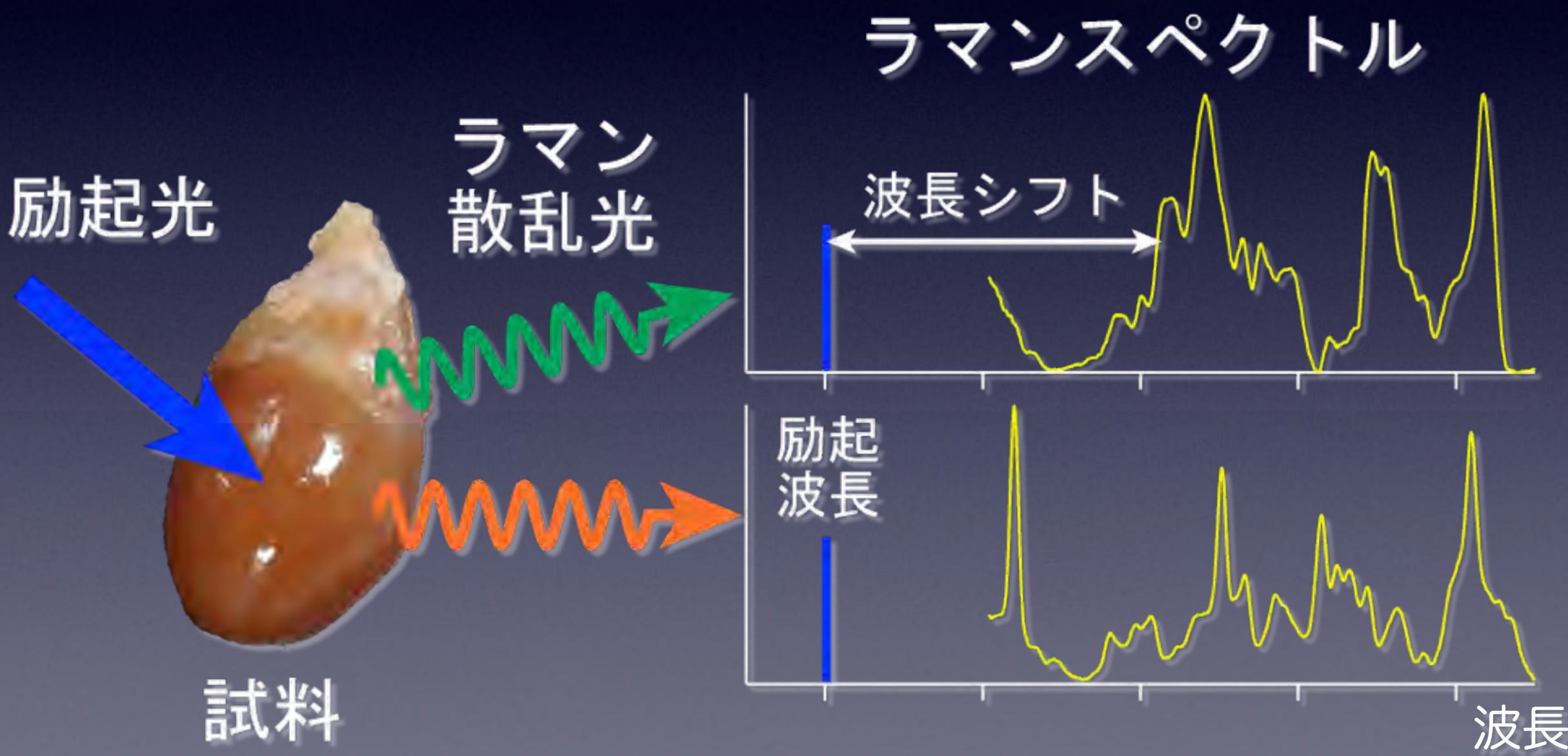
High speed Raman imaging



Raman spectroscopy

細胞・組織内分子の分子振動を計測（振動分光法）

分子振動：分子の種類や構造を反映



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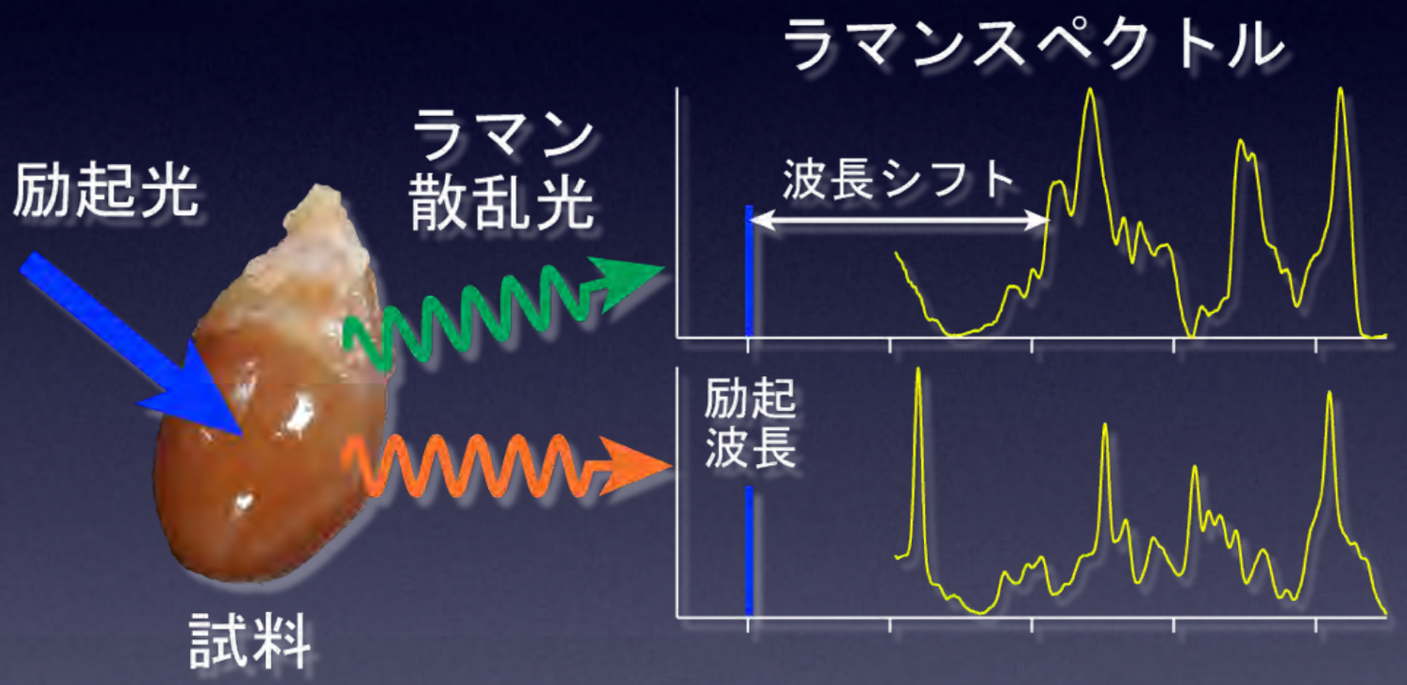
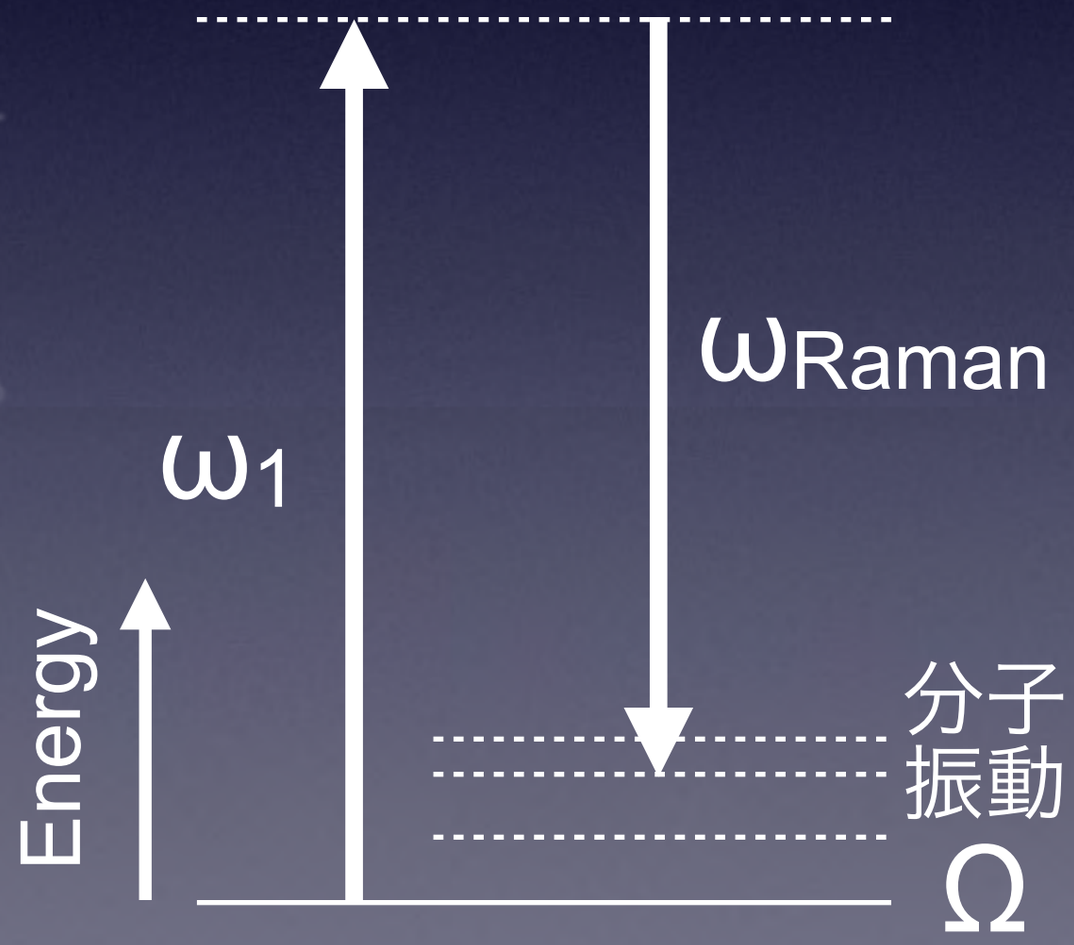


High intensity

$\omega_1 - \Omega = \omega_{Raman}$

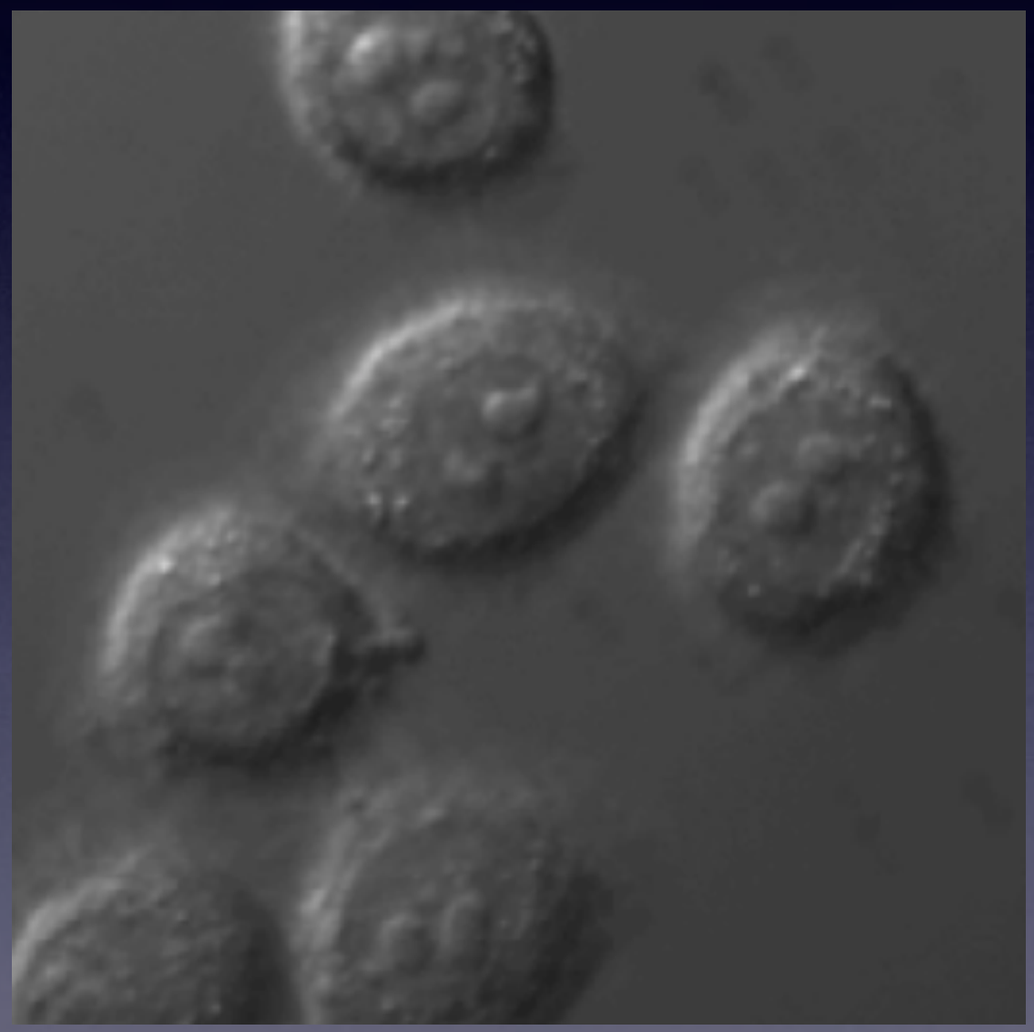


ラマン散乱分光法で何を見たいのか？

～ 医学・医療応用 ～

Our goal

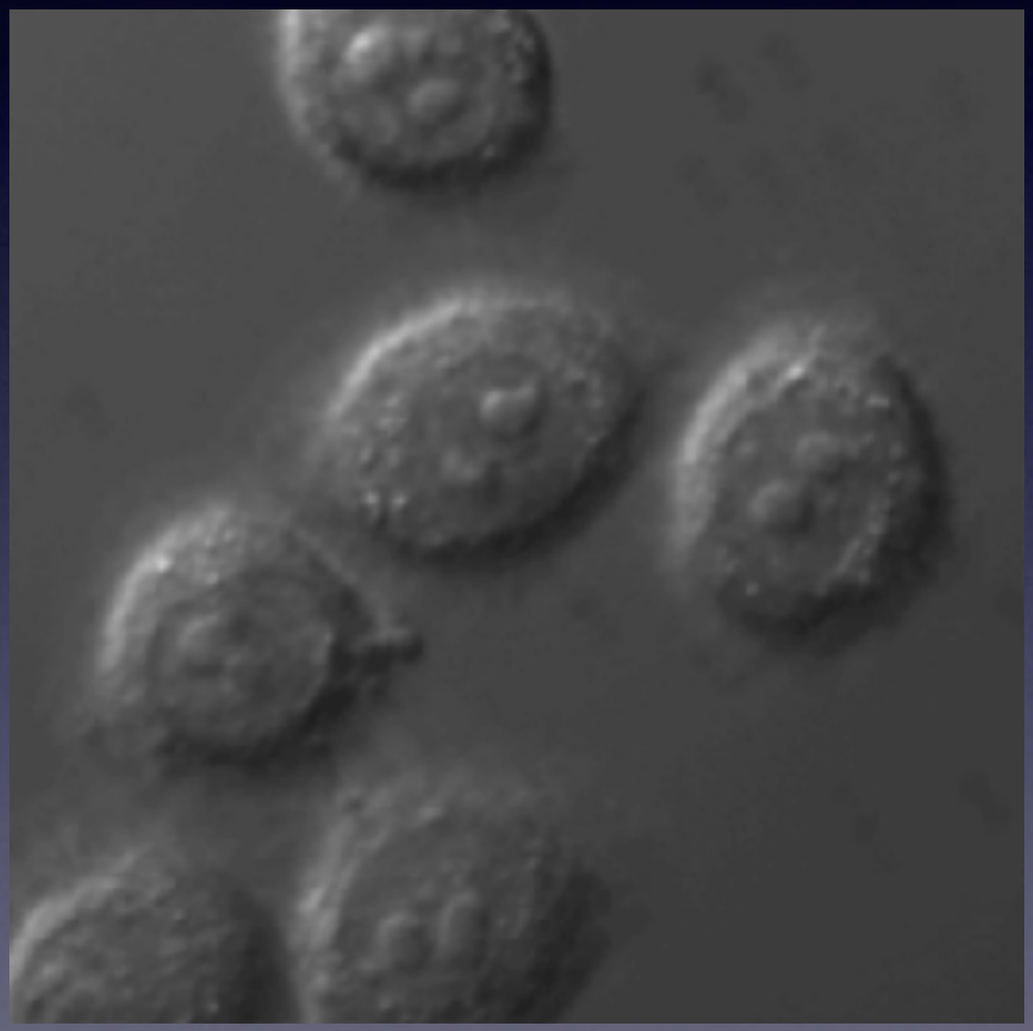
Imaging method
to visualize molecular information



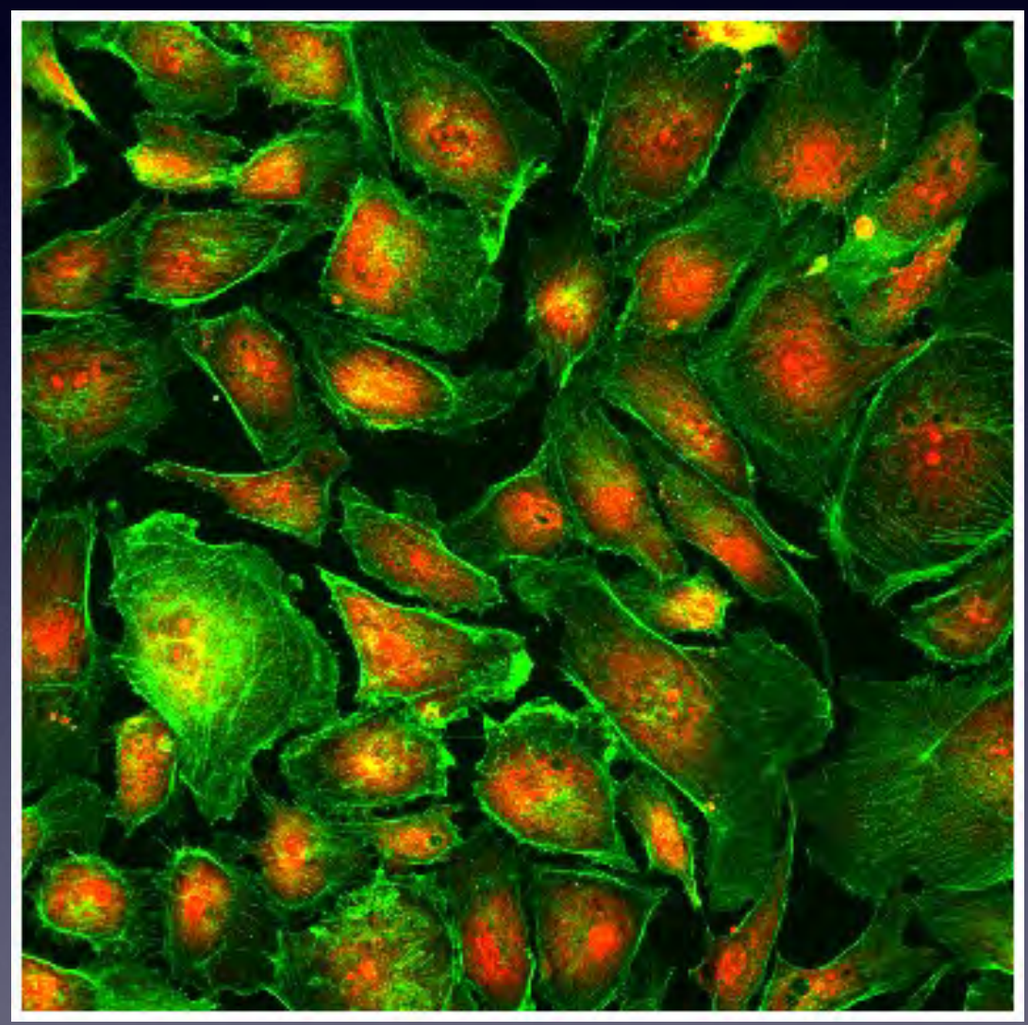
White/Black image

Our goal

Imaging method
to visualize molecular information



White/Black image

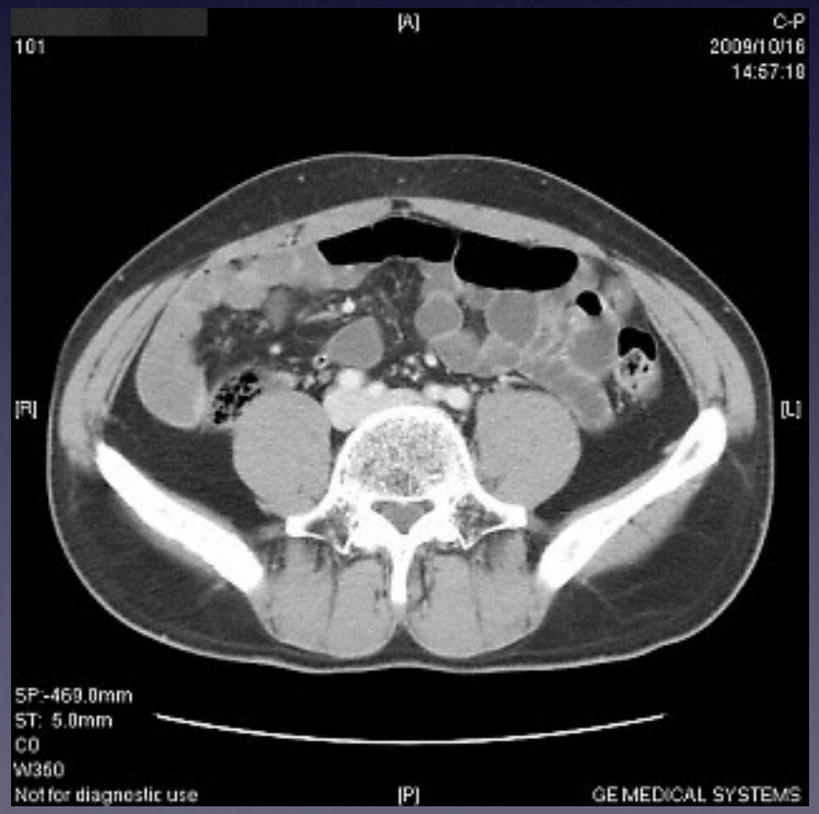


Color image!!

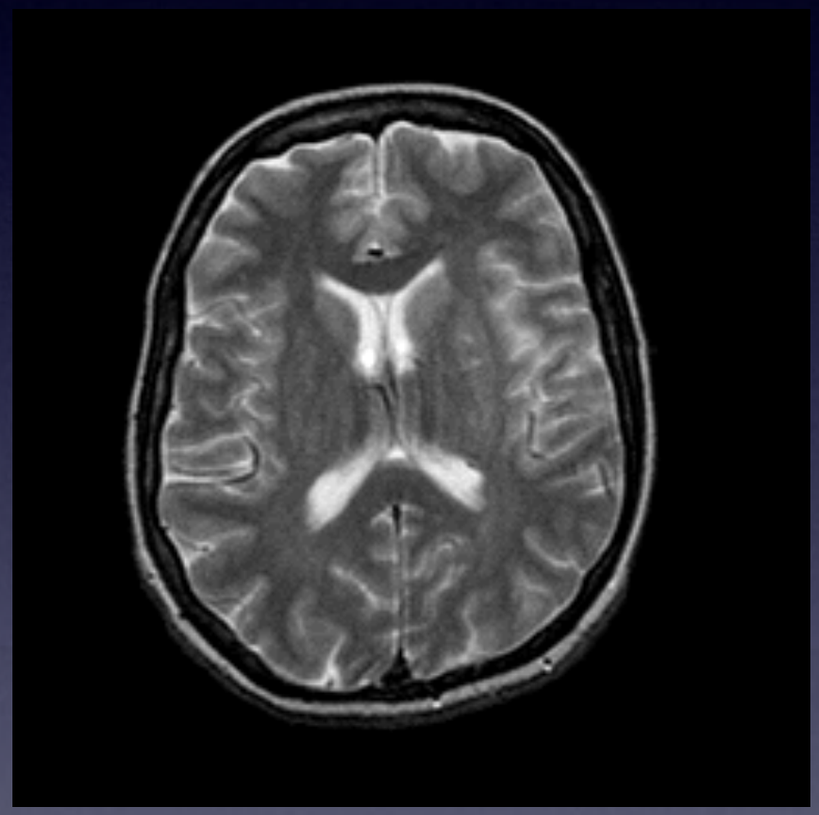
従来技術で

何を見ることができないのか？

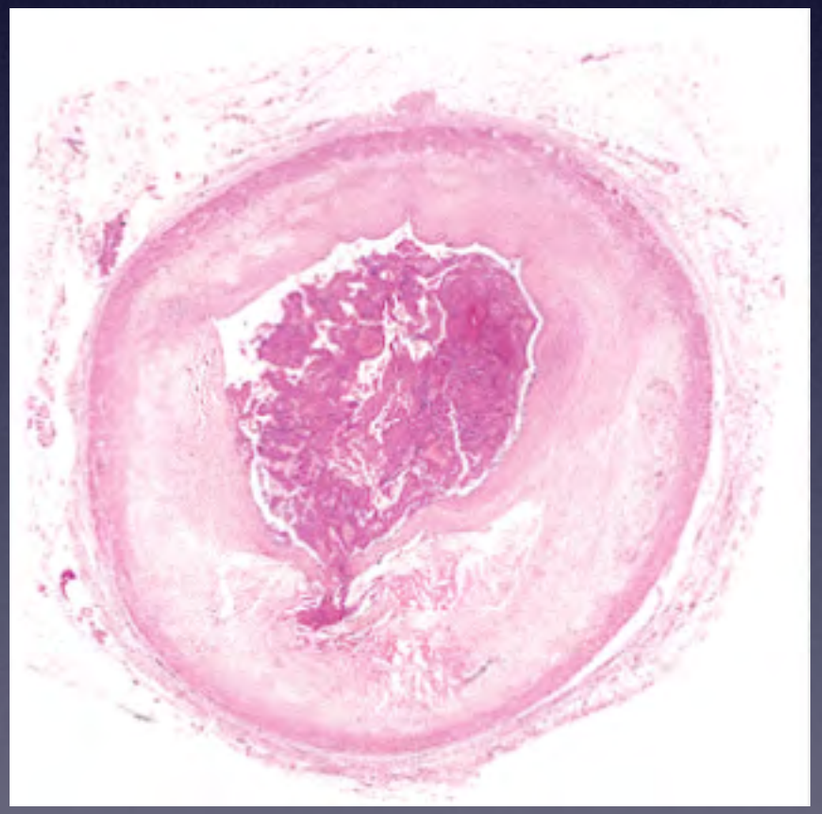
従来技術で
何を見ることができないのか？



CT



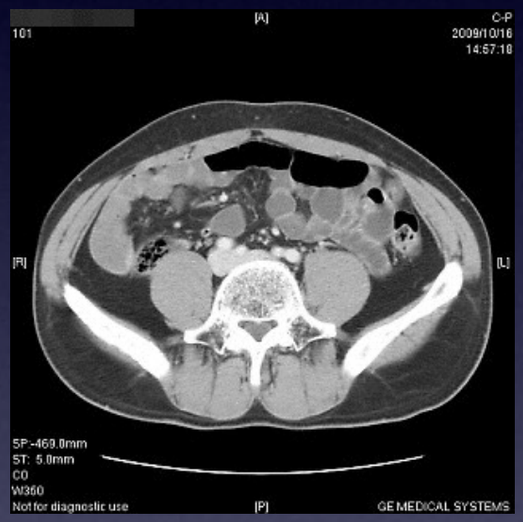
MRI



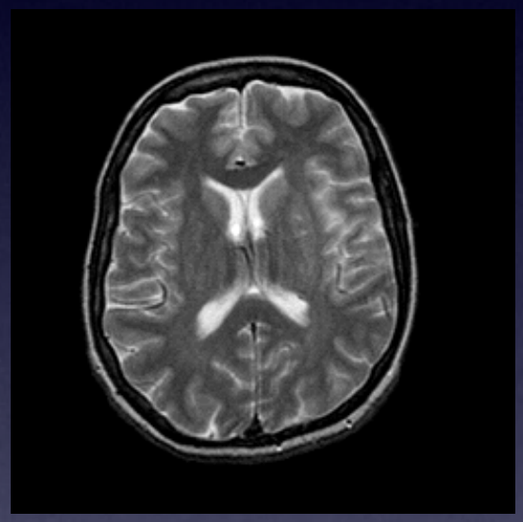
染色

従来技術で
何を見ることができないのか？

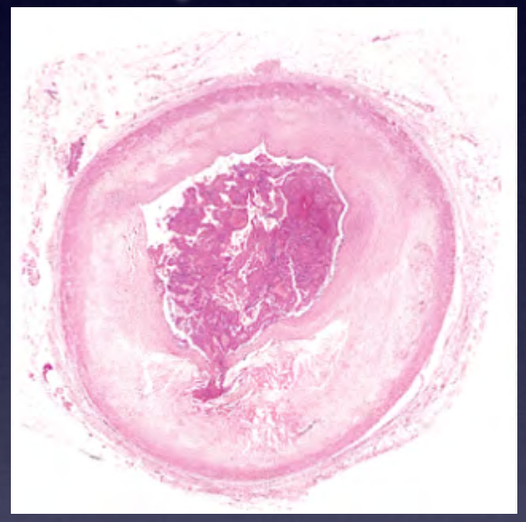
CT



MRI

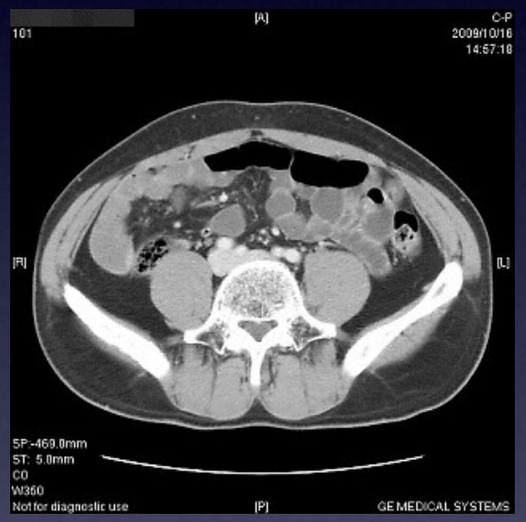


染色

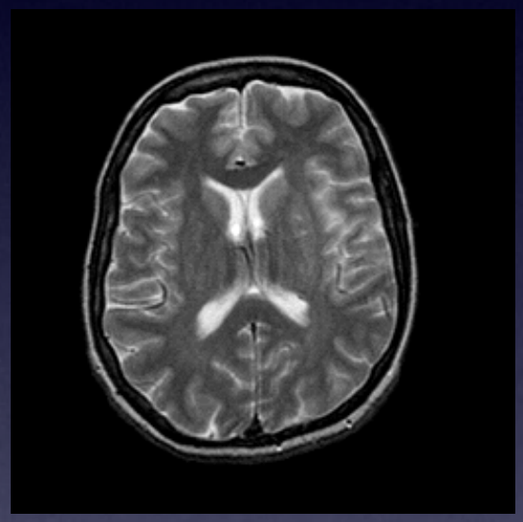


従来技術で
何を見ることができないのか？

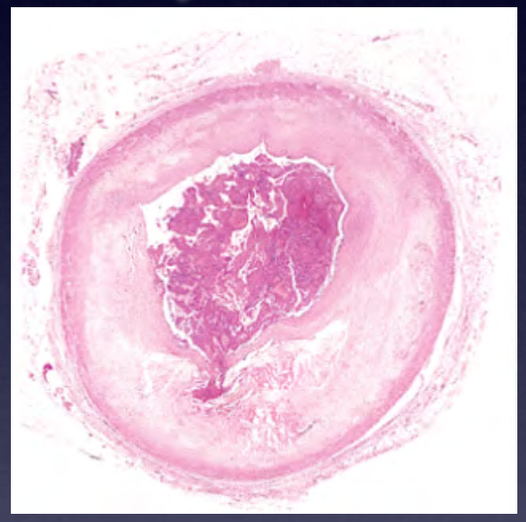
CT



MRI



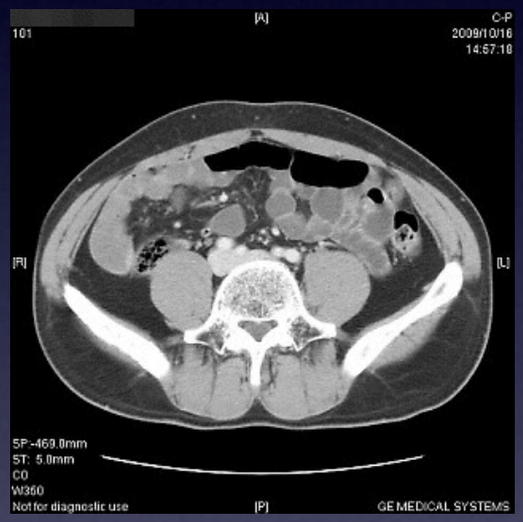
染色



- ×放射線被曝
- ×装置が大きい
- ×低コントラスト

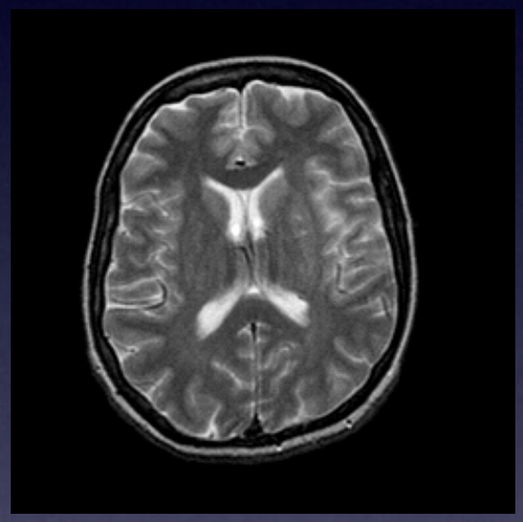
従来技術で
何を見ることができないのか？

CT



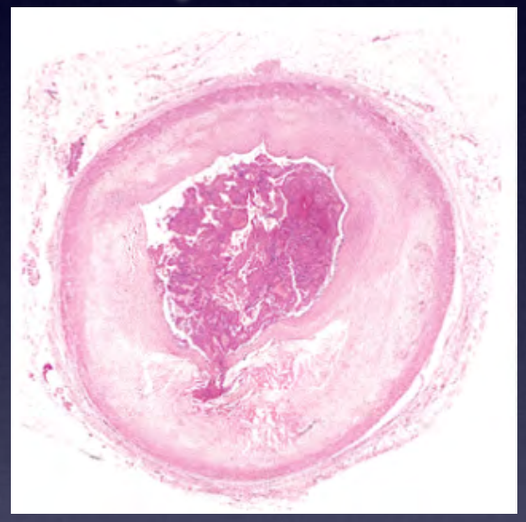
×放射線被曝
×装置が大きい
×低コントラスト

MRI



×時間がかかる
×装置が大きい
×強い磁場

染色

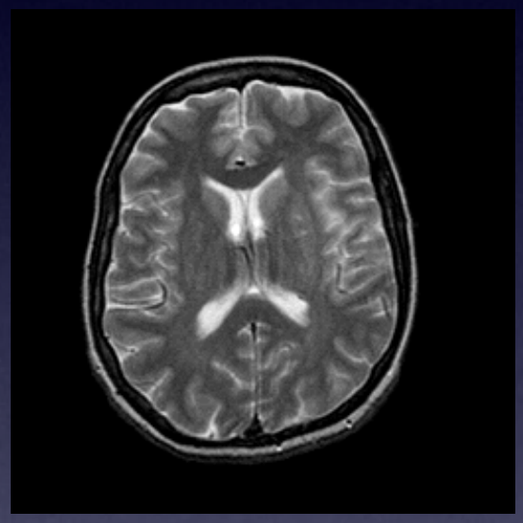


従来技術で
何を見ることができないのか？

CT

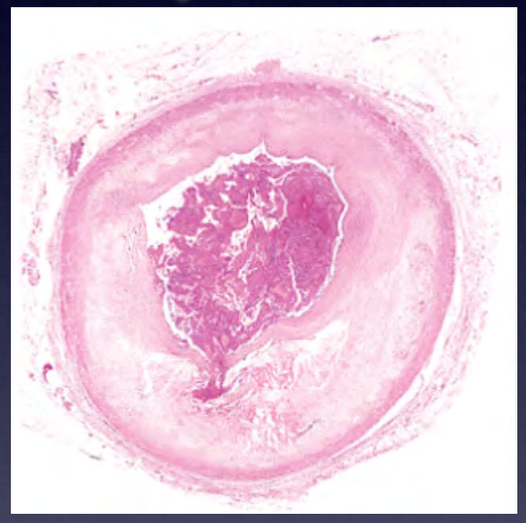
- ×放射線被曝
- ×装置が大きい
- ×低コントラスト

MRI



- ×時間がかかる
- ×装置が大きい
- ×強い磁場

染色



- ×病理医が不可欠
- ×破壊的検査
- ×時間がかかる

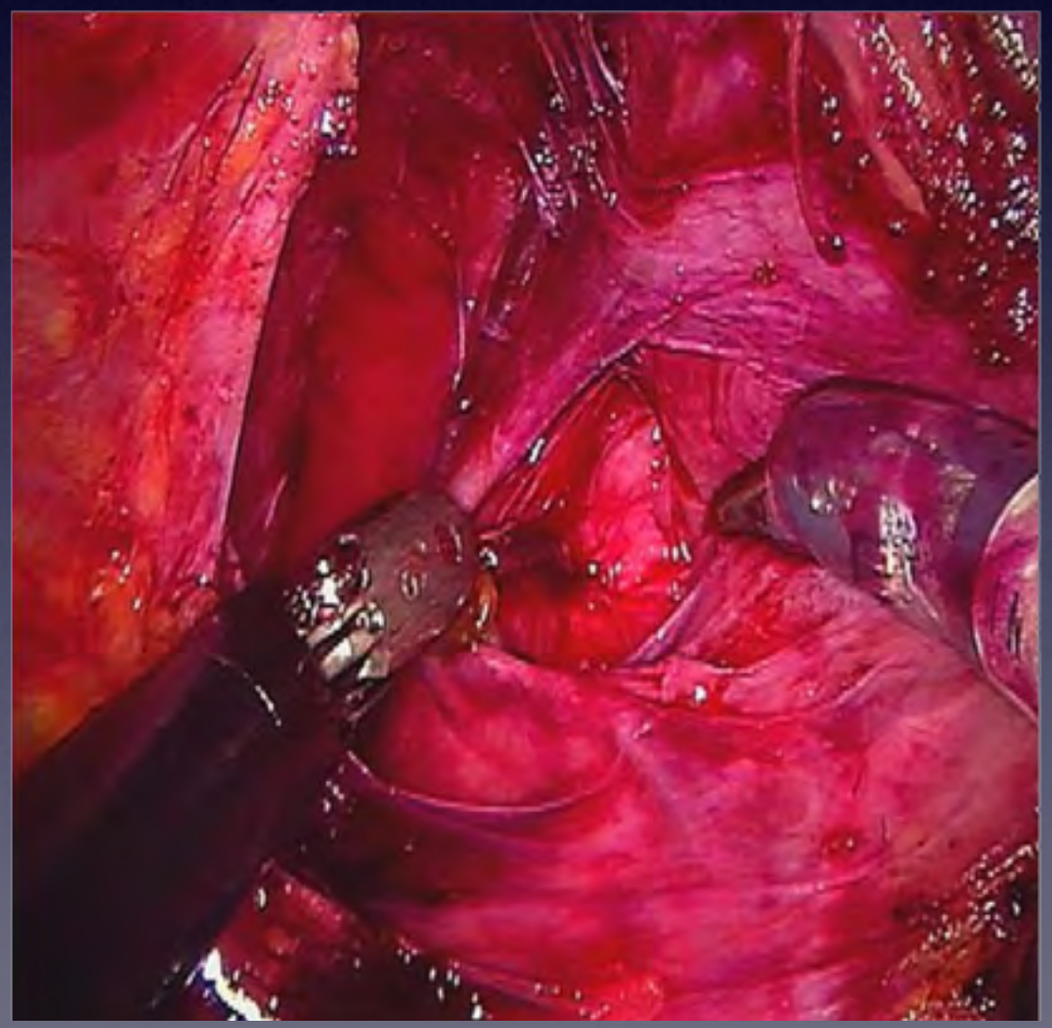
Medical applications I

- Peripheral nerve detection -

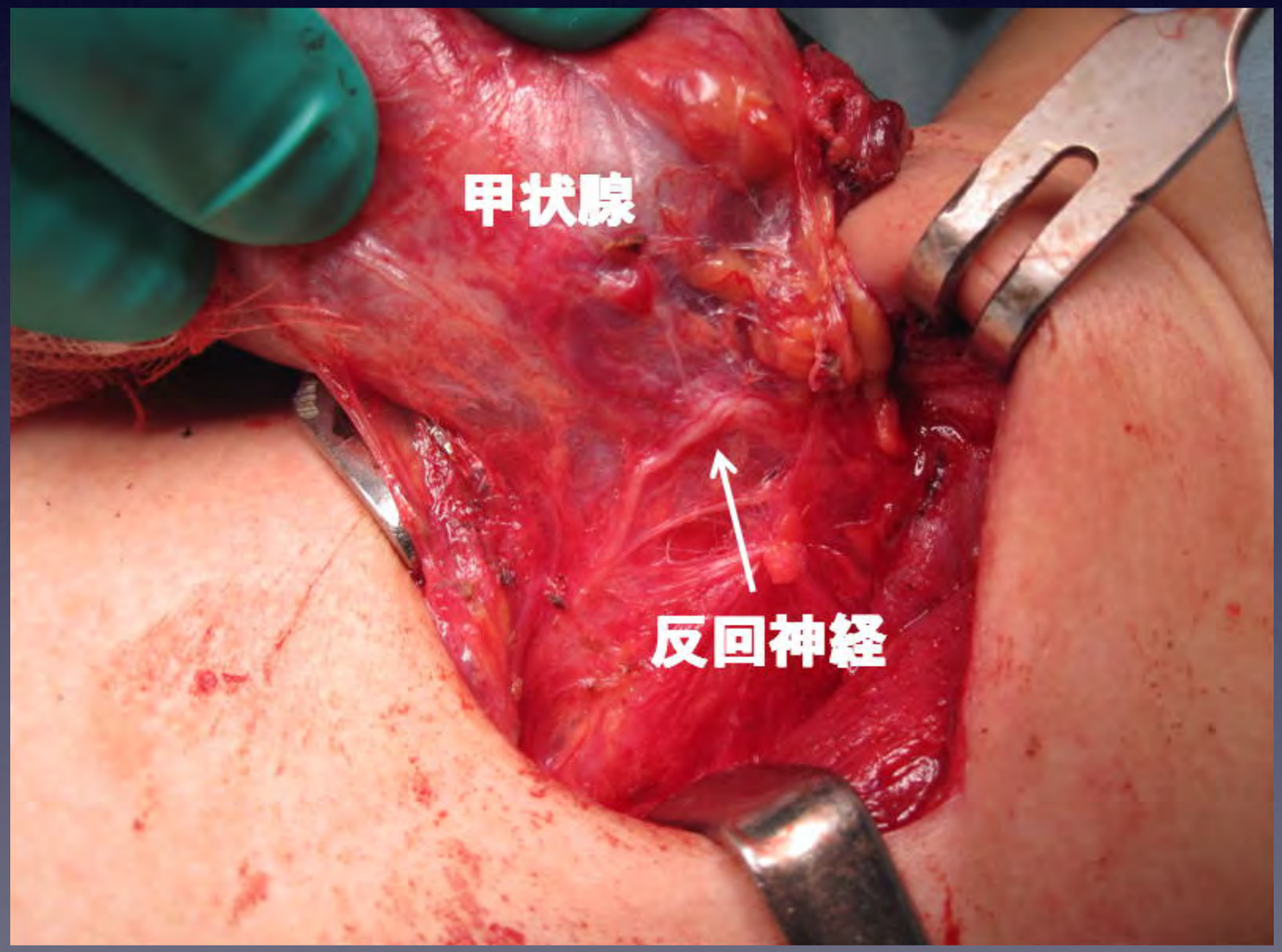
Introduction

Nerve-sparing surgery

Preservation of peripheral nerves during surgery



Prostate cancer

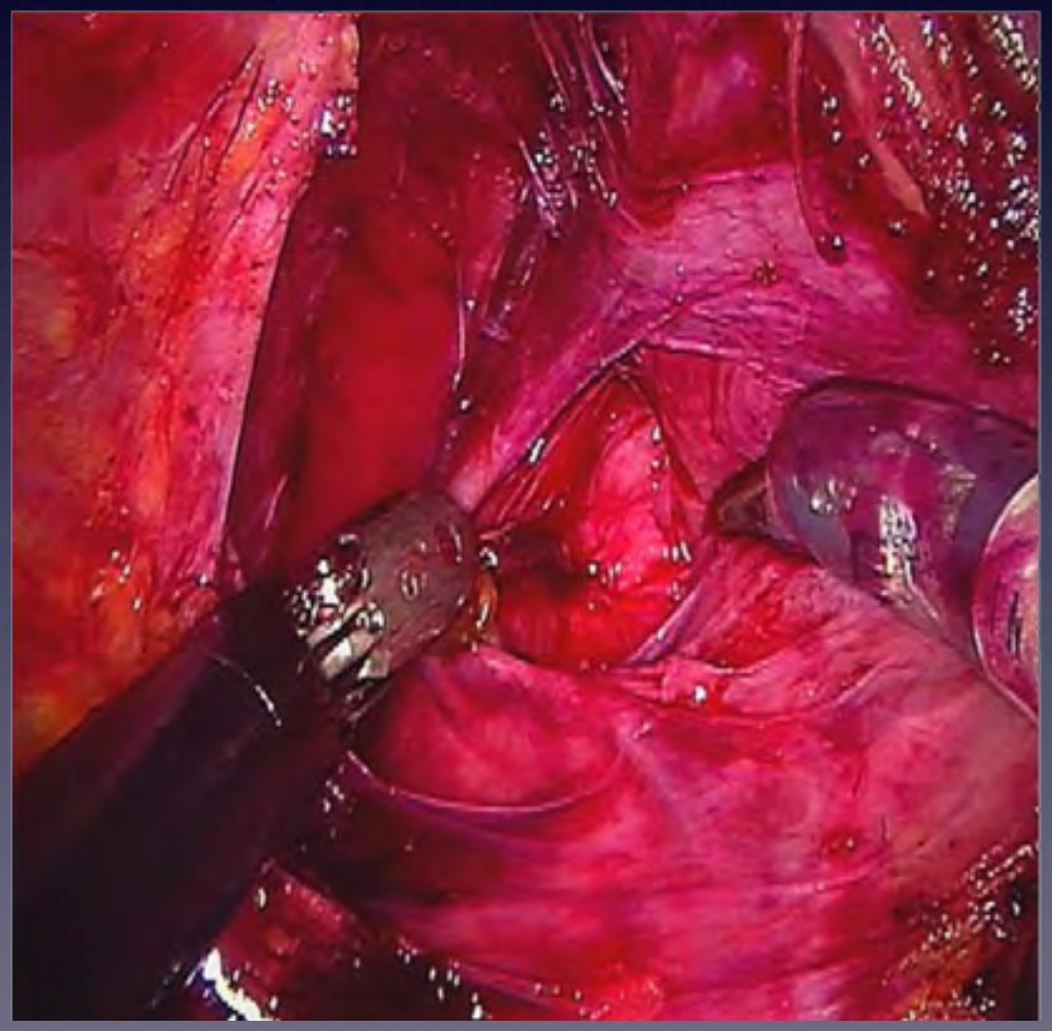


Thyroid surgery

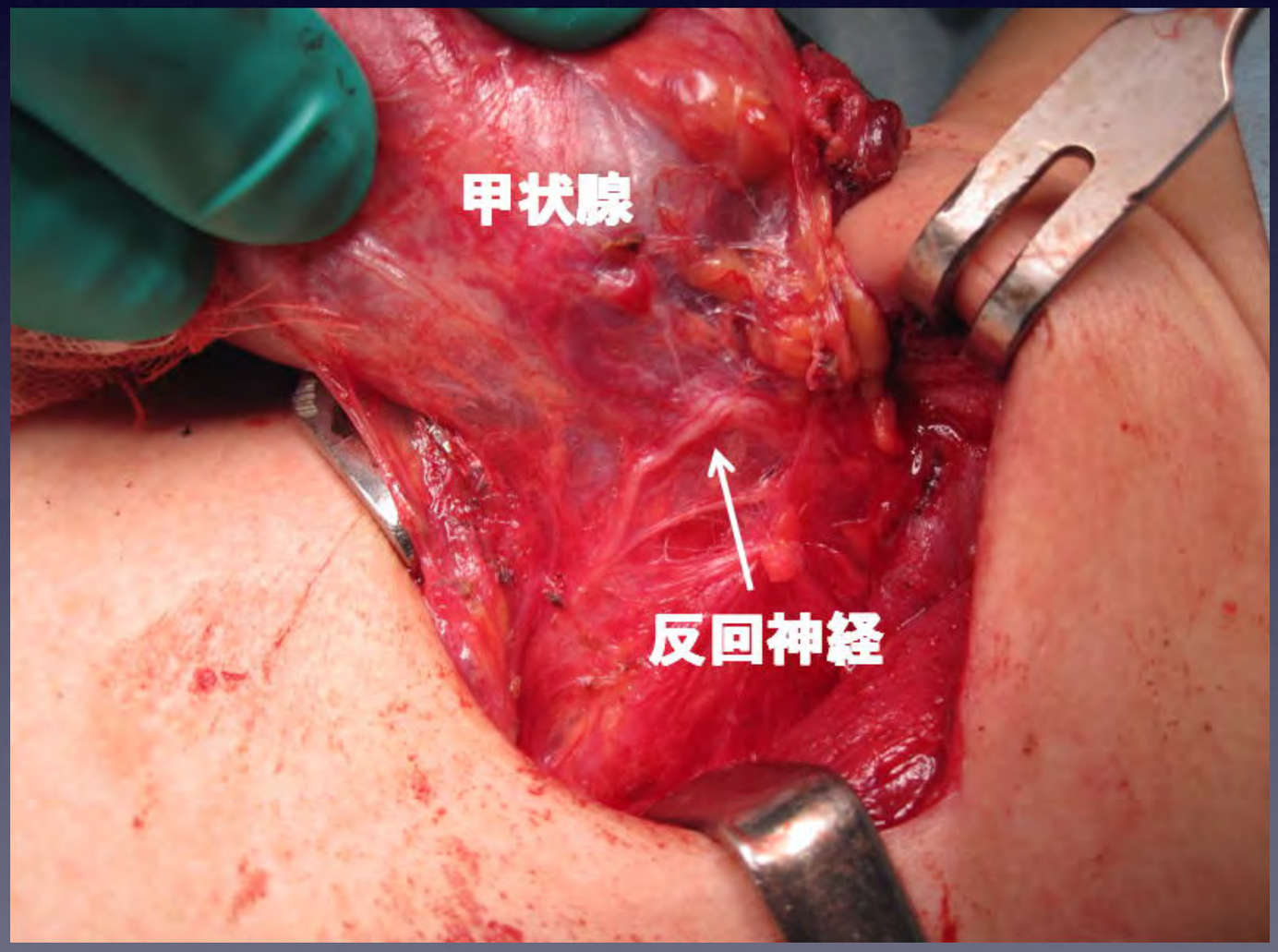
Introduction

Nerve-sparing surgery

Preservation of only large peripheral nerves during surgery
(that can be identified by human eye or under white light imaging)



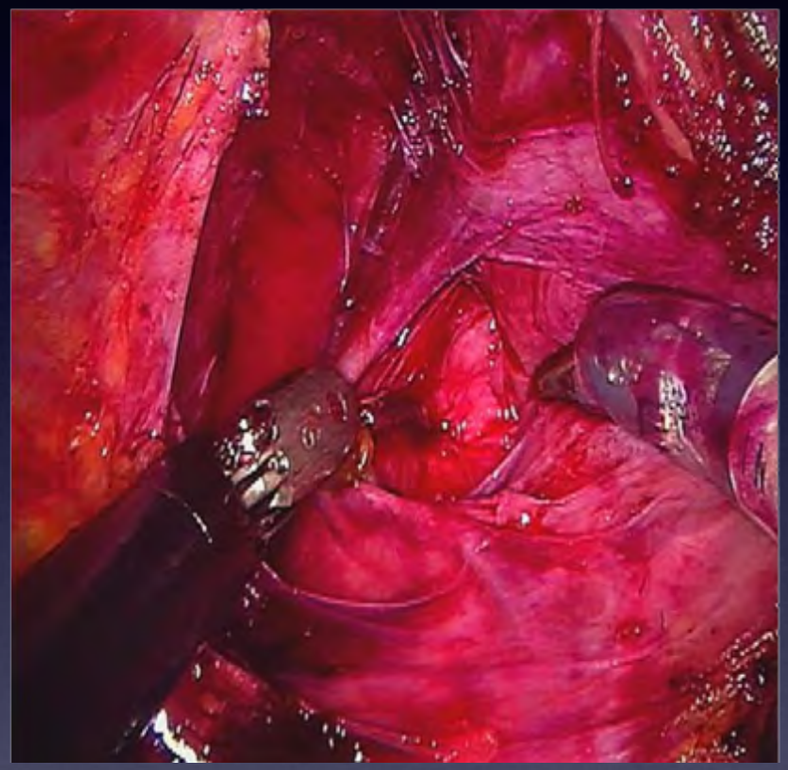
Prostate cancer

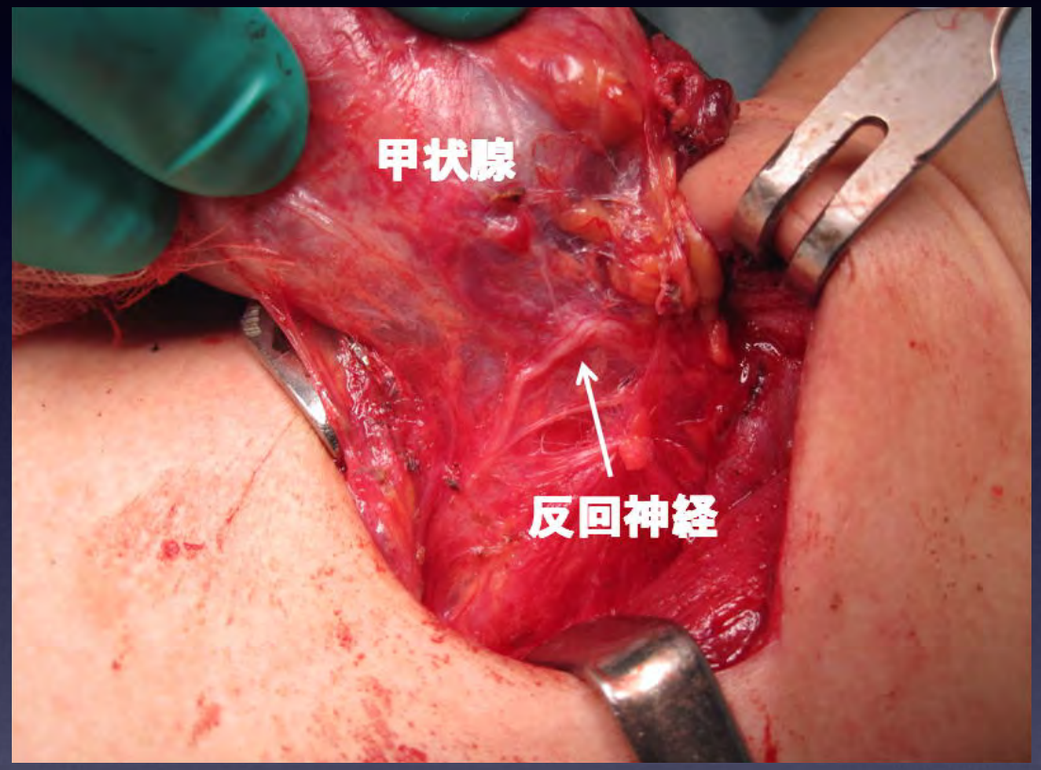


Thyroid surgery

Purpose

“In situ detection of peripheral nerves”





Purpose

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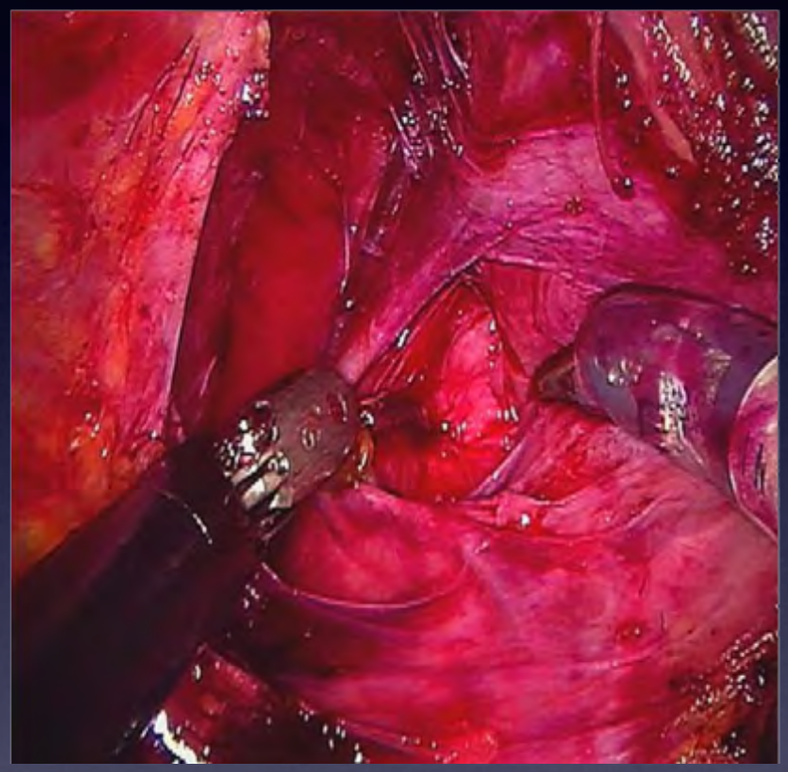
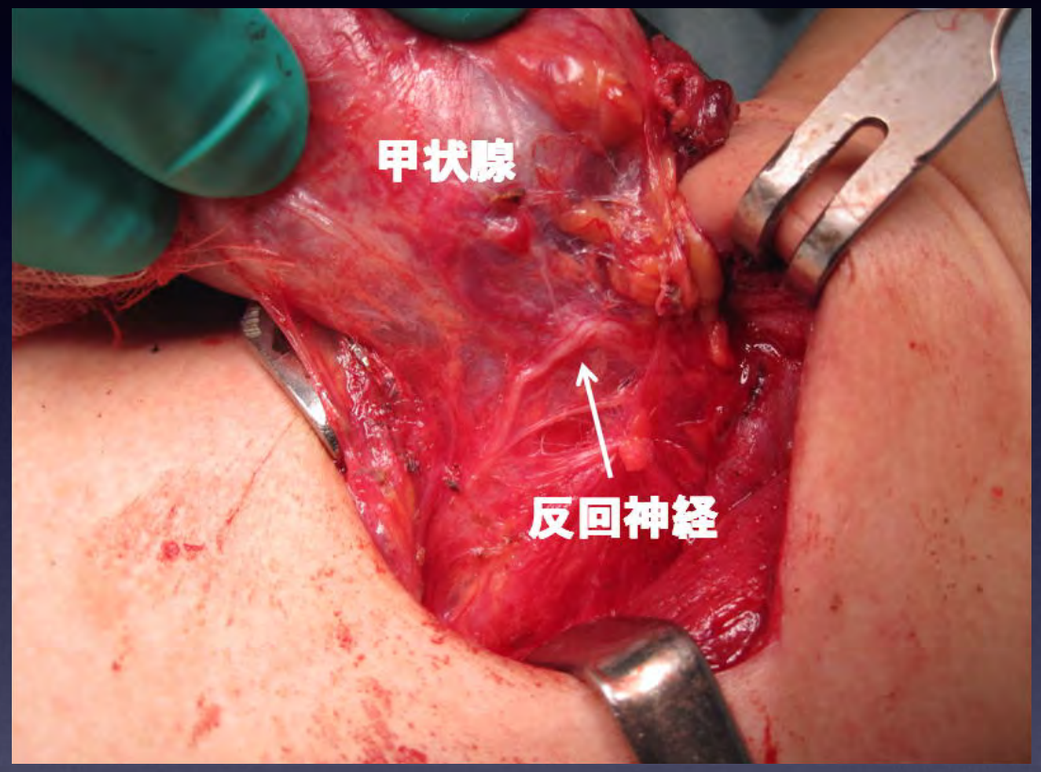
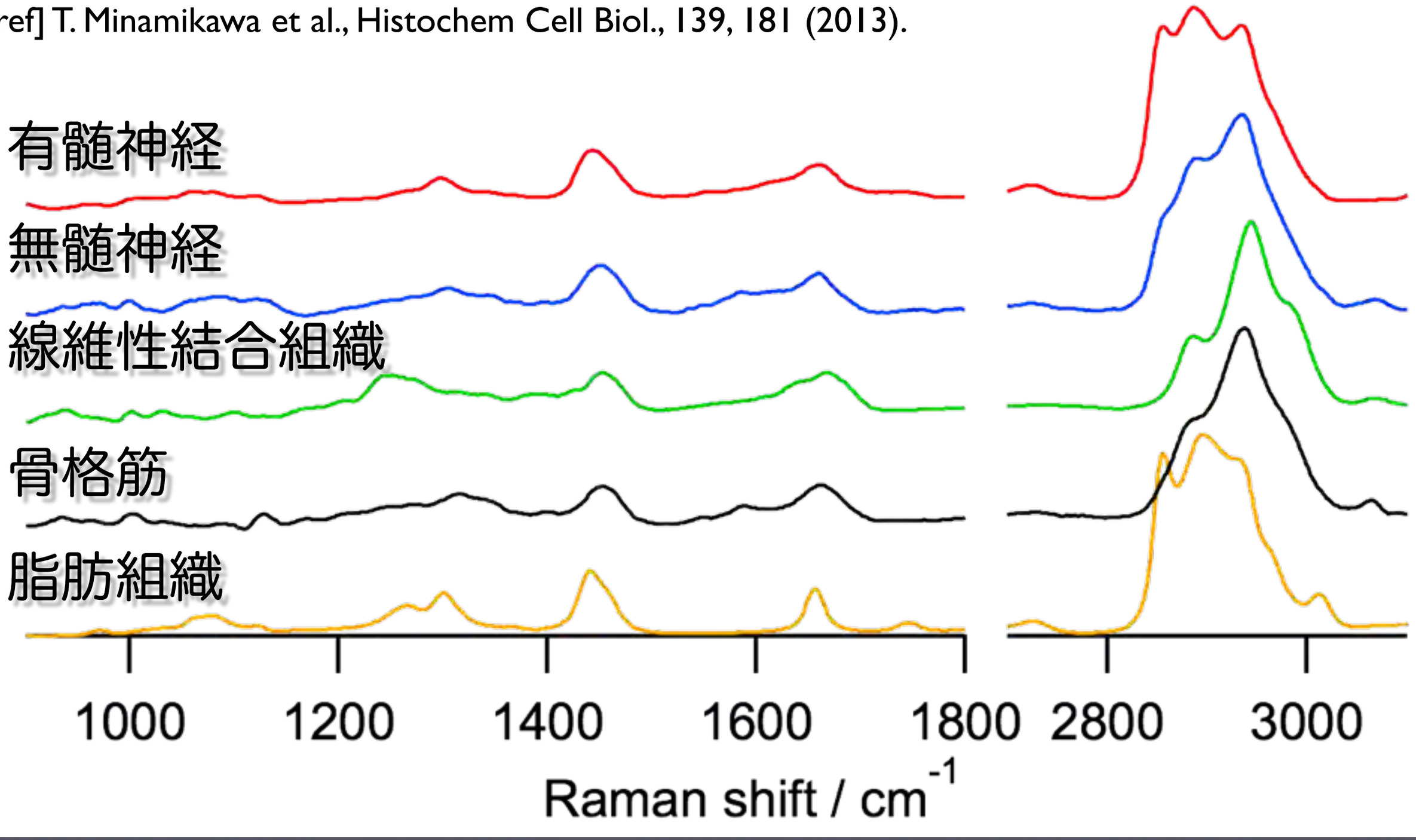




Label-free observation of peripheral nerves by Raman spectroscopy

Raman spectra of peripheral nerves
(Sectioned sample)

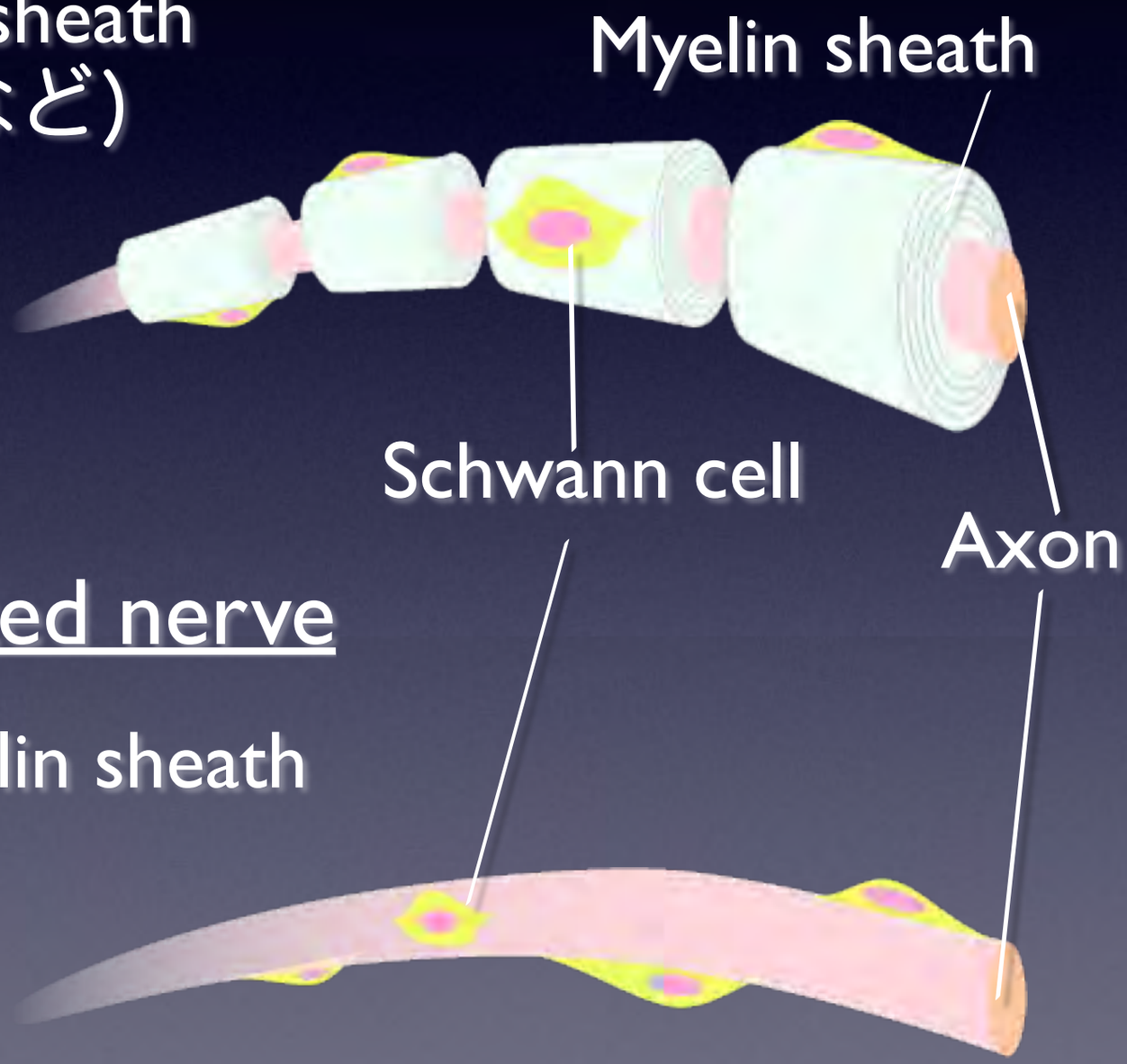
[ref] T. Minamikawa et al., Histochem Cell Biol., 139, 181 (2013).



Type of peripheral nerves

有髄神経 (Myelinated nerve)

Nerve fiber with myelin sheath
(運動神経，感覚神経など)

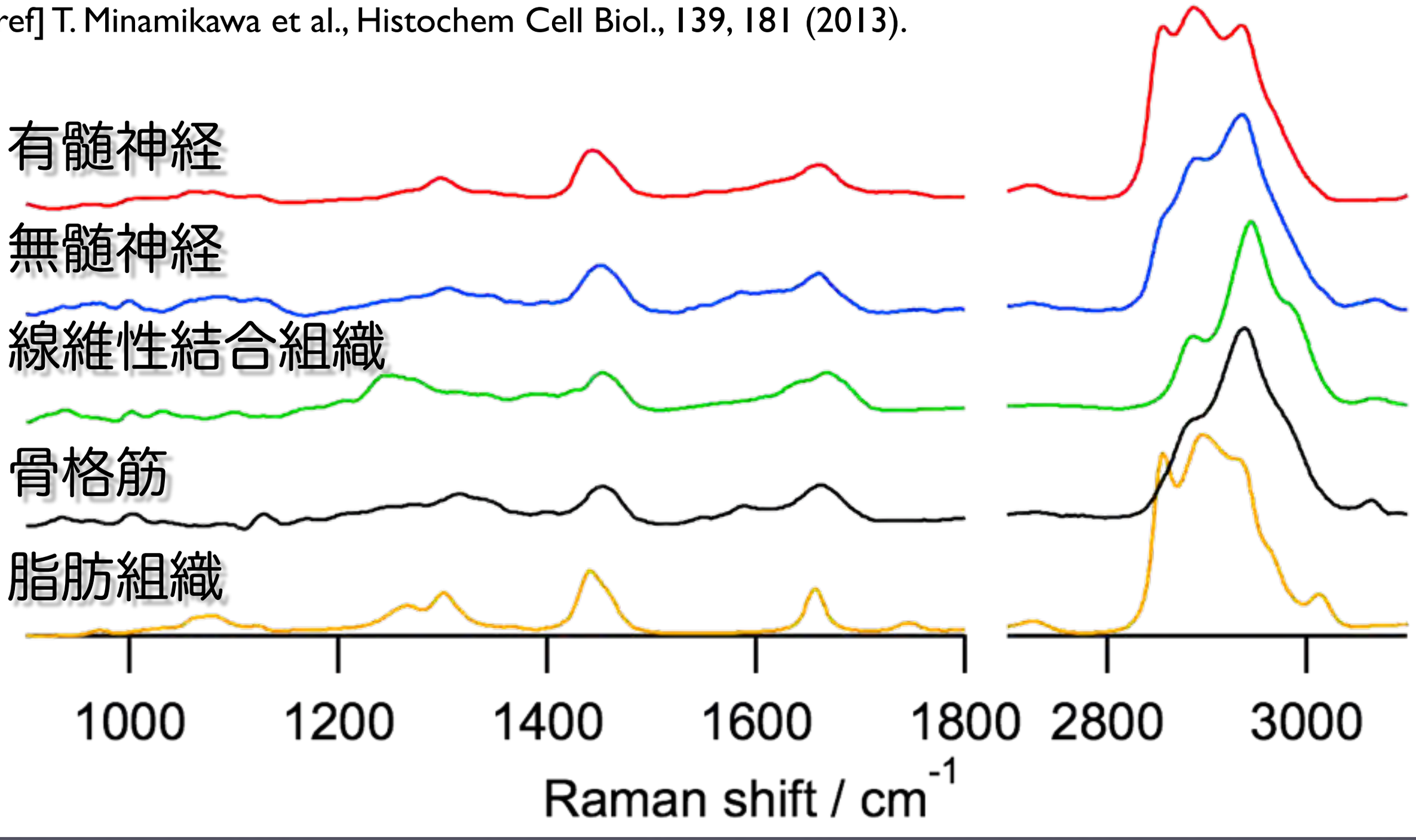


無髄神経 Unmyelinated nerve

Nerve fiber without myelin sheath
(自律神経など)

Raman spectra of peripheral nerves
(Sectioned sample)

[ref] T. Minamikawa et al., Histochem Cell Biol., 139, 181 (2013).



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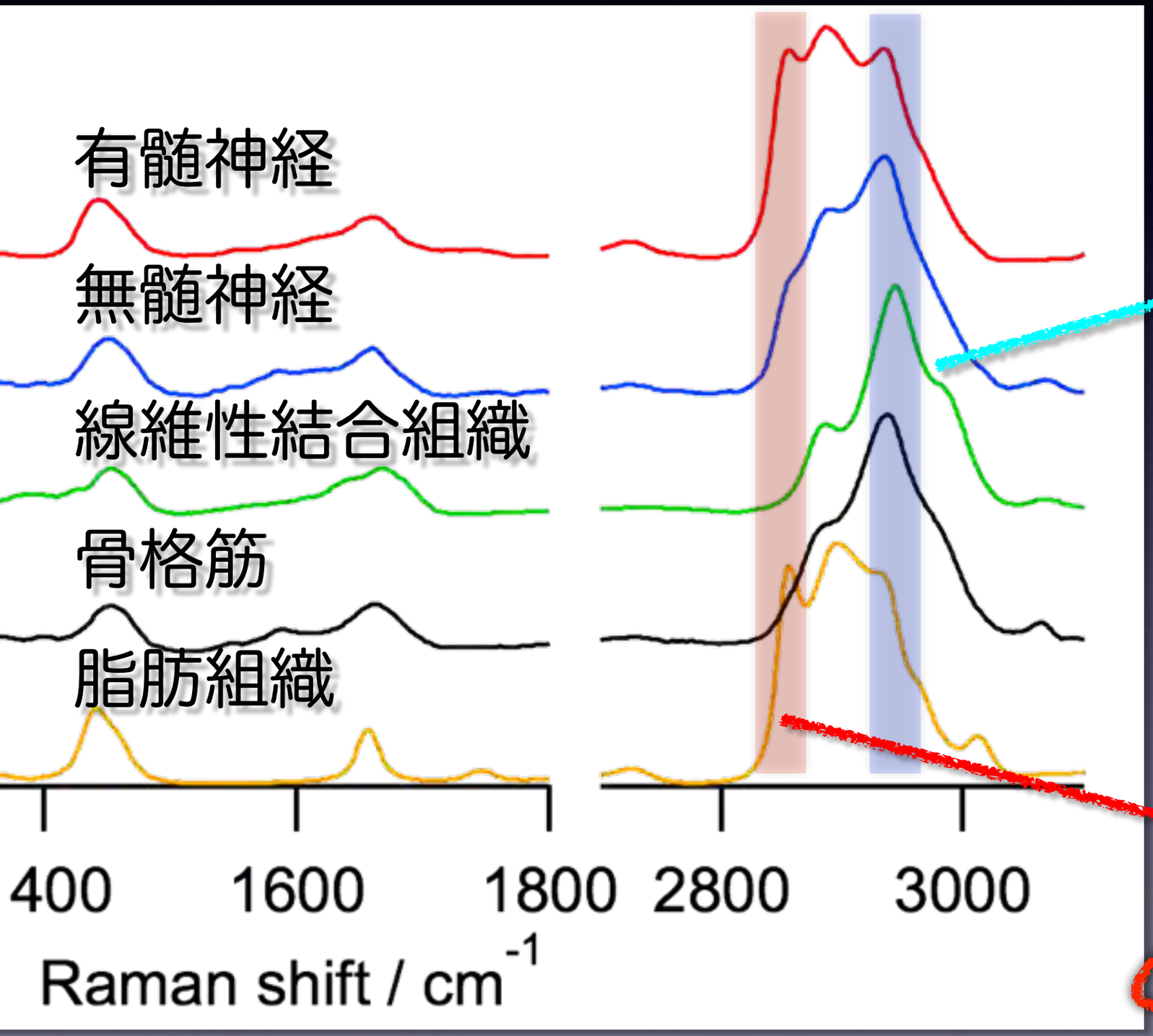
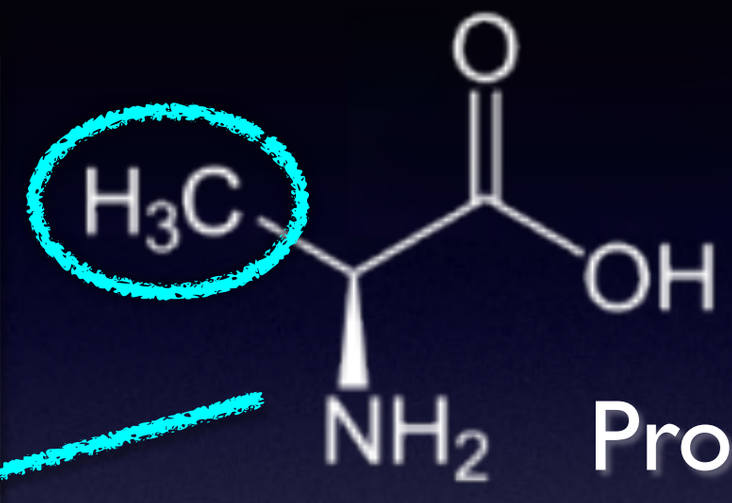
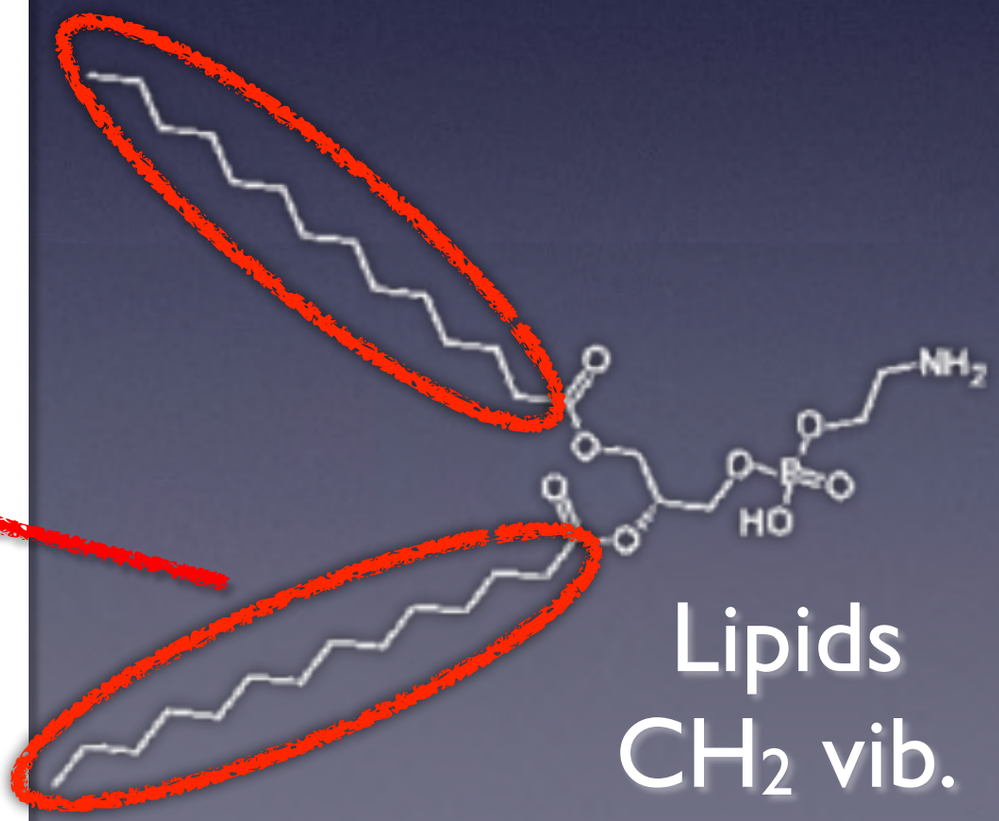




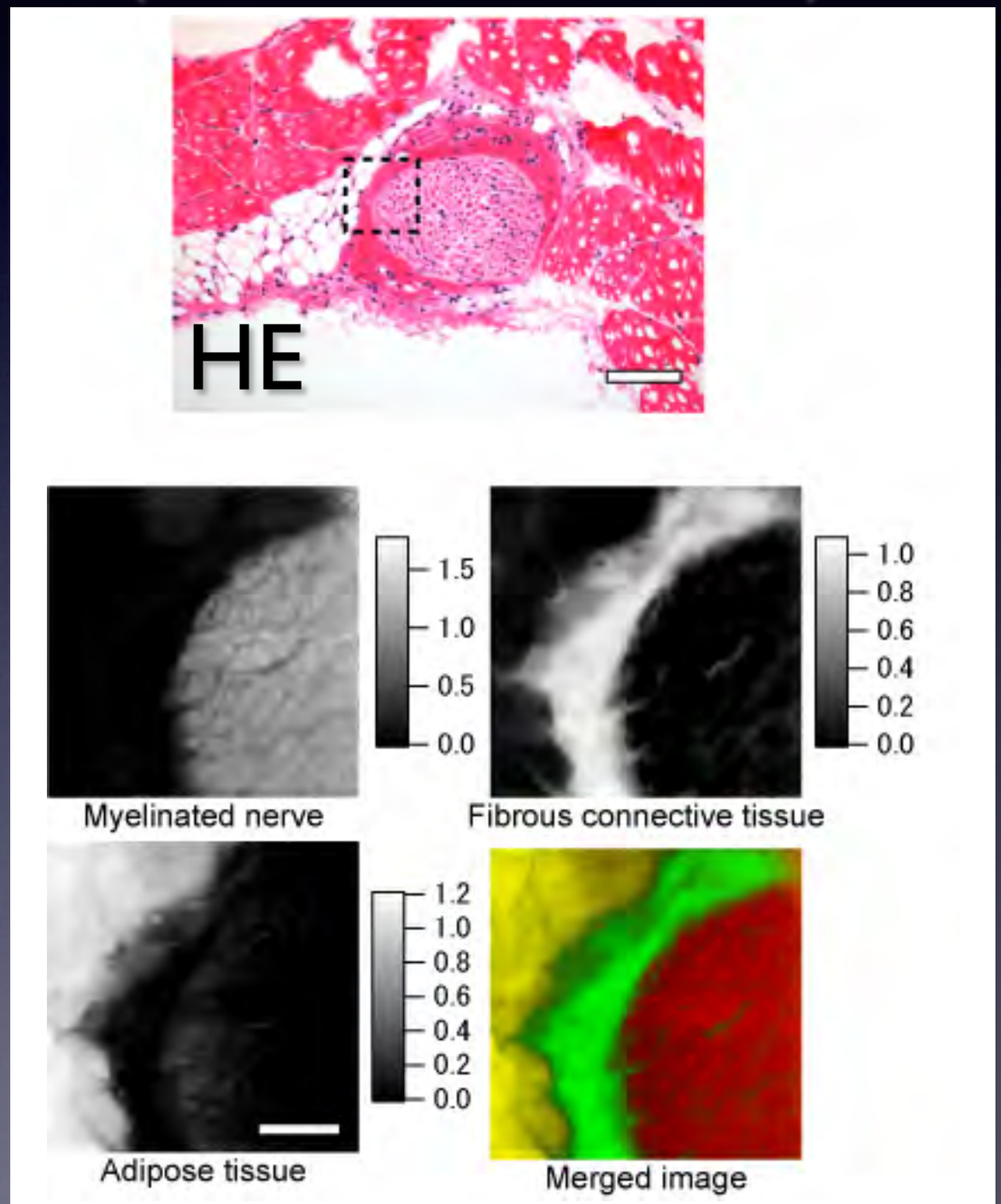
Proteins
CH_3 vib.



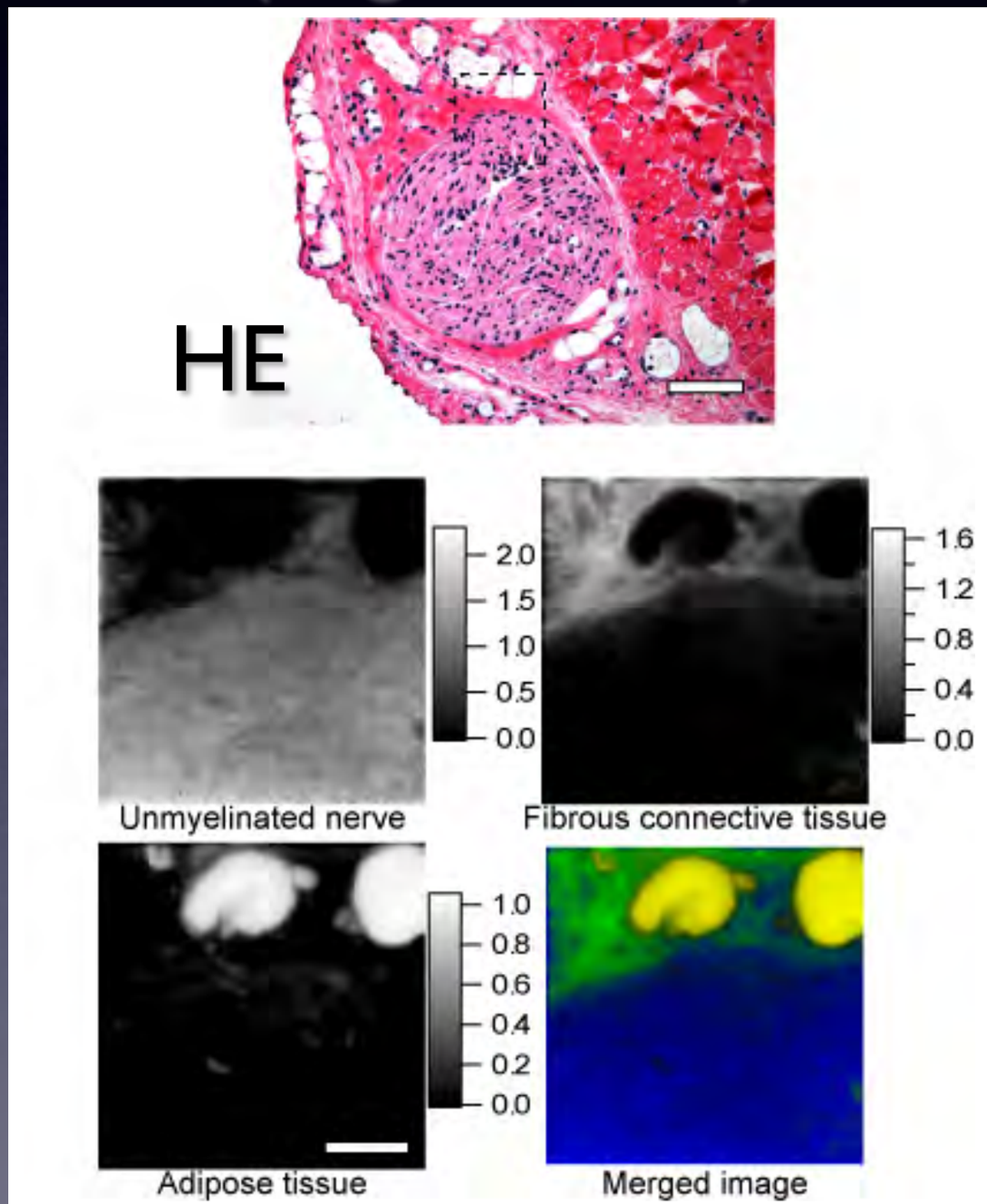
Lipids
CH_2 vib.

Raman imaging of nerves (Wistar rats)

Myelinated nerve (Intercostal nerve)



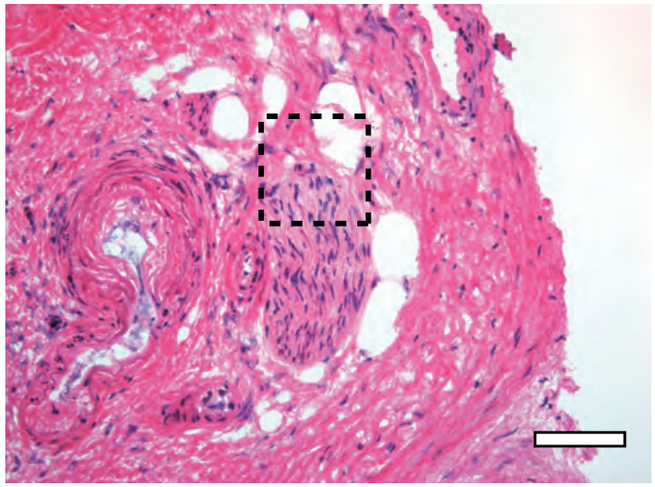
Unmyelinated nerve (Vagus nerve)



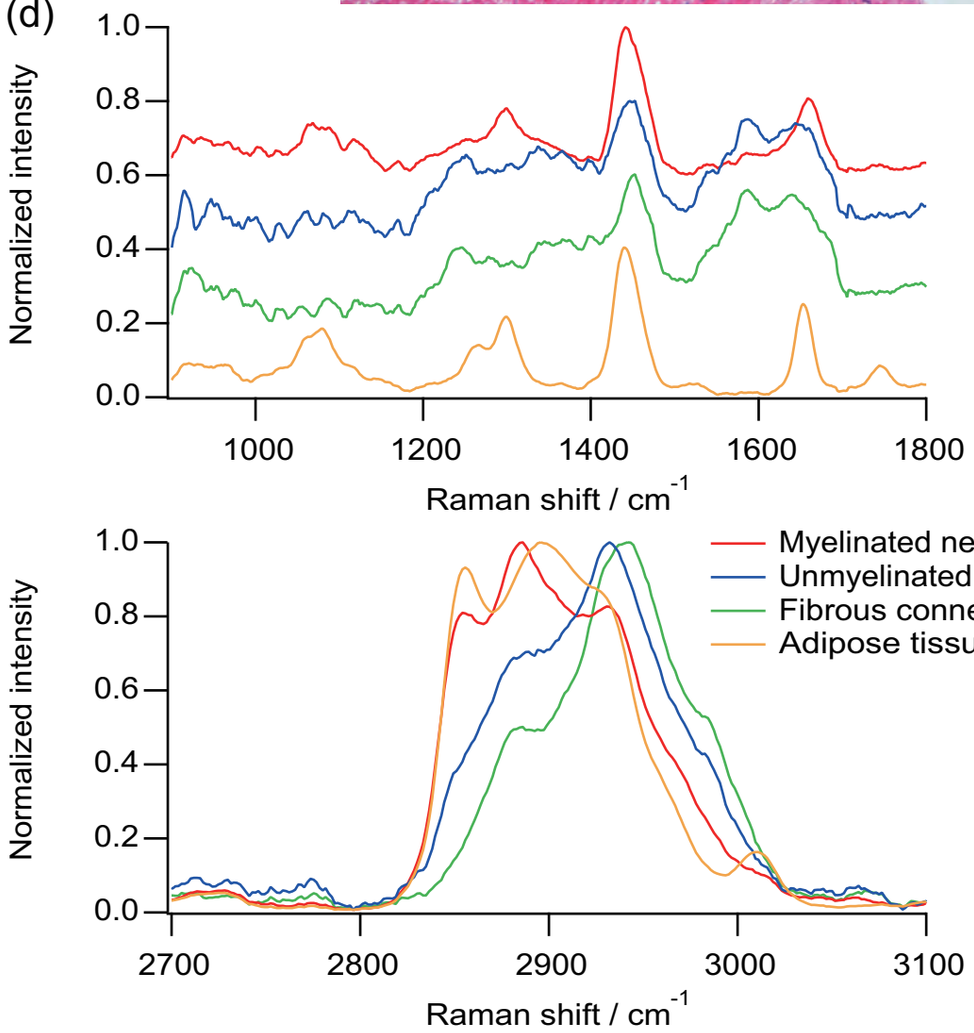
[ref] T. Minamikawa et al., Histochem Cell Biol., 139, 181 (2013).

Raman imaging of human periprostate

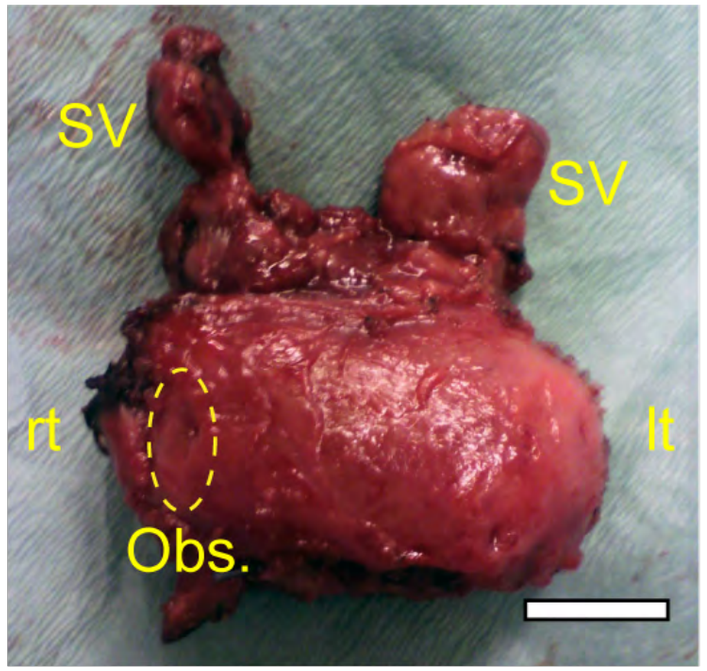
HE



(d)



(a)



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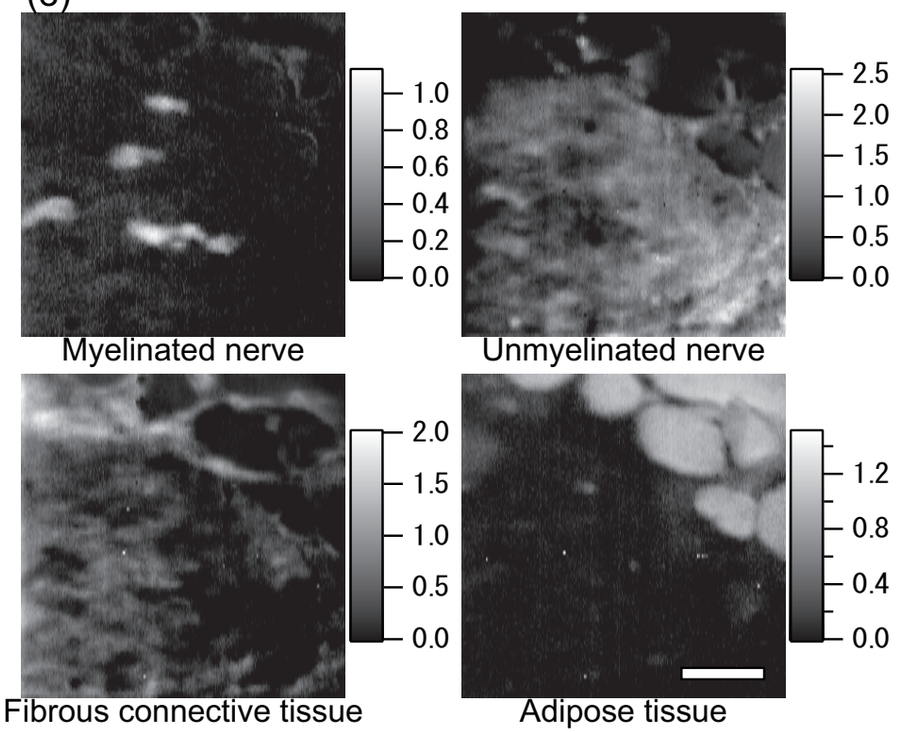
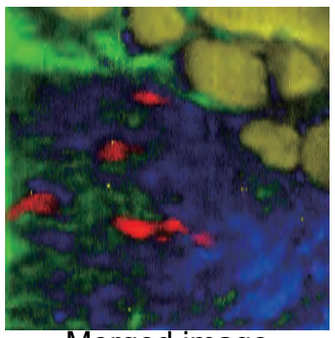




Merged image

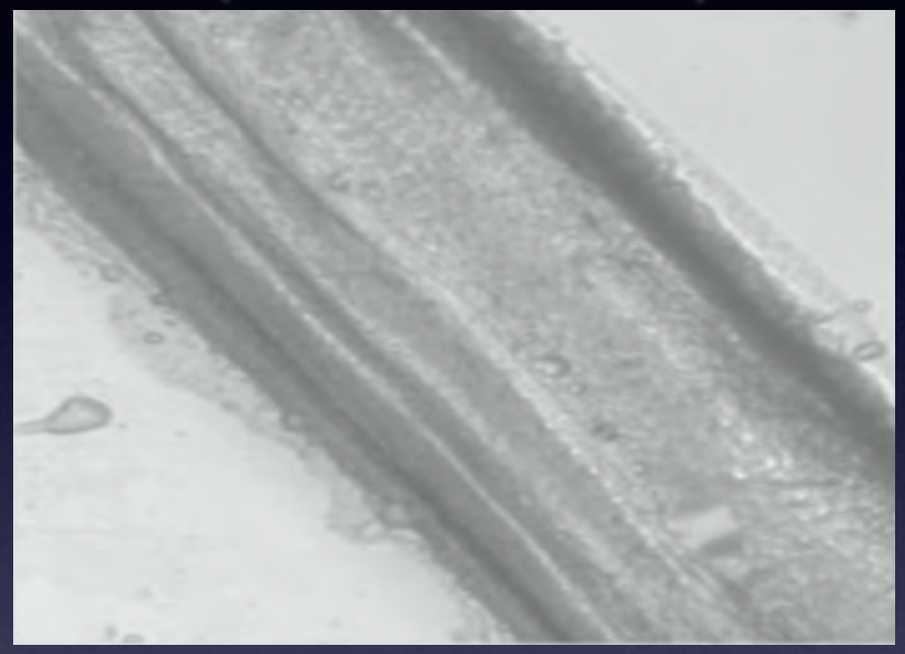
[ref] T. Minamikawa et al., Histochem Cell Biol., 139, 181 (2013).

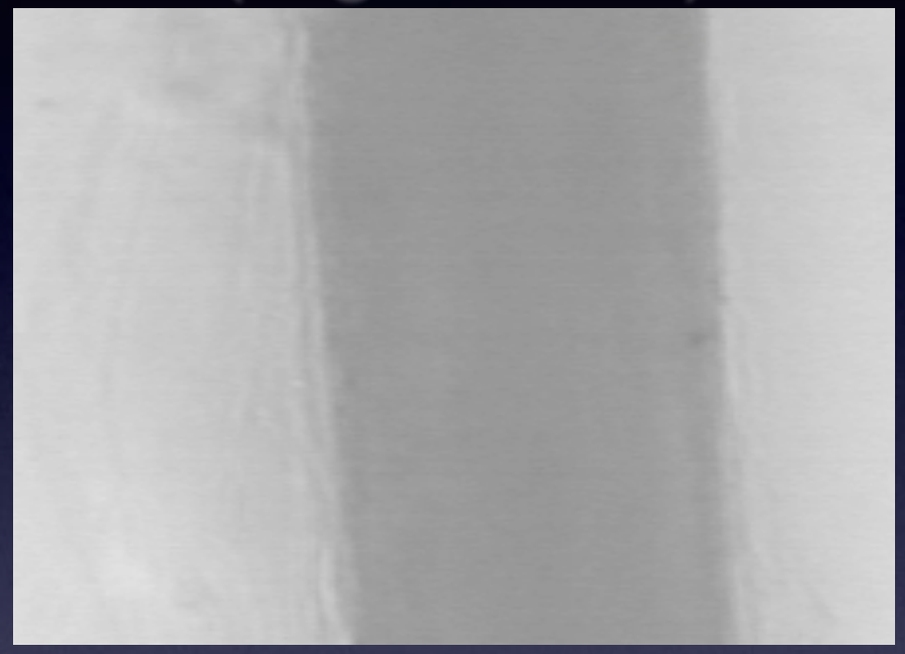
Ex vivo imaging

Myel nerve
(Sciatic nerve)

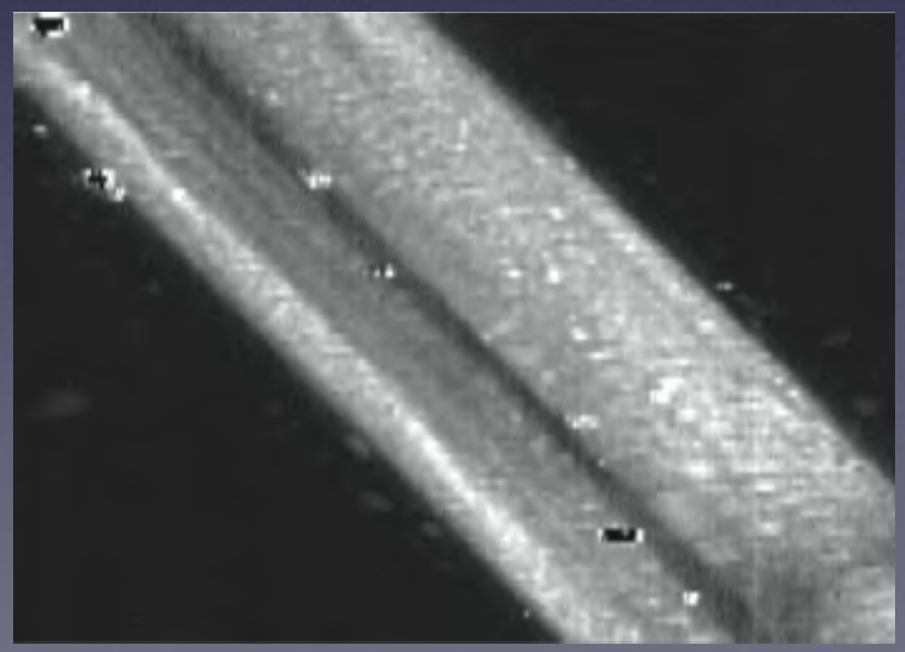
Unmyel nerve
(Vagus nerve)

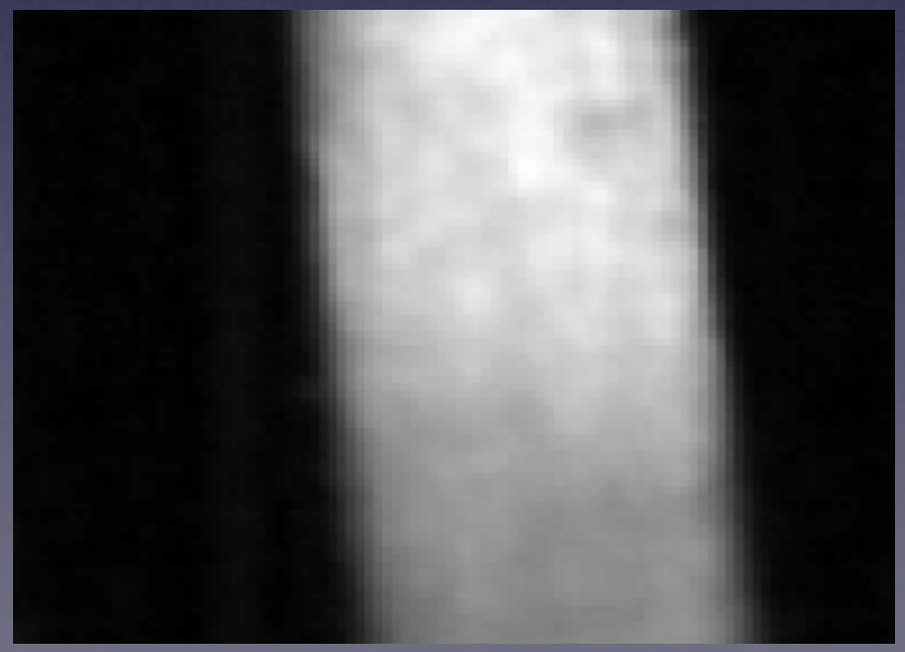
White light





Raman
(2700-3100 cm^{-1})



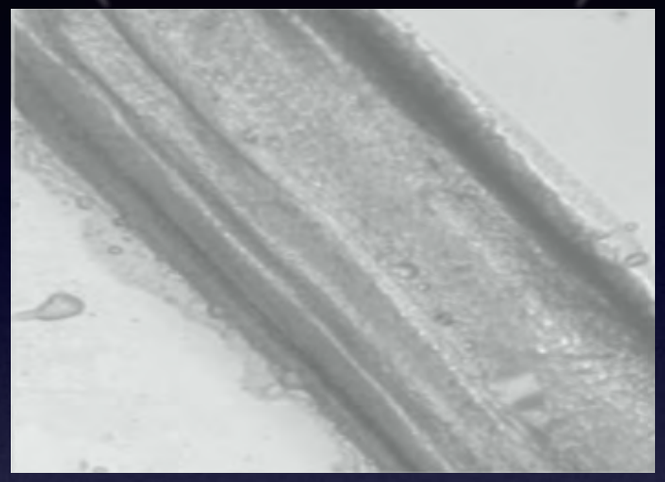


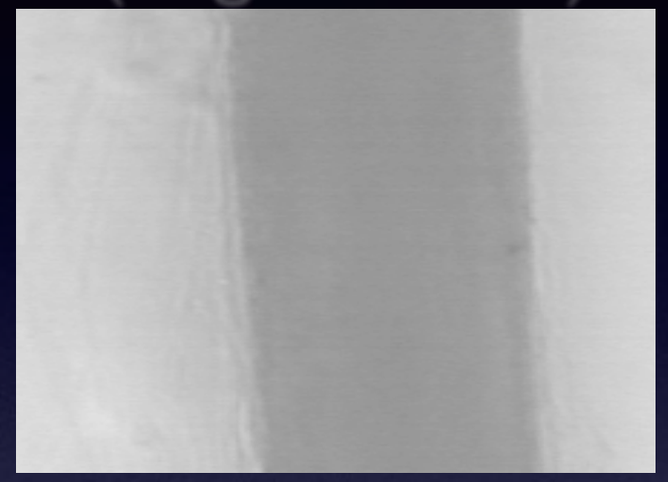
Ex vivo imaging

Myel nerve
(Sciatic nerve)

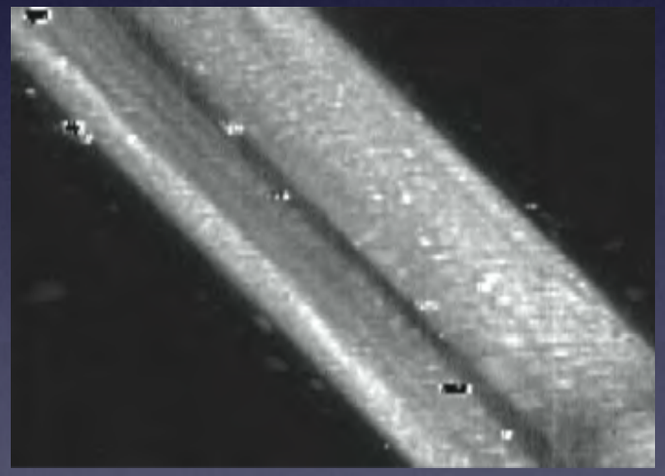
Unmyel nerve
(Vagus nerve)

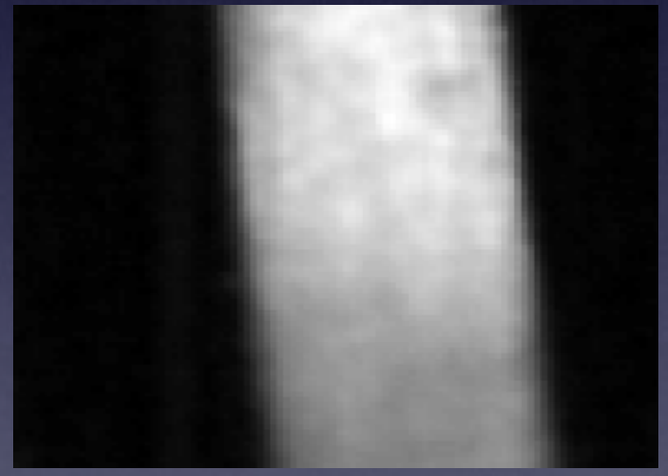
White light





Raman
(2700-3100 cm^{-1})





Prediction

R: Myel
B: Unmyel
W: Others

Prediction

Prediction

R: Myel

B: Unmyel

W: Others

	Myel	Unmyel	Adipose	Collagenous	Muscle
Sensitivity (%)	95.5	88.3	96.5	89.2	88.2
Specificity (%)	99.4	93.5	100	98.0	98.6

T. Minamikawa et al., (submitted), 関西イノベーション総合特区→医療機器化へ

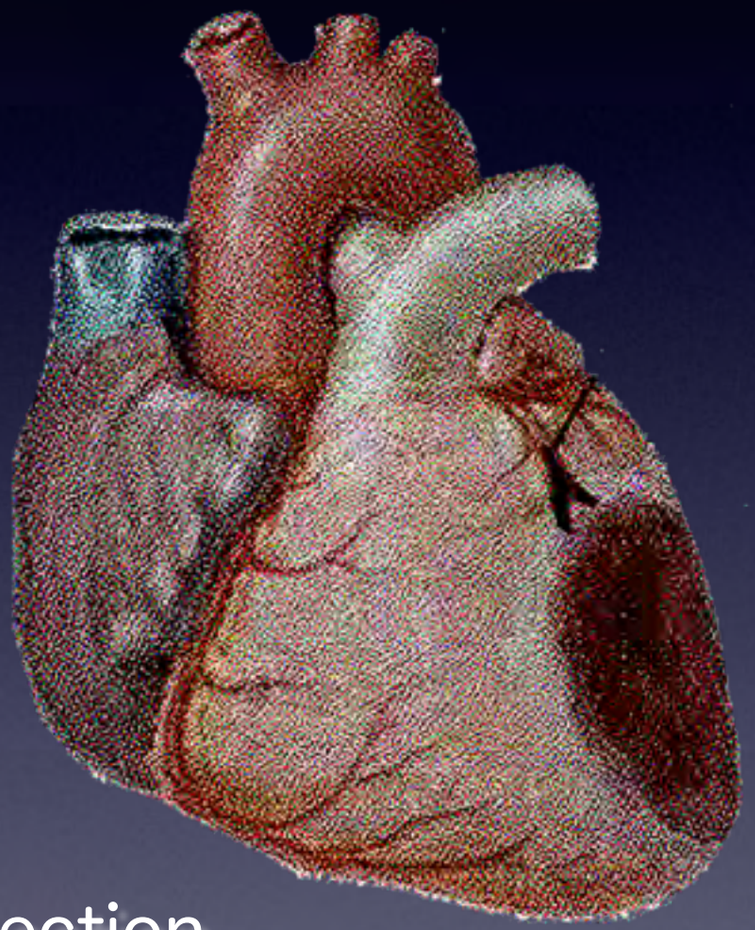
Medical applications II

- Myocardial infarction -

Myocardial infarction (MI)

Death of cardiomyocytes due to prolonged ischemia

Severe heart dysfunction



Treatment of MI

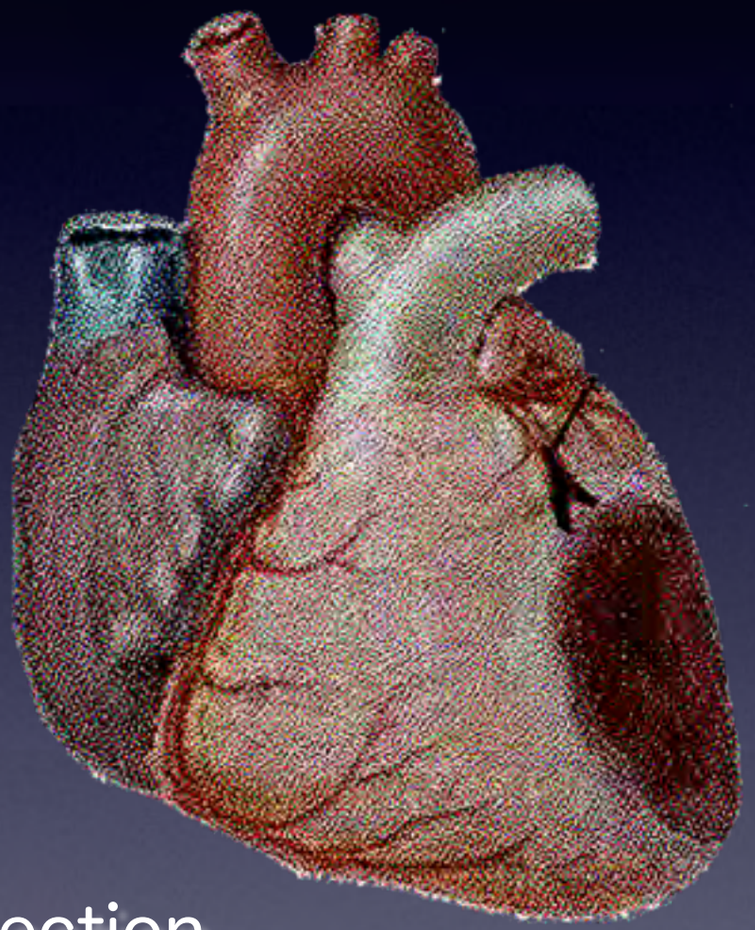
Resection
(SVR: Surgical ventricular restoration)

Revascularization
(CABG: Coronary artery bypass grafting)

Myocardial infarction (MI)

Death of cardiomyocytes due to prolonged ischemia

Severe heart dysfunction



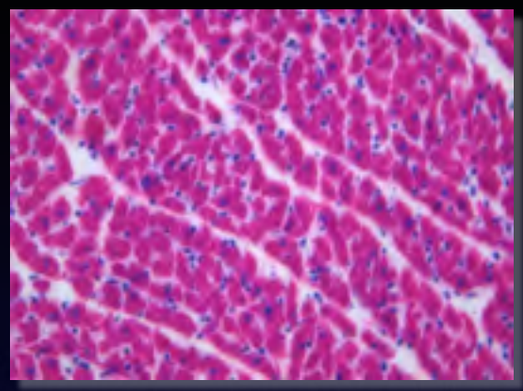
Treatment of MI

Assessment of MI

Resection
(SVR: Surgical ventricular restoration)

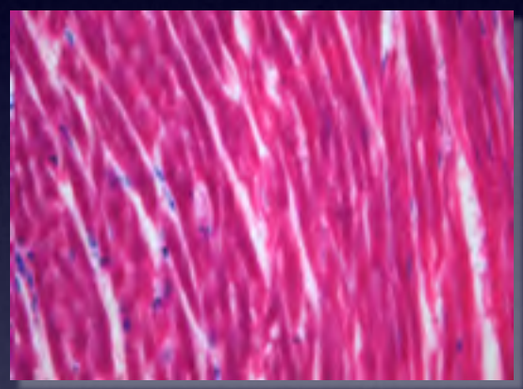
Revascularization
(CABG: Coronary artery bypass grafting)

Development of myocardial infarction



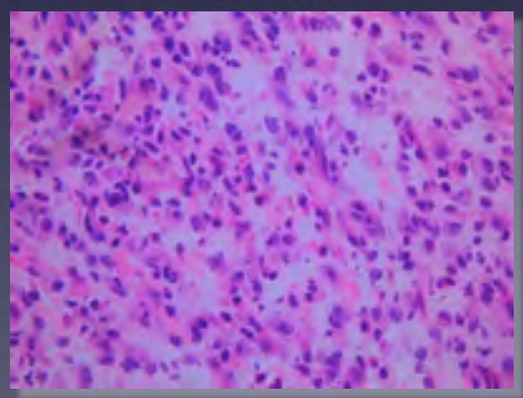
Normal

Living cardiomyocytes



Necrosis

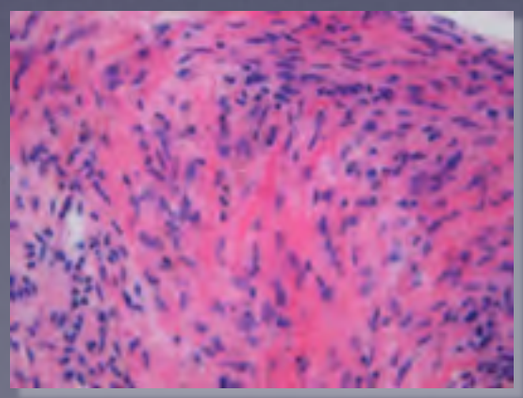
Dead cardiomyocytes



Granulation

Inflammatory cells

Angiogenesis etc



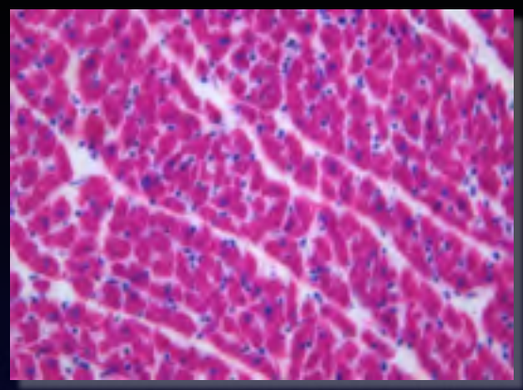
Fibrosis

Collagen

Ischemia

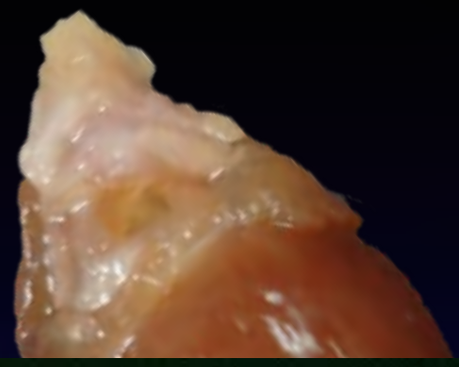
Infarction!!

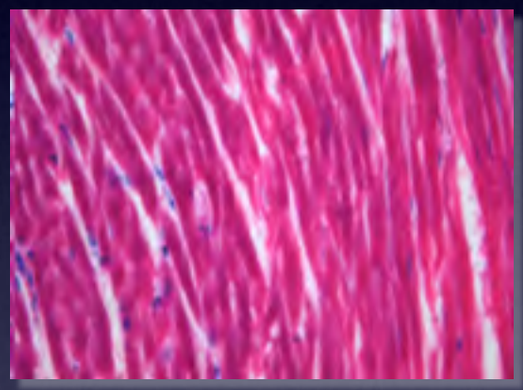
Development of myocardial infarction



Normal

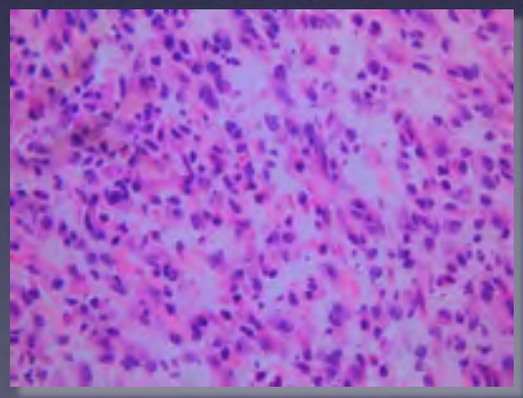
Living cardiomyocytes





Ne

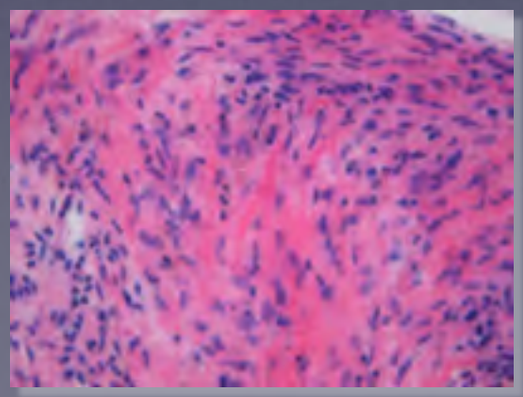
D



Gr

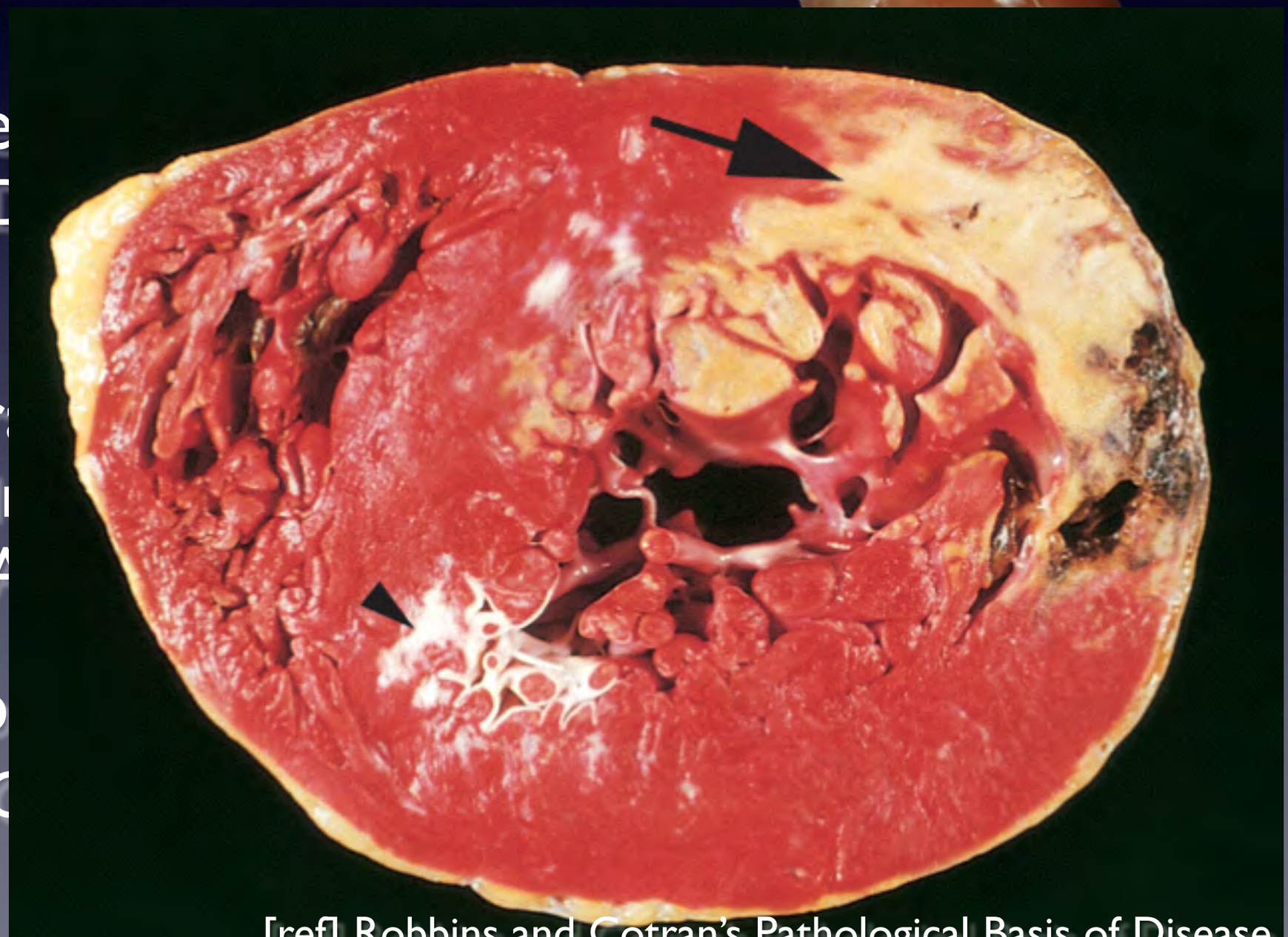
I

A



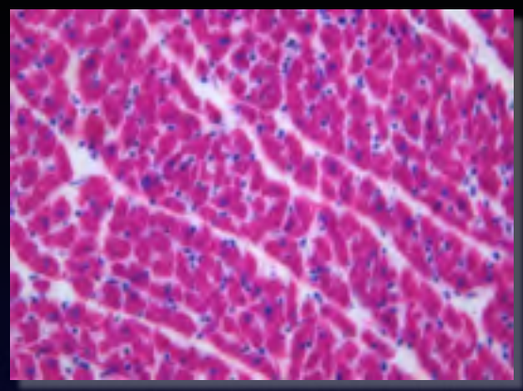
Fib

C



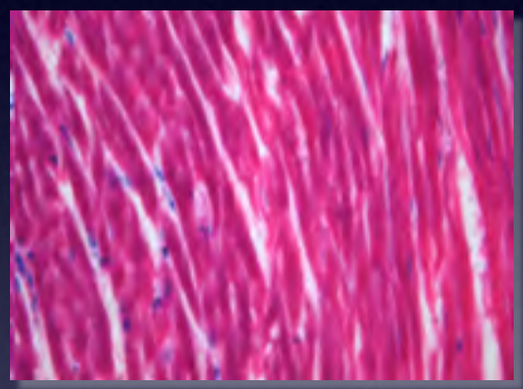
[ref] Robbins and Cotran's Pathological Basis of Disease

Development of myocardial infarction



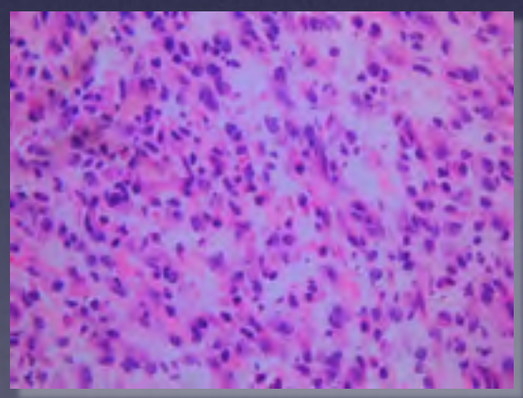
Normal

Living cardiomyocytes



Necrosis

Dead cardiomyocytes



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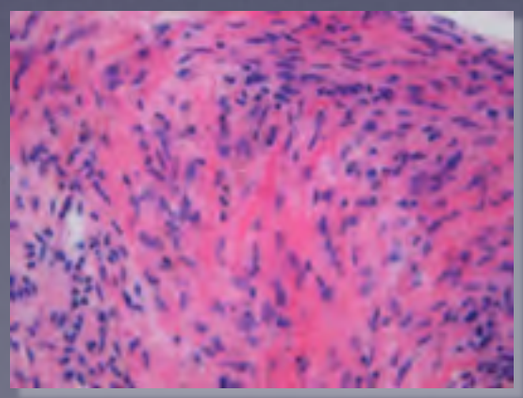
Inflammatory cells

Angiogenesis etc



Fibrosis

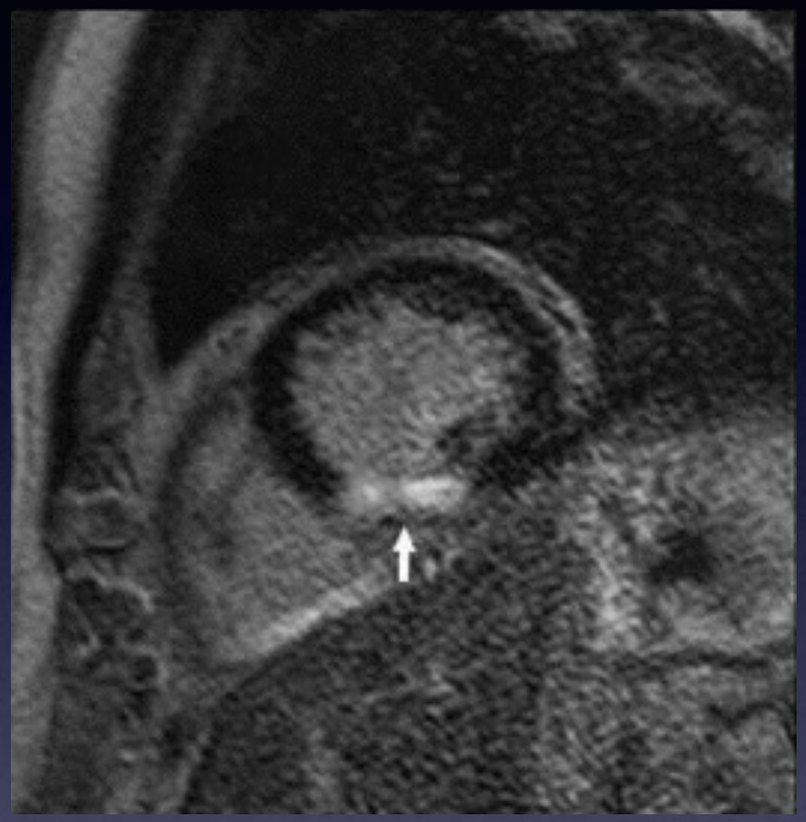
Collagen

Ischemia

Infarction!!

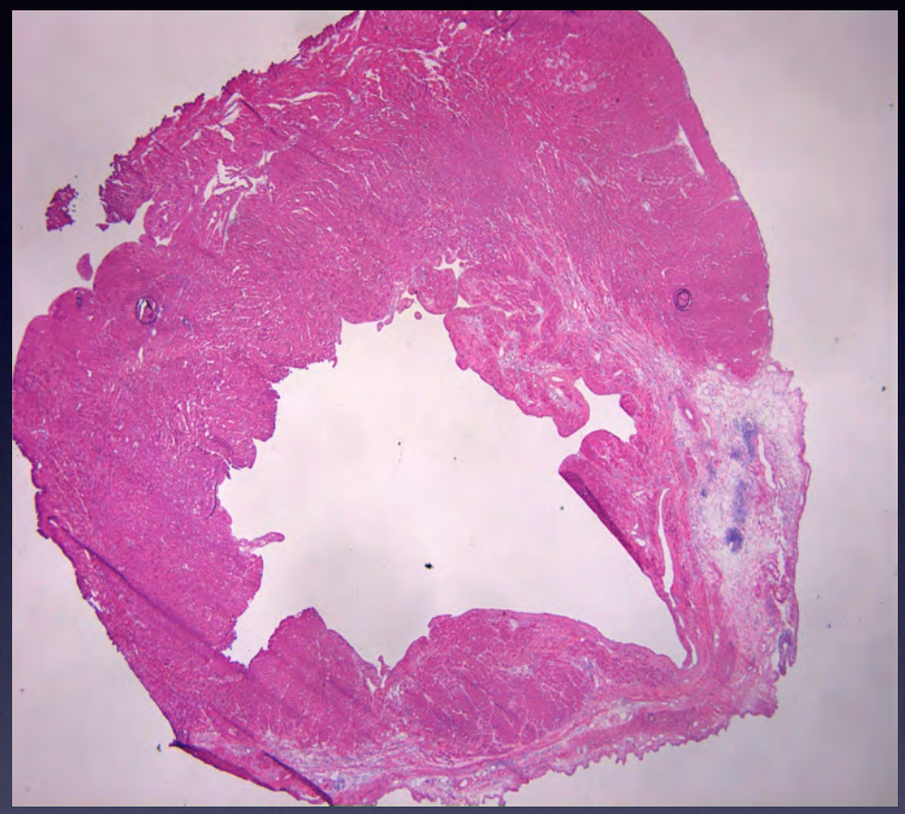
Conventional methods for assessing MI

MRI



Visualization of infarcted region
In vivo assessment
Requires contrast agents
Limits in histological analysis
Requires large system

Histopathological analysis



Visualization of histology
Ex vivo assessment
Requires invasive biopsy
Requires sectioning and staining

[ref] M. Ogawa et al. Gen. Thorac. Cardiovasc. Surg, (2007)

Myocardial infarction model

Female Wistar rat
(8-wks-old)

Ligated at left anterior descending branch

Excised at each post-ligation time

Normal : Normal myocardial tissue
2 days : Coagulation necrosis
5 days : Coagulation necrosis and granulation
21 days : Fibrosis

All animal experiments were conducted with the approval of and in accordance with guidelines from the Committee for Animal Research, Kyoto Prefectural University of Medicine, Japan.

Raman spectra of infarcted hearts

Normal

Necrosis

Day 2

Day 5

Granulation

Fibrosis

600 800 1000 1200 1400 1600 2800 3000

Raman shift / cm^{-1}

*Mean ± SD

[1] N.N Muranishi et al., Anal. Chem, (2014).

Raman spectra of infarcted hearts

Normal

Necrosis

Day 2

Day 5

Granulation

Fibrosis

600 800 1000 1200 1400 1600 2800 3000

Raman shift / cm^{-1}

*Mean ± SD

750 cm^{-1} : Cytochrome c [2]

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Raman spectra of infarcted hearts

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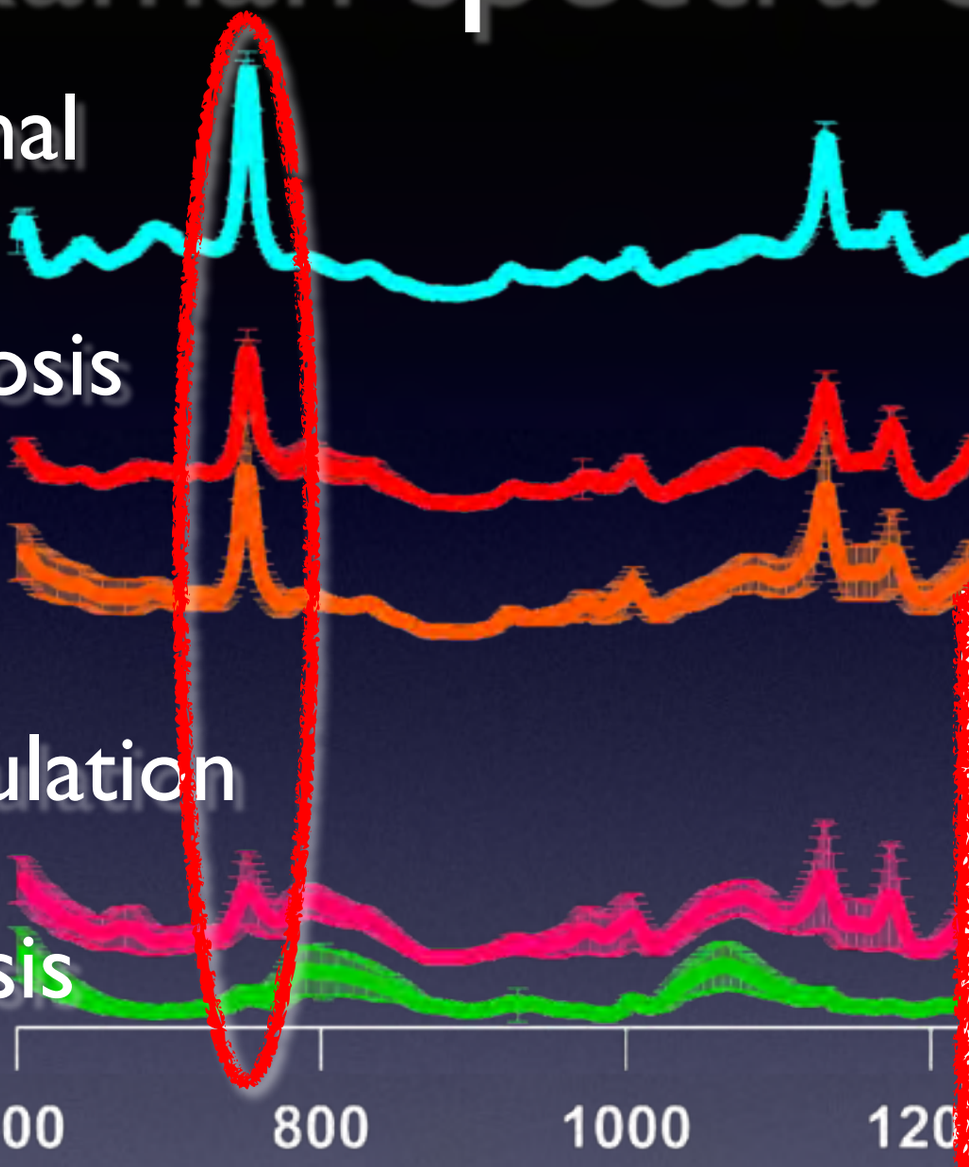
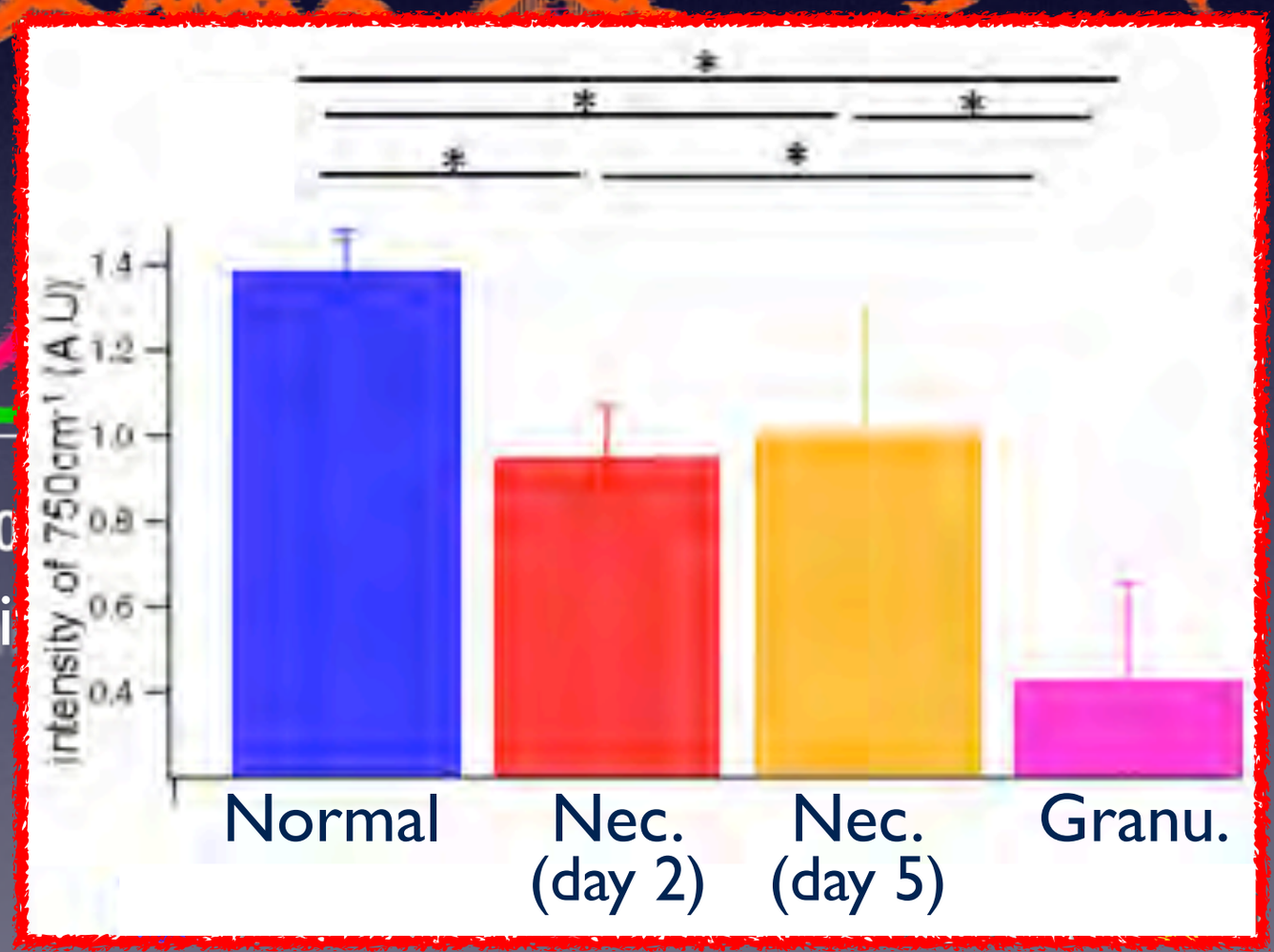


750 cm^{-1} : Cytochrome c [2]



[1] N.N Muranishi et al., Anal. Chem, (2014).

Raman spectra of infarcted hearts

Normal

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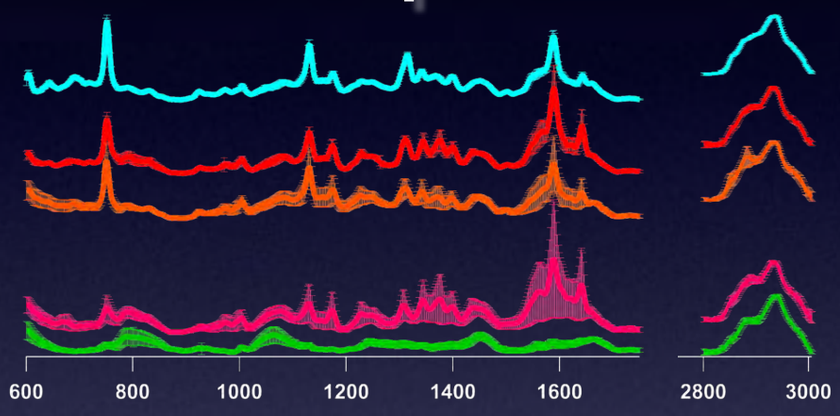
Partial least-squares regression discriminant analysis (PLS-DA)

✓ Raman spectrum



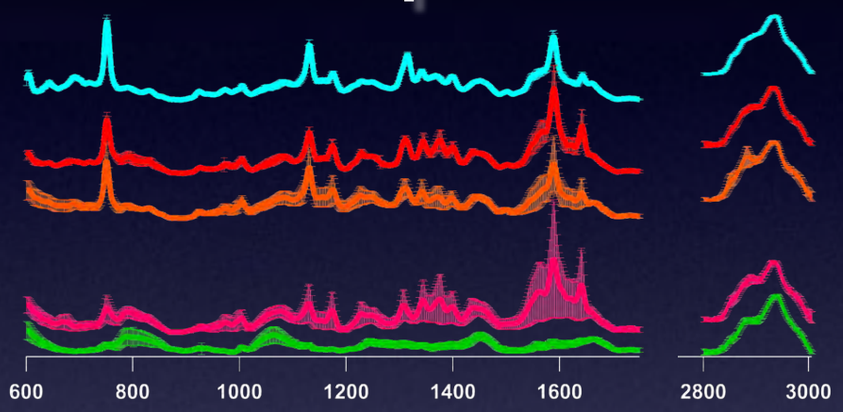
✓ Tissue types

- Normal
- Necrosis
- Granulation
- Fibrosis

[1] N.N Muranishi et al., Anal. Chem, (2014).

Partial least-squares regression discriminant analysis (PLS-DA)

- ✓ Raman spectrum

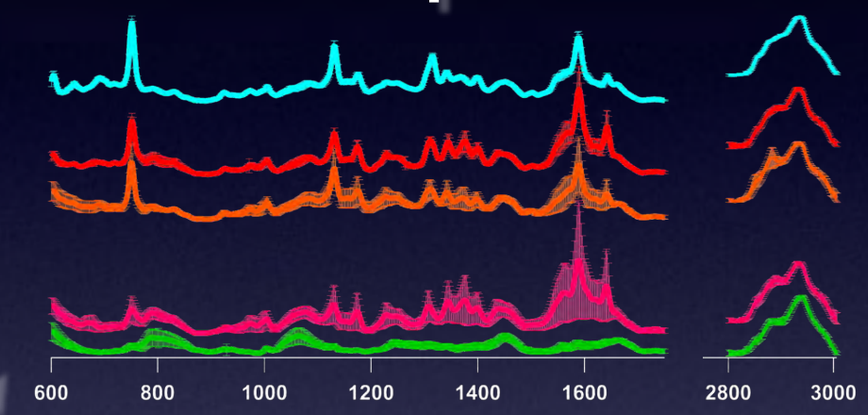


- ✓ Tissue types
  - Normal
  - Necrosis
  - Granulation
  - Fibrosis

[1] N.N Muranishi et al., Anal. Chem, (2014).

Partial least-squares regression discriminant analysis (PLS-DA)

✓ Raman spectrum



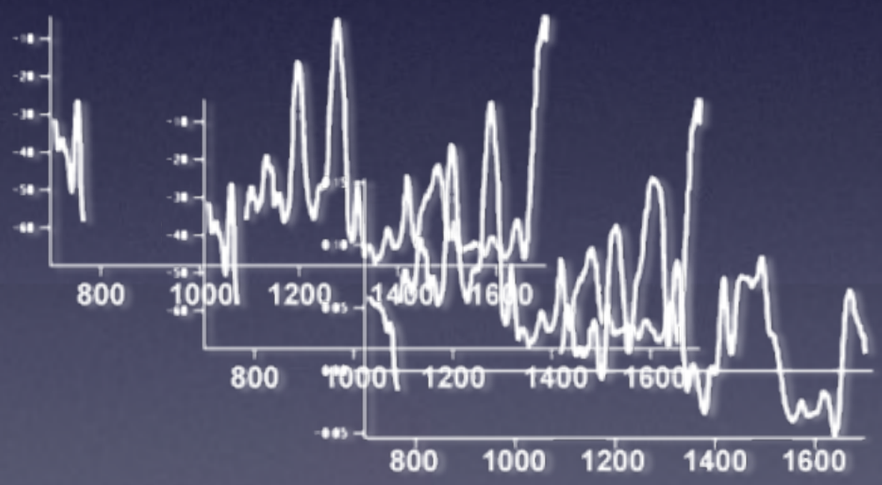
✓ Tissue types

- Normal
- Necrosis
- Granulation
- Fibrosis

✓ PLS

Extract featured components

- Latent variables (LV)
- Scores

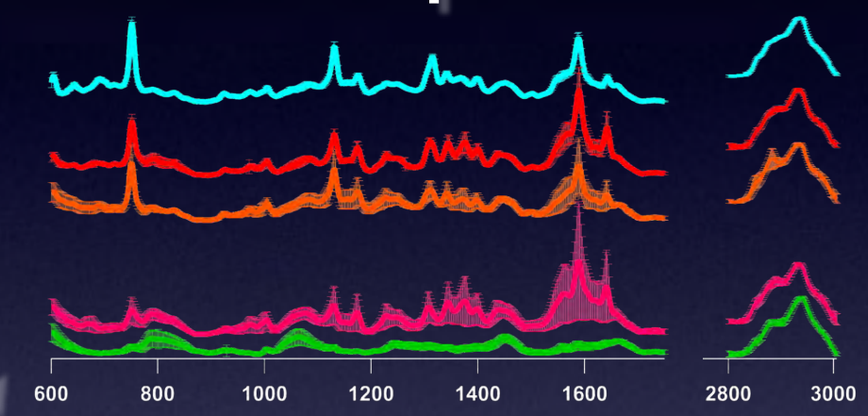


LV1, LV2, LV3…

[1] N.N Muranishi et al., Anal. Chem, (2014).

Partial least-squares regression discriminant analysis (PLS-DA)

✓Raman spectrum



✓Tissue types

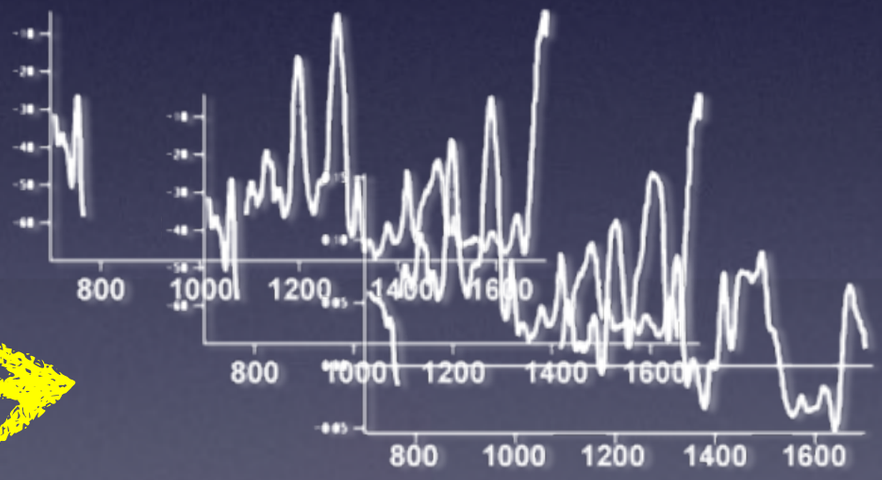
- Normal
- Necrosis
- Granulation
- Fibrosis

✓PLS

Extract featured components

- Latent variables (LV)
- Scores

Related!

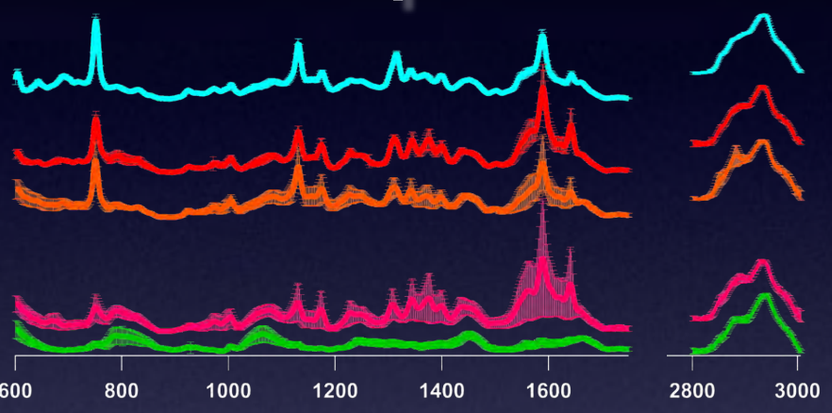


LV1, LV2, LV3…

[1] N.N Muranishi et al., Anal. Chem, (2014).

Partial least-squares regression discriminant analysis (PLS-DA)

✓Raman spectrum



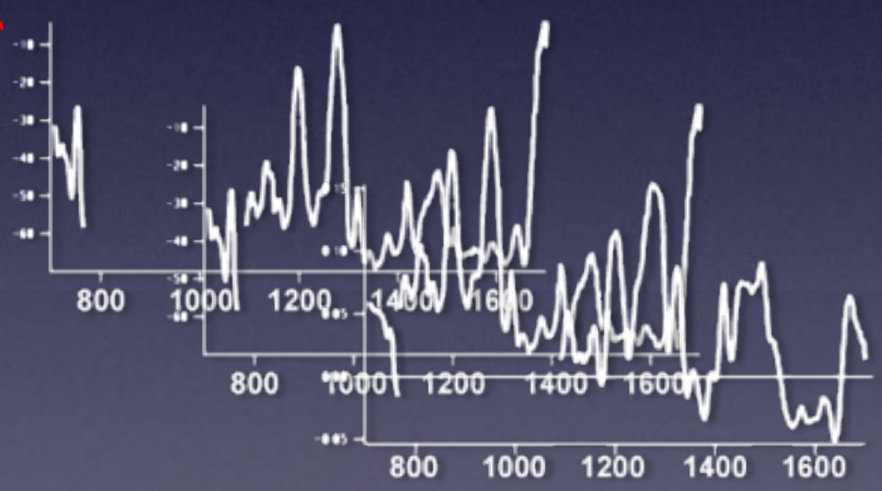
✓Tissue types

- Normal
- Necrosis
- Granulation
- Fibrosis

✓PLS

Extract featured components

- Latent variables (LV)
- Scores



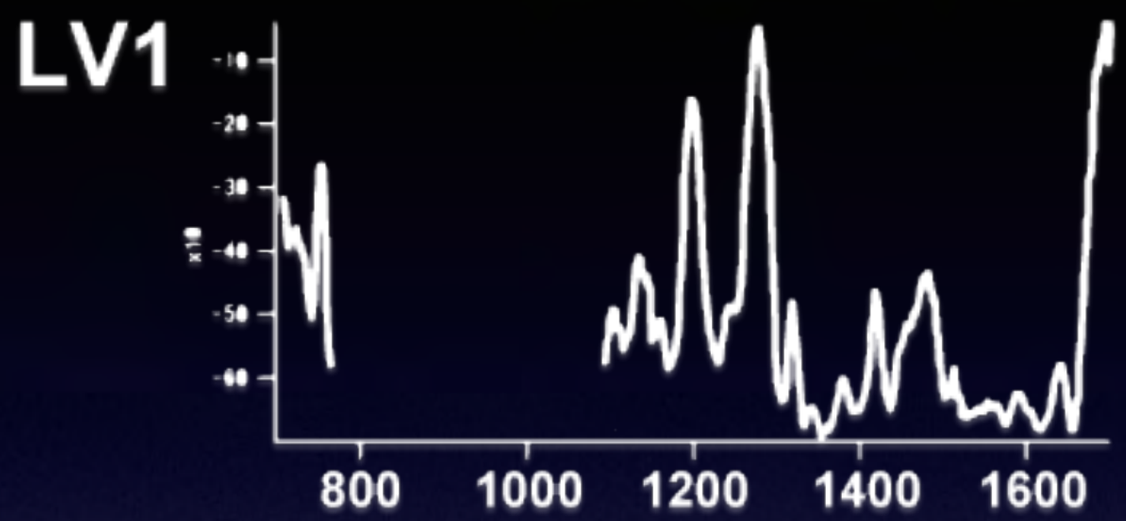
LV1, LV2, LV3…

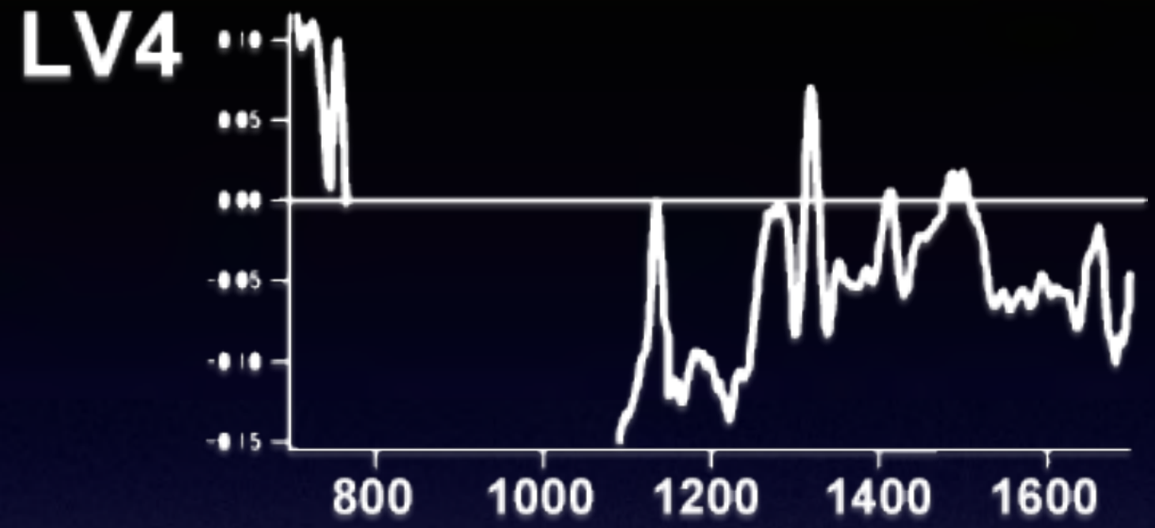
✓Linear DA

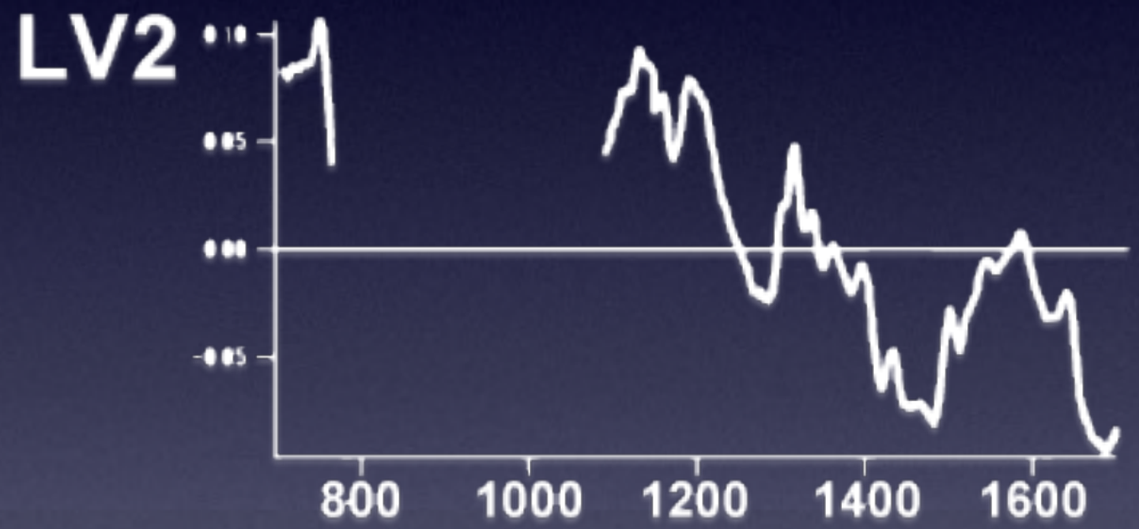
Prediction model of tissue types

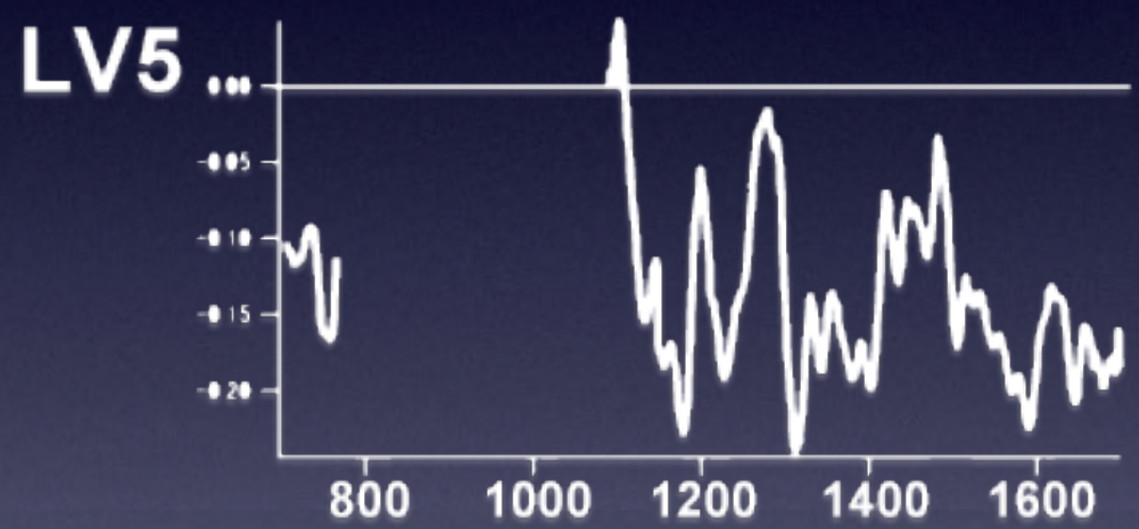
[1] N.N Muranishi et al., Anal. Chem, (2014).

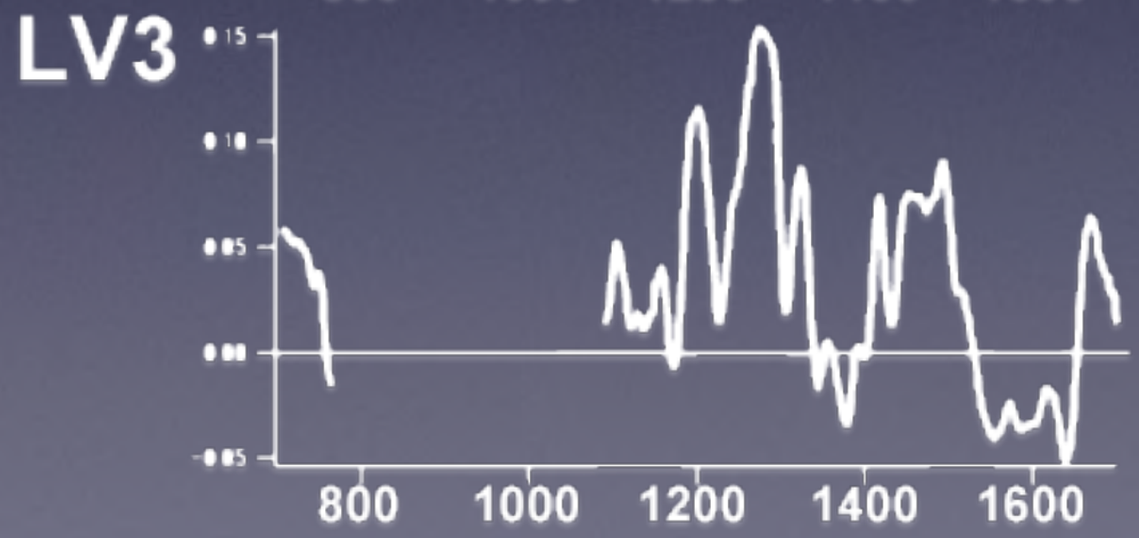
Latent variables of PLS-DA

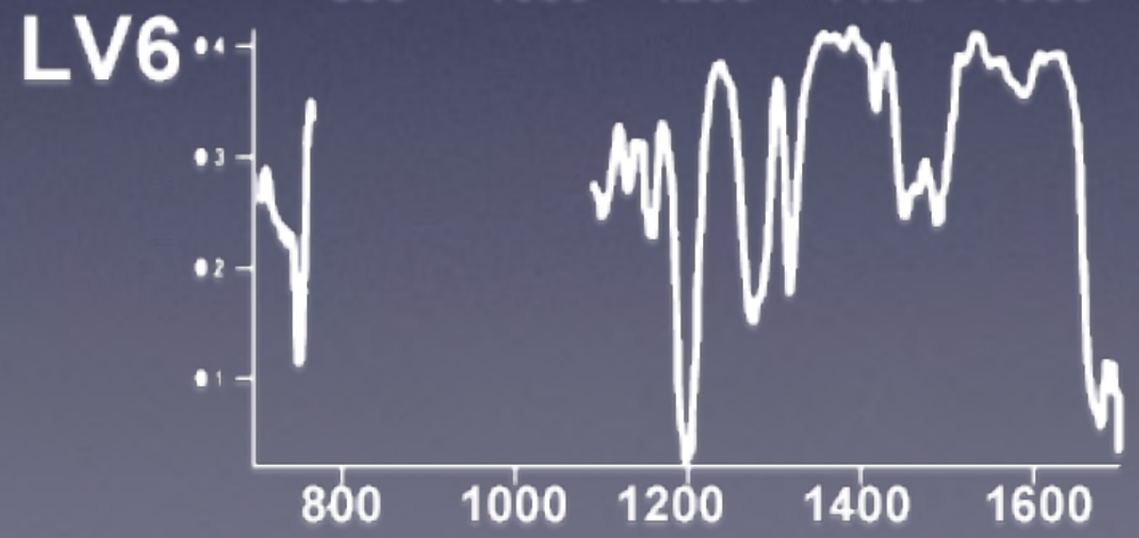






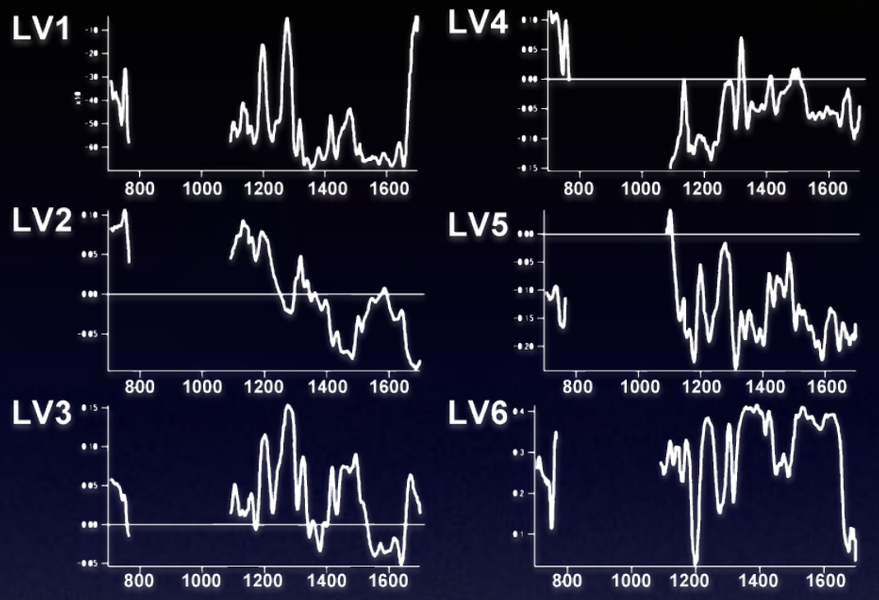


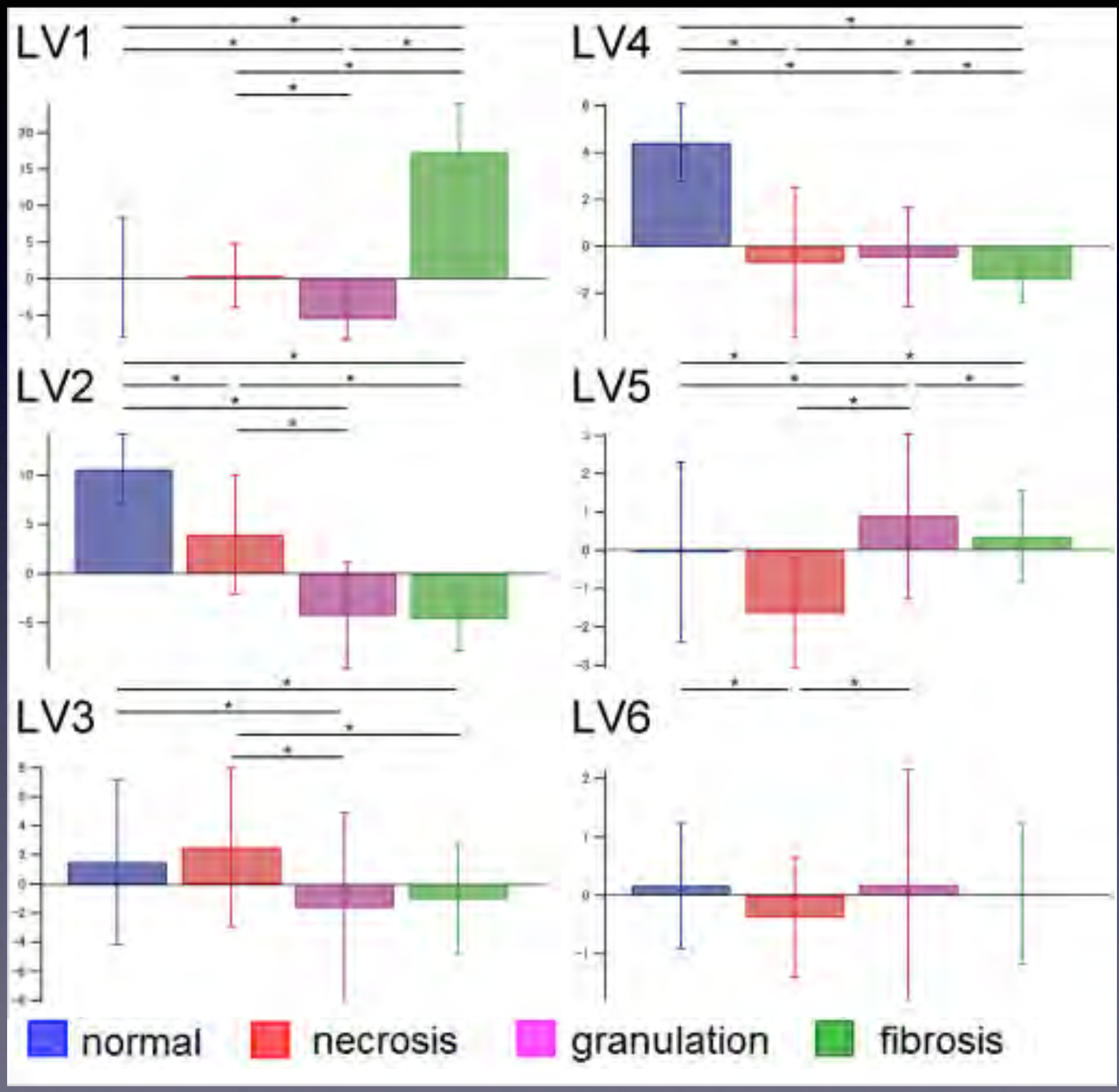




[1] N.N Muranishi et al., Anal. Chem, (2014).

Scores of PLS-DA

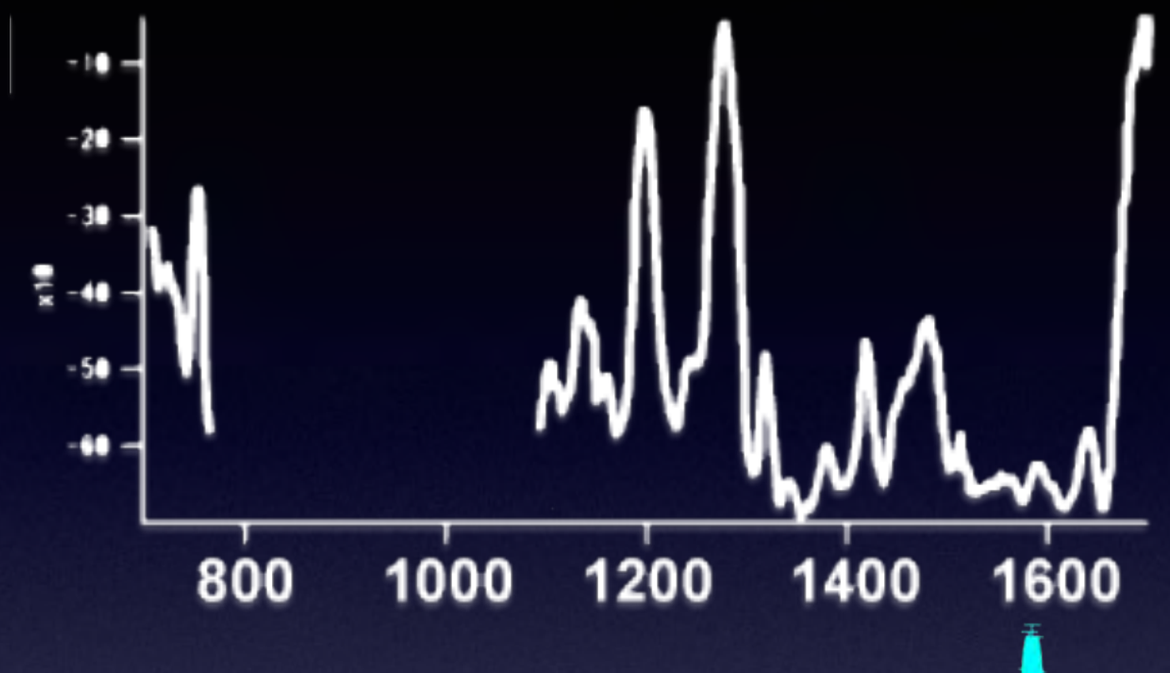


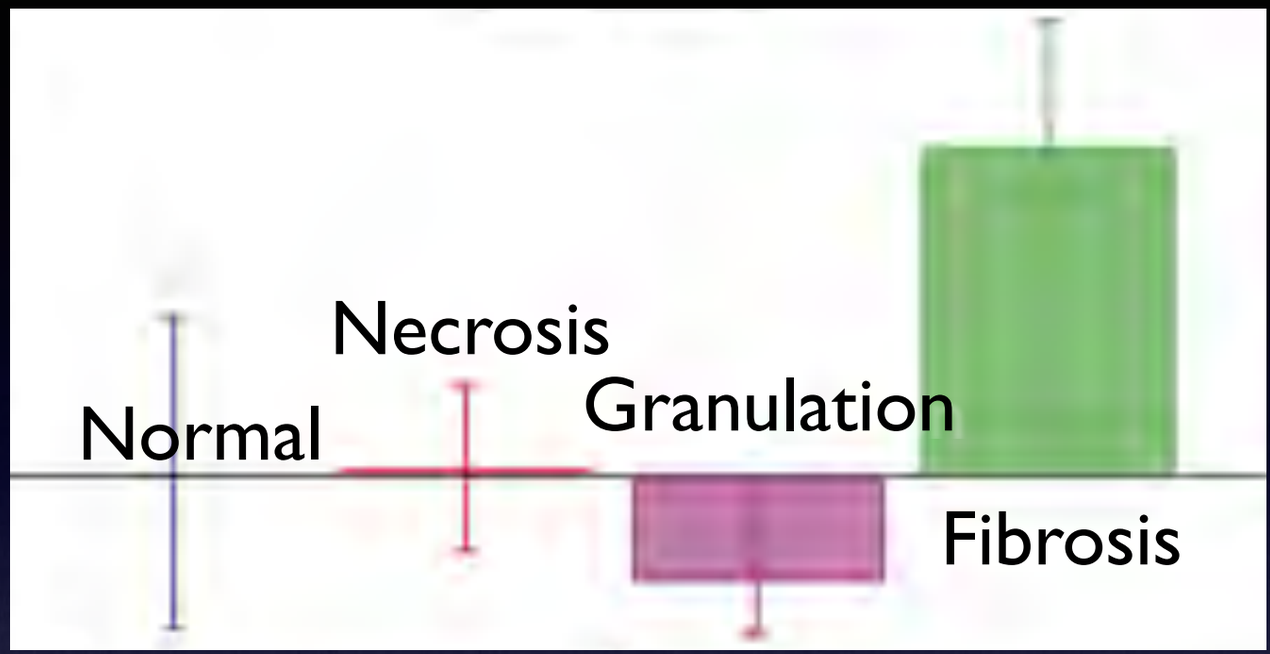


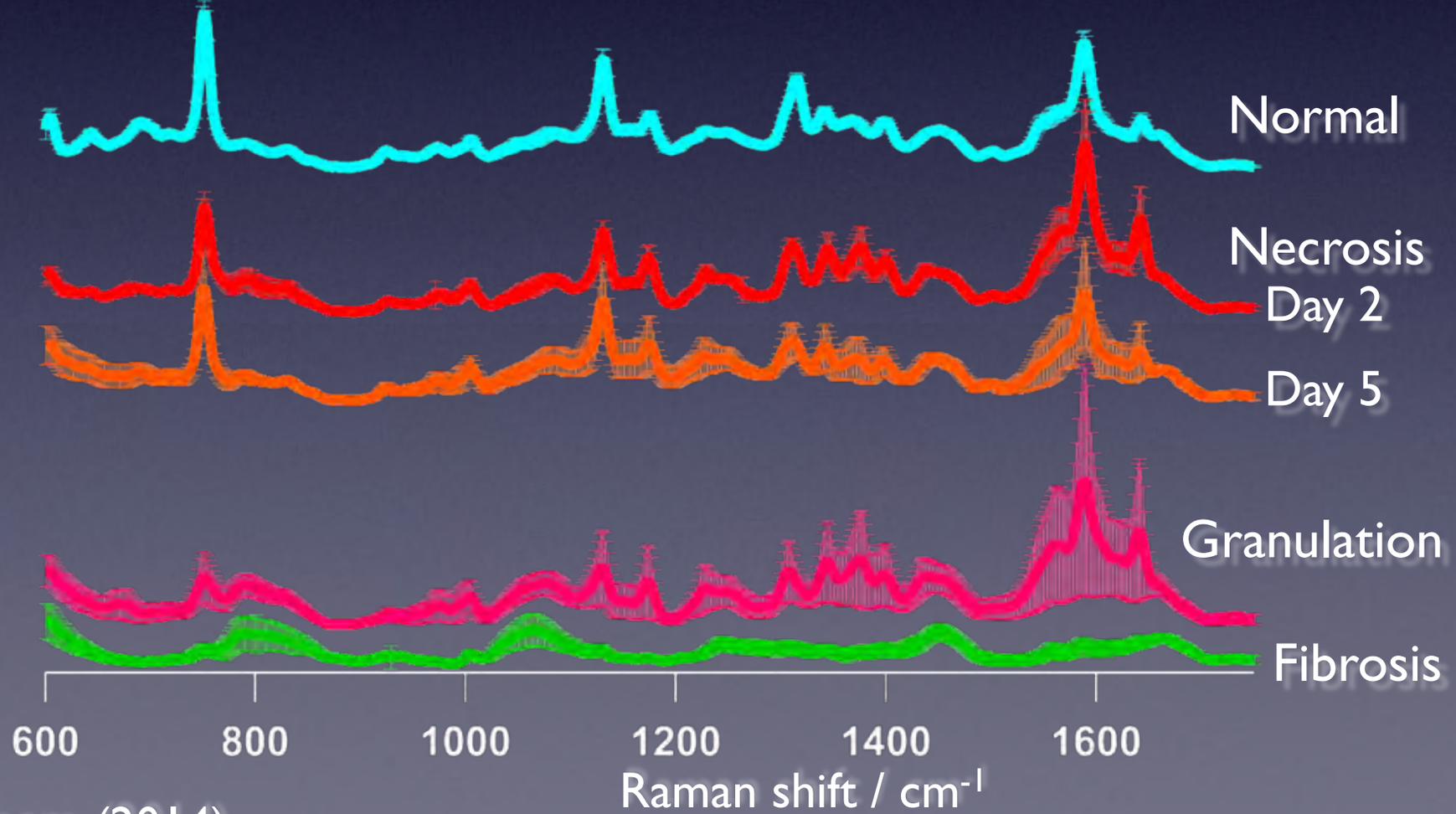
* Significant difference

[1] N.N Muranishi et al., Anal. Chem, (2014).

LVI







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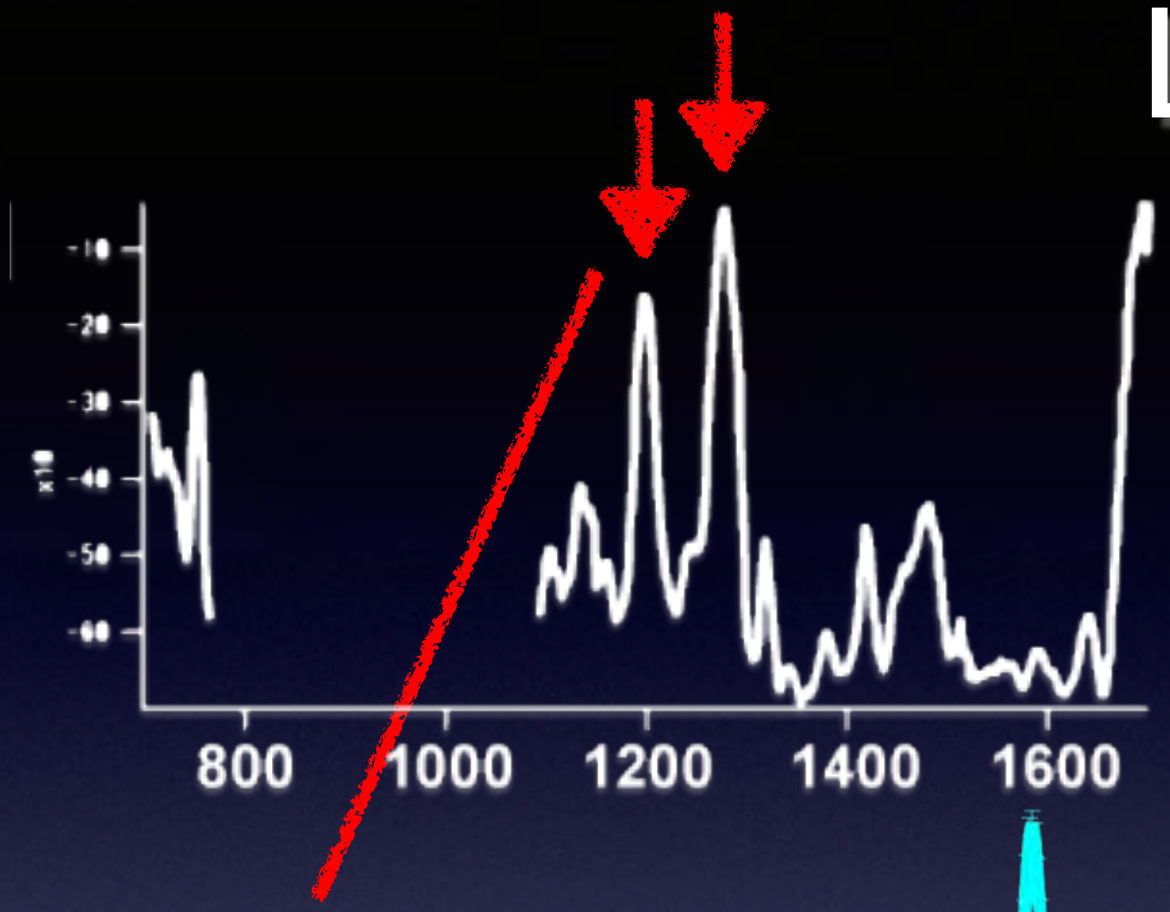
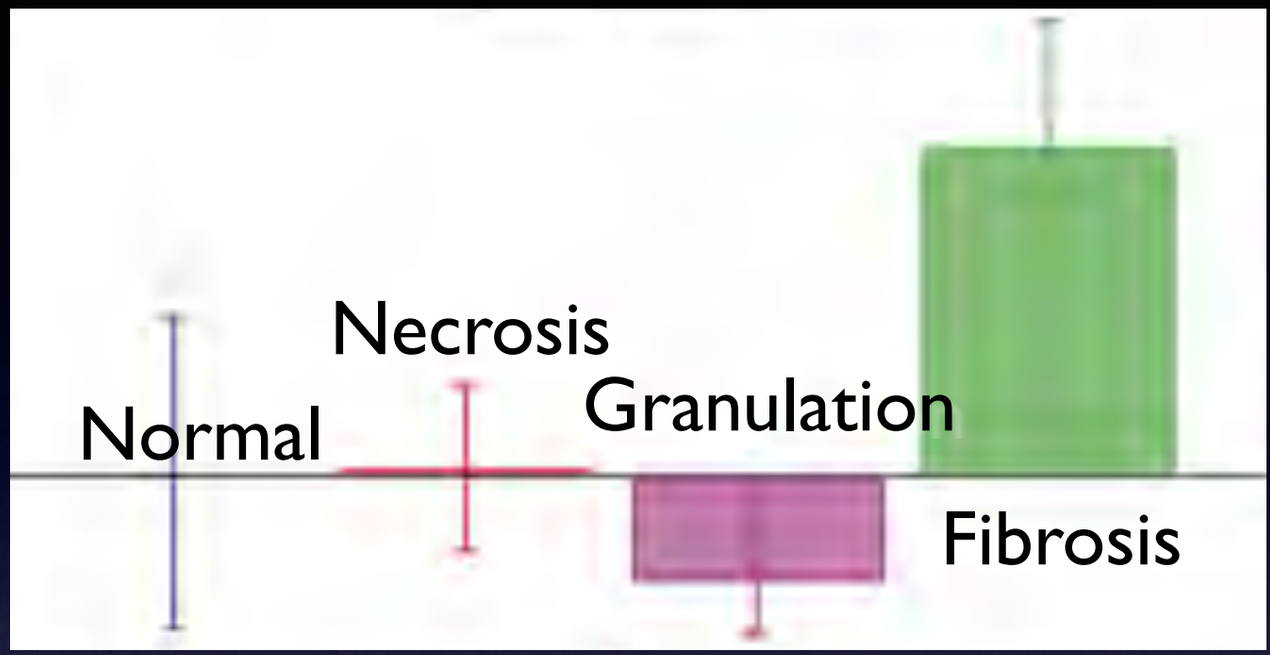
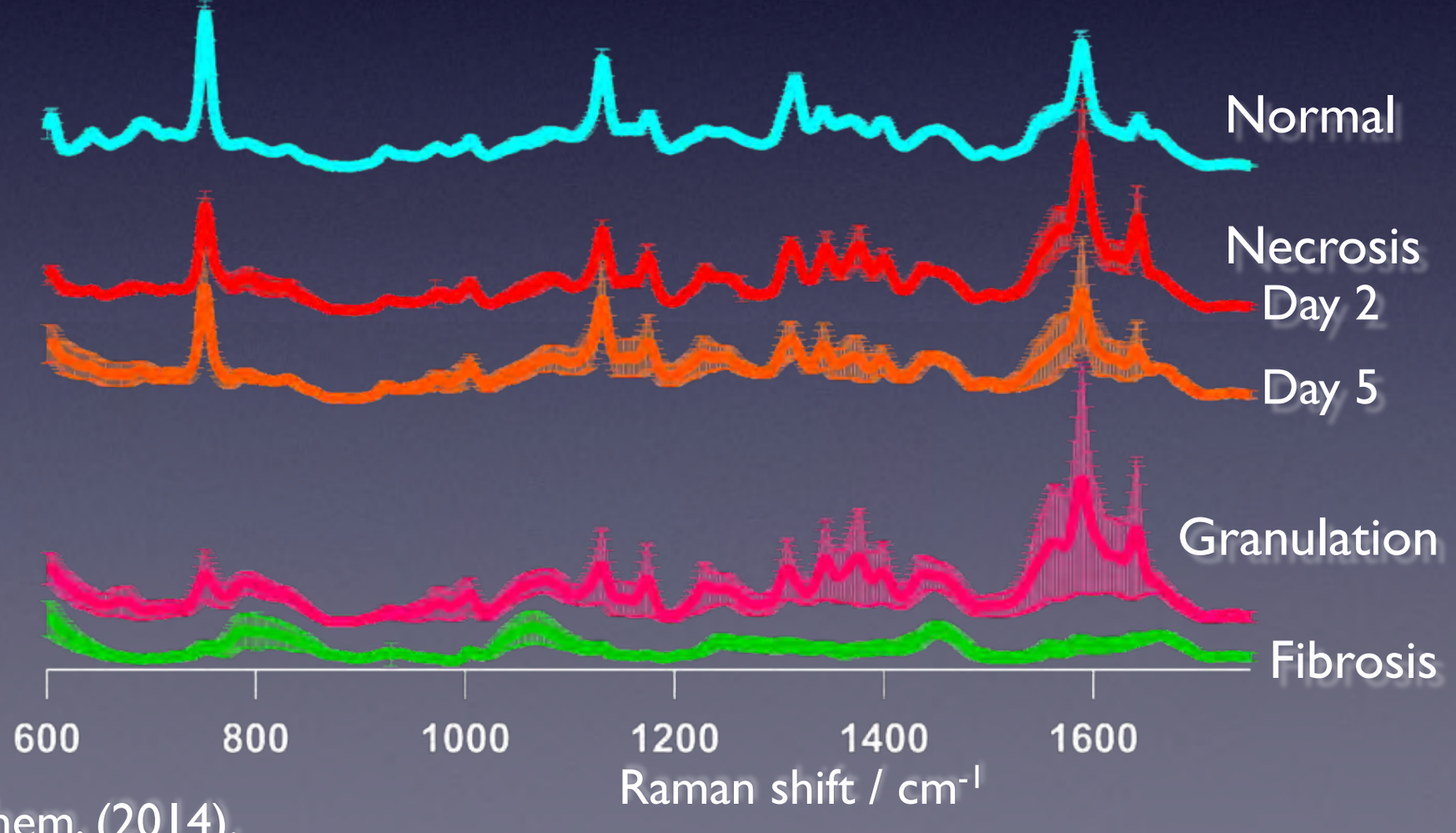
LVI



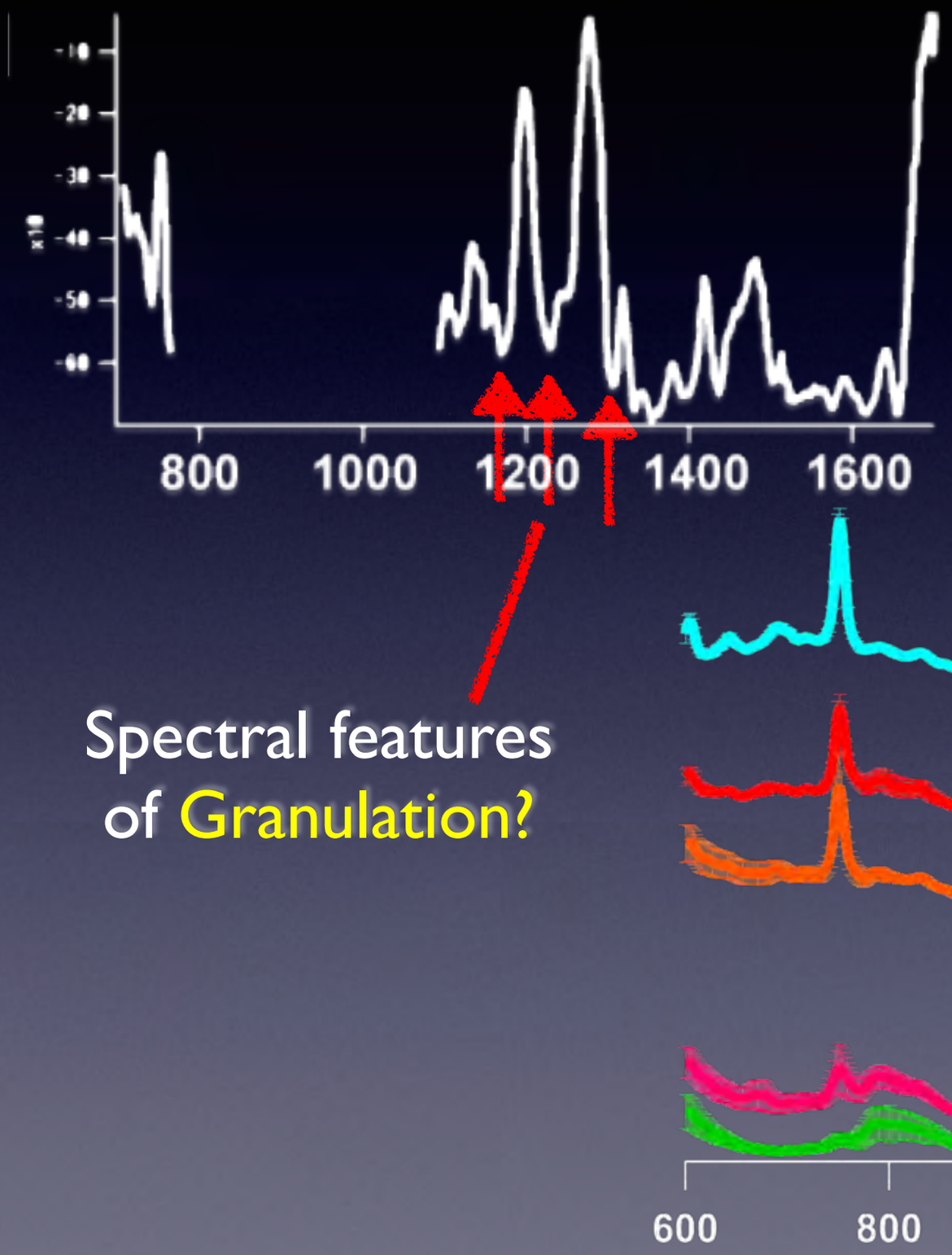
Representative Raman bands of collagen type-I



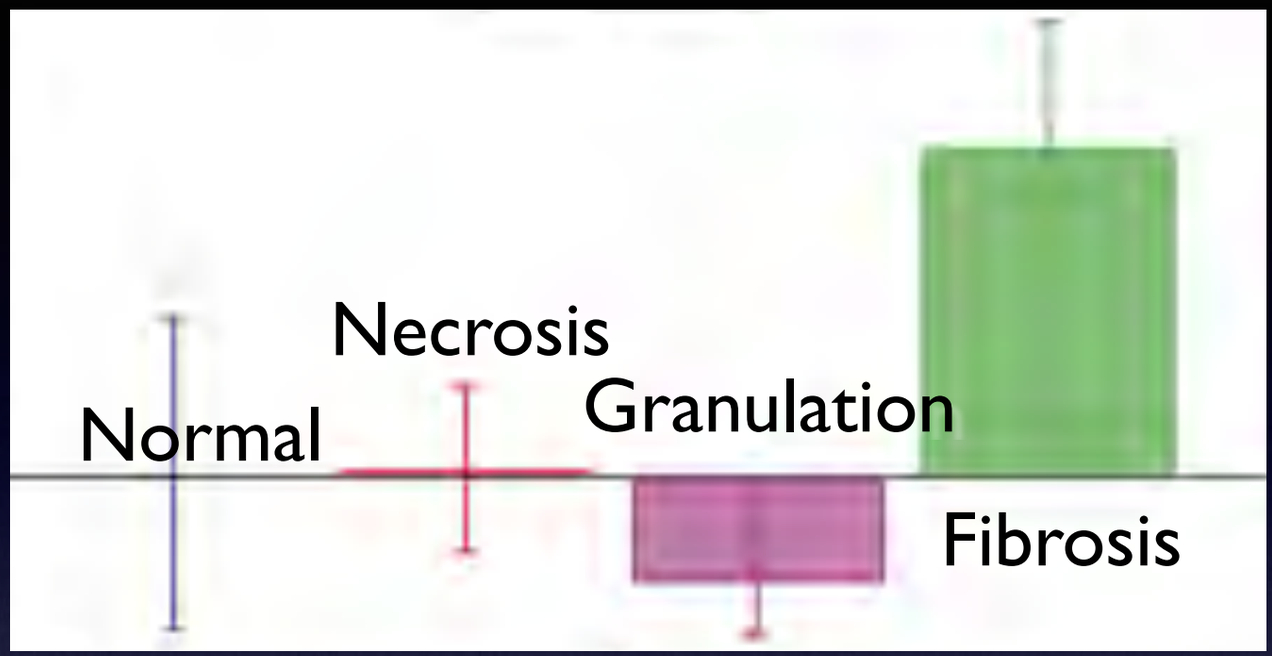


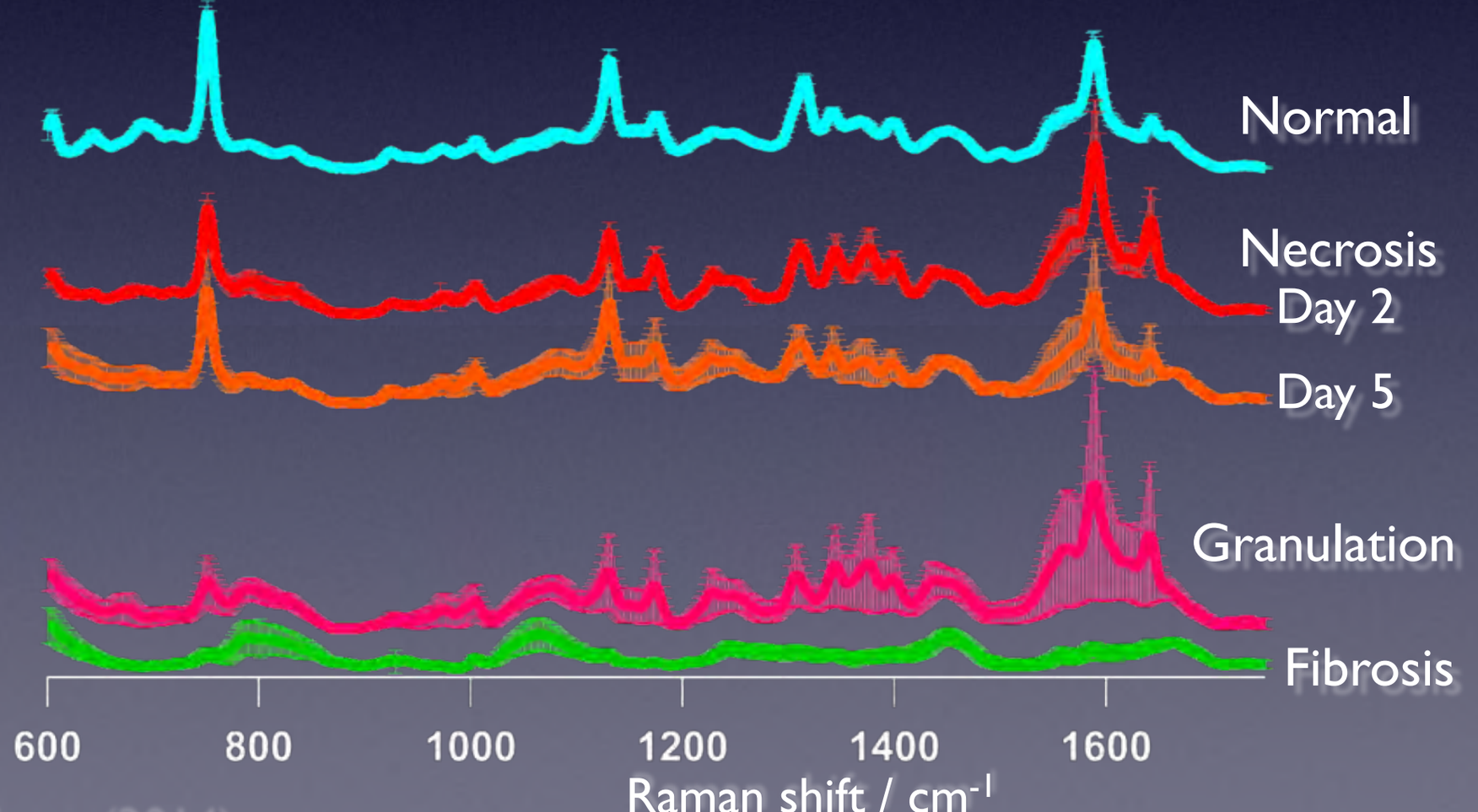
[1] N.N Muranishi et al., Anal. Chem, (2014).

LVI



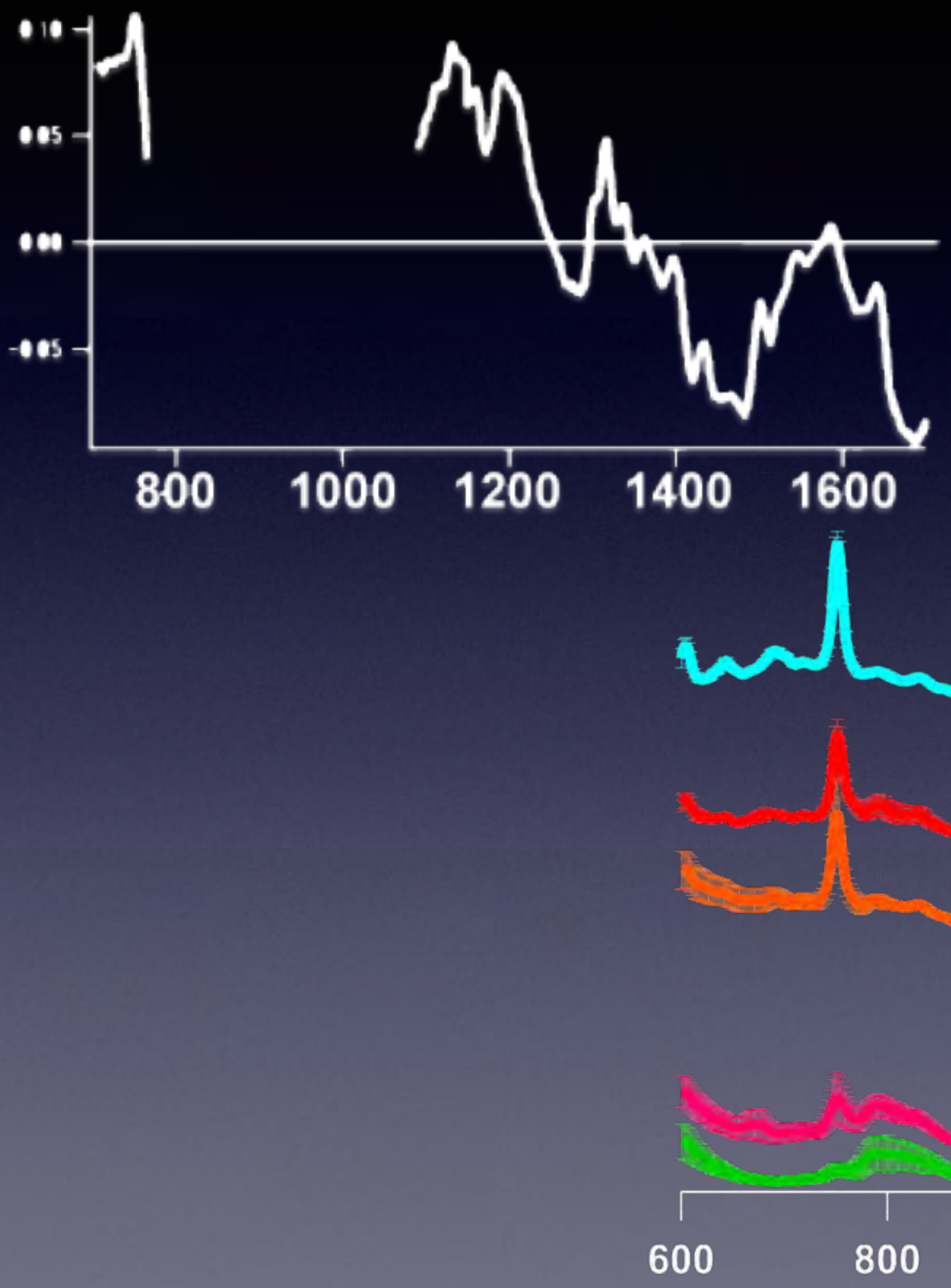
Spectral features of Granulation?

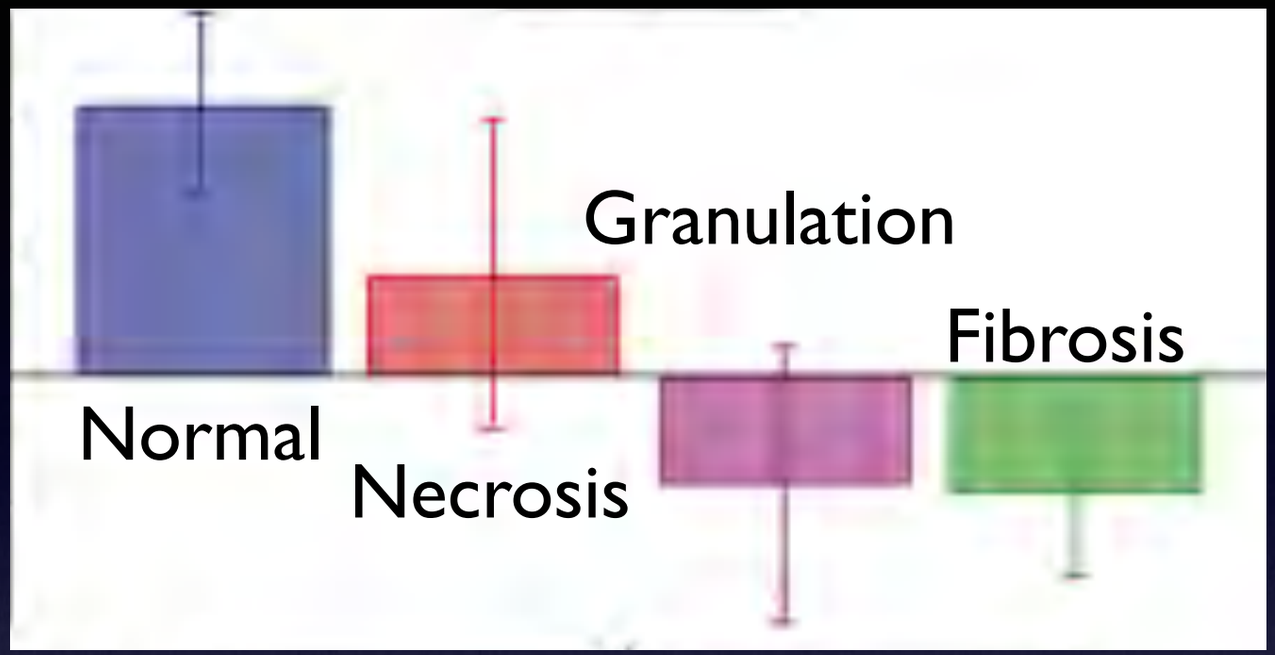


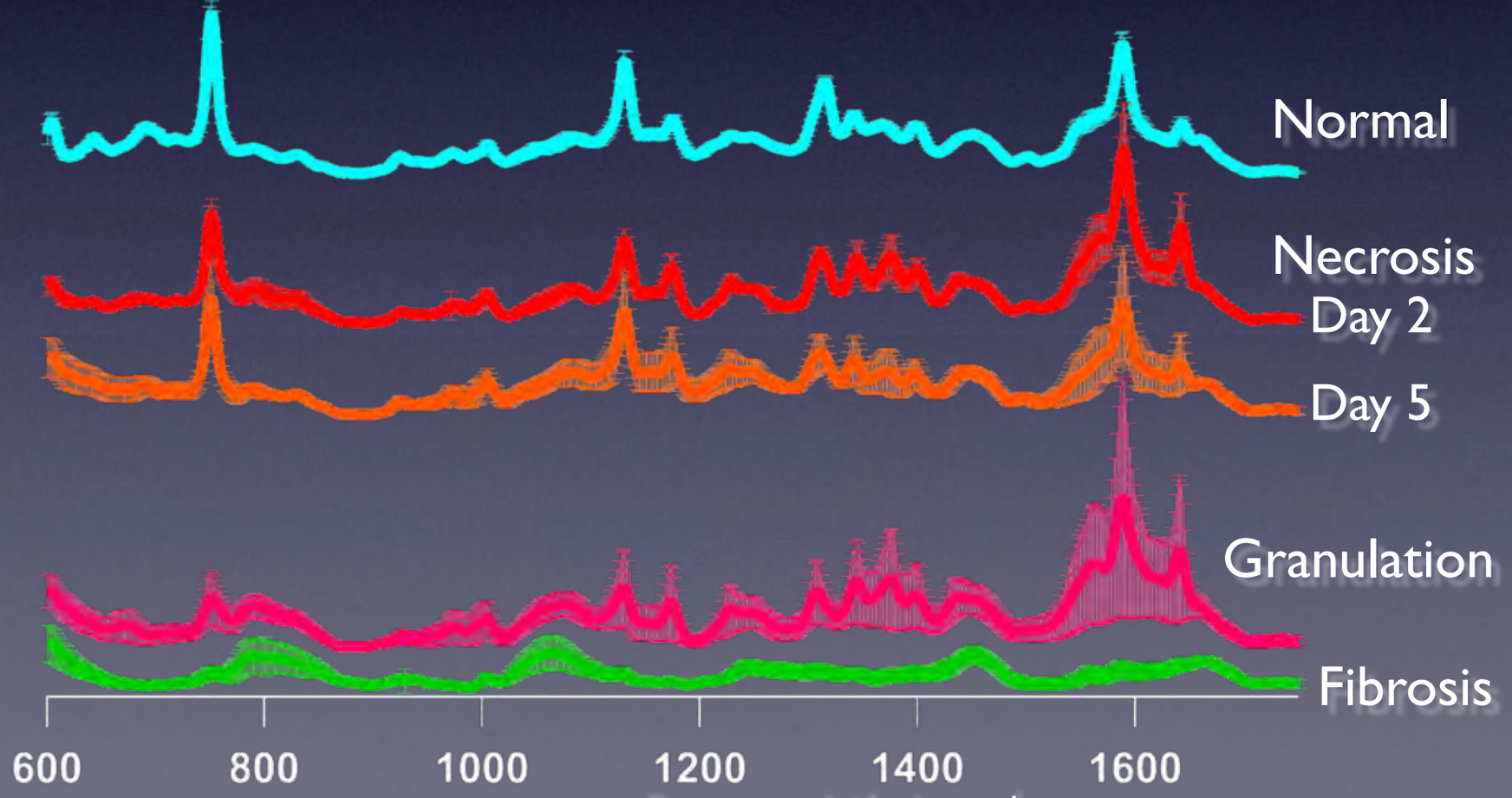


[1] N.N Muranishi et al., Anal. Chem, (2014).

LV2

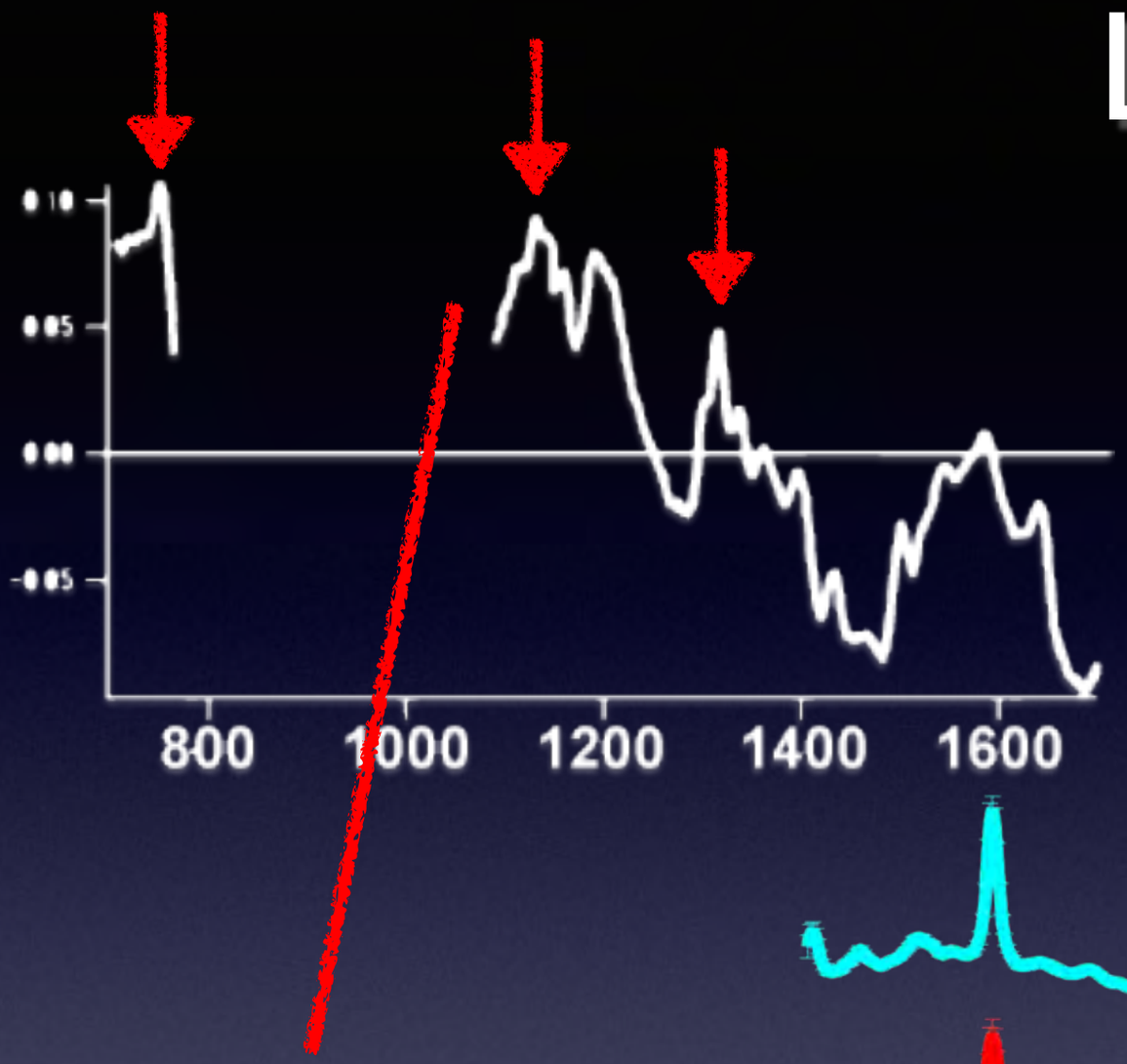




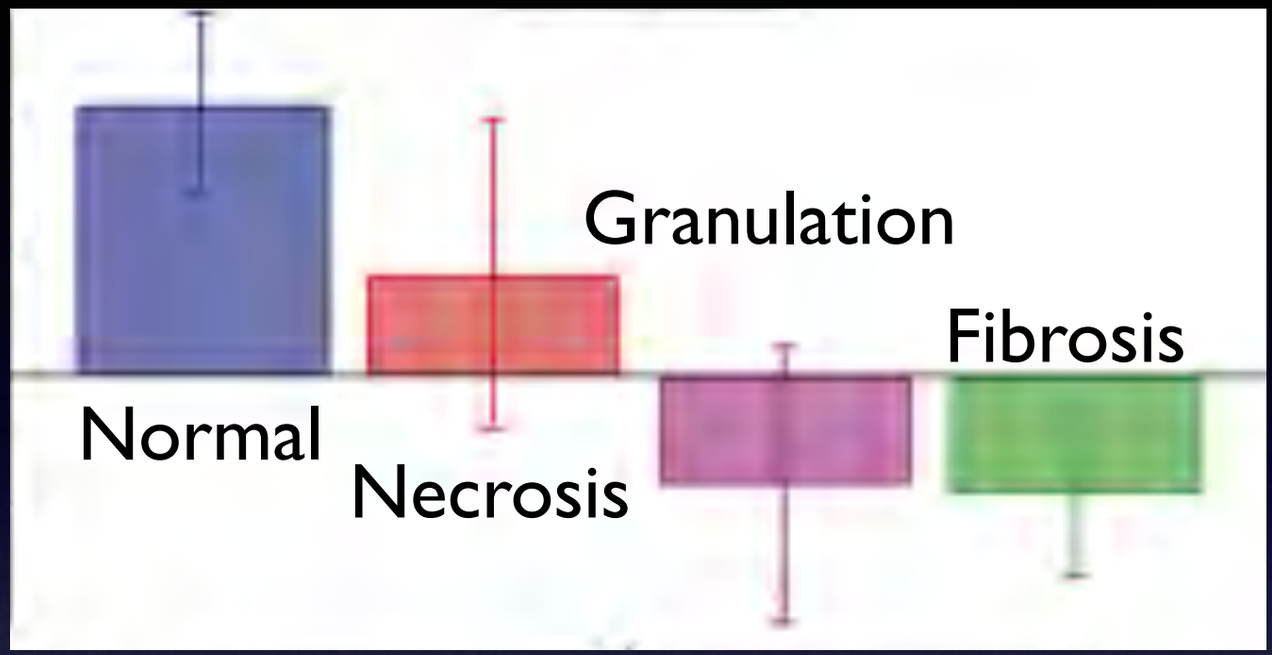


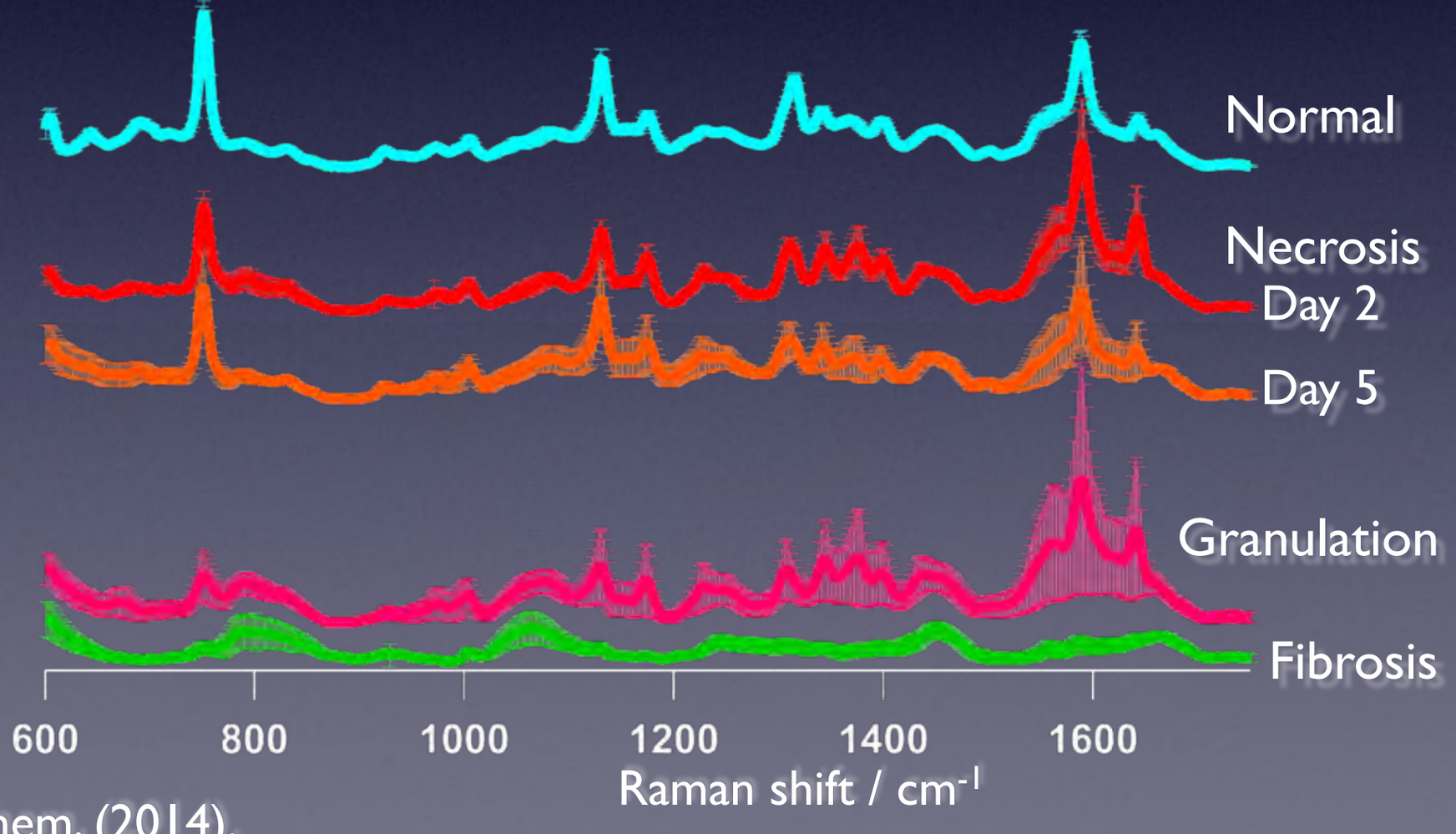
[1] N.N Muranishi et al., Anal. Chem, (2014).

LV2



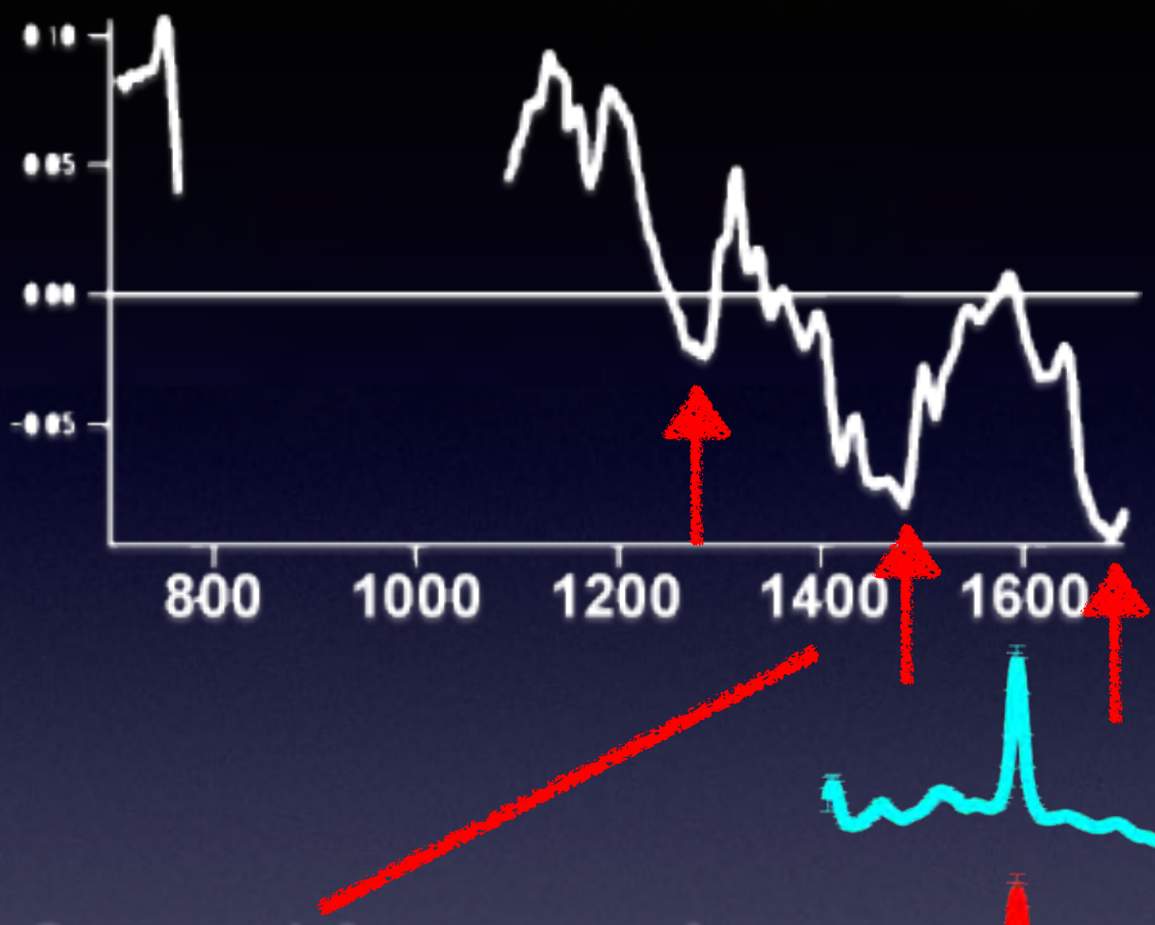
Representative
Raman bands of
Cytochrome c

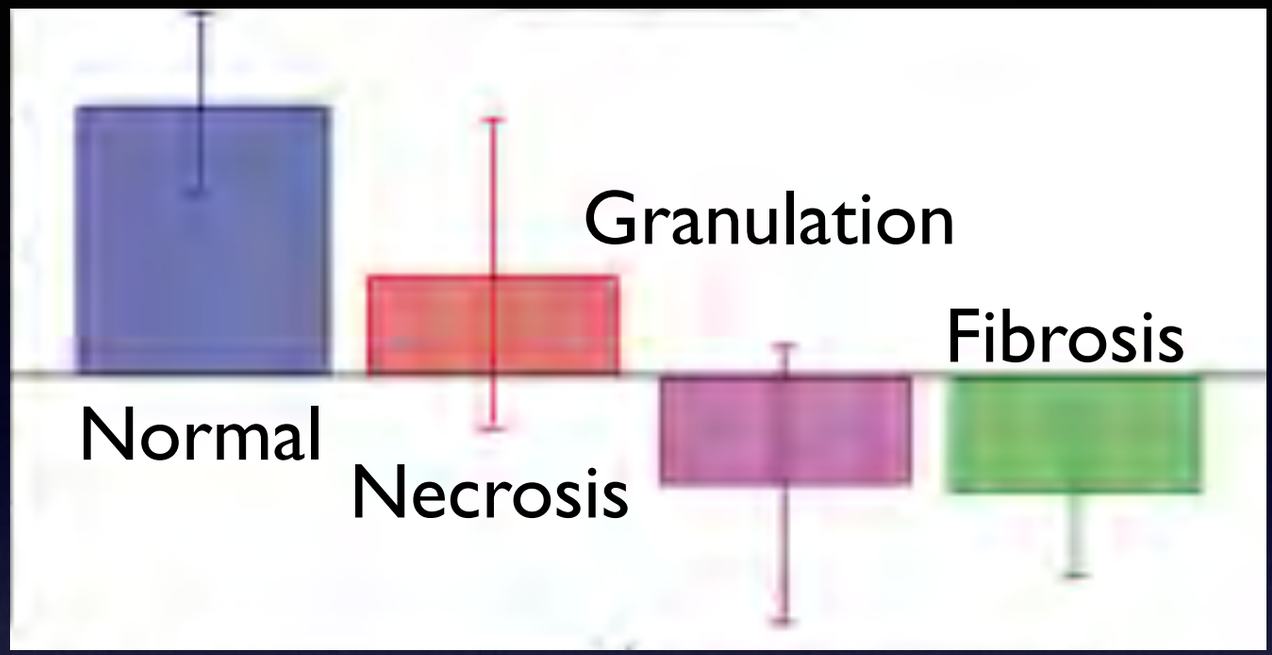




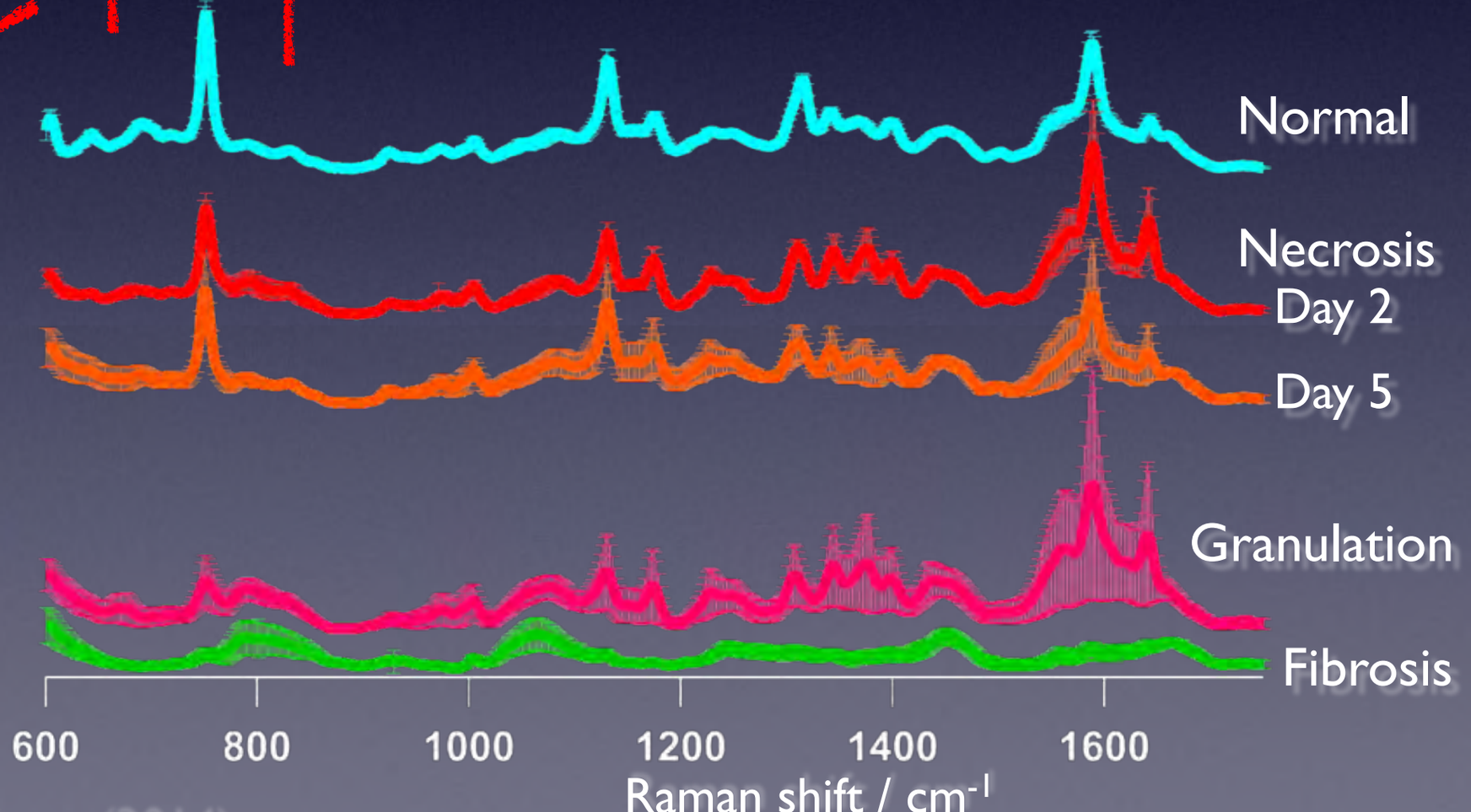
[1] N.N Muranishi et al., Anal. Chem, (2014).

LV2



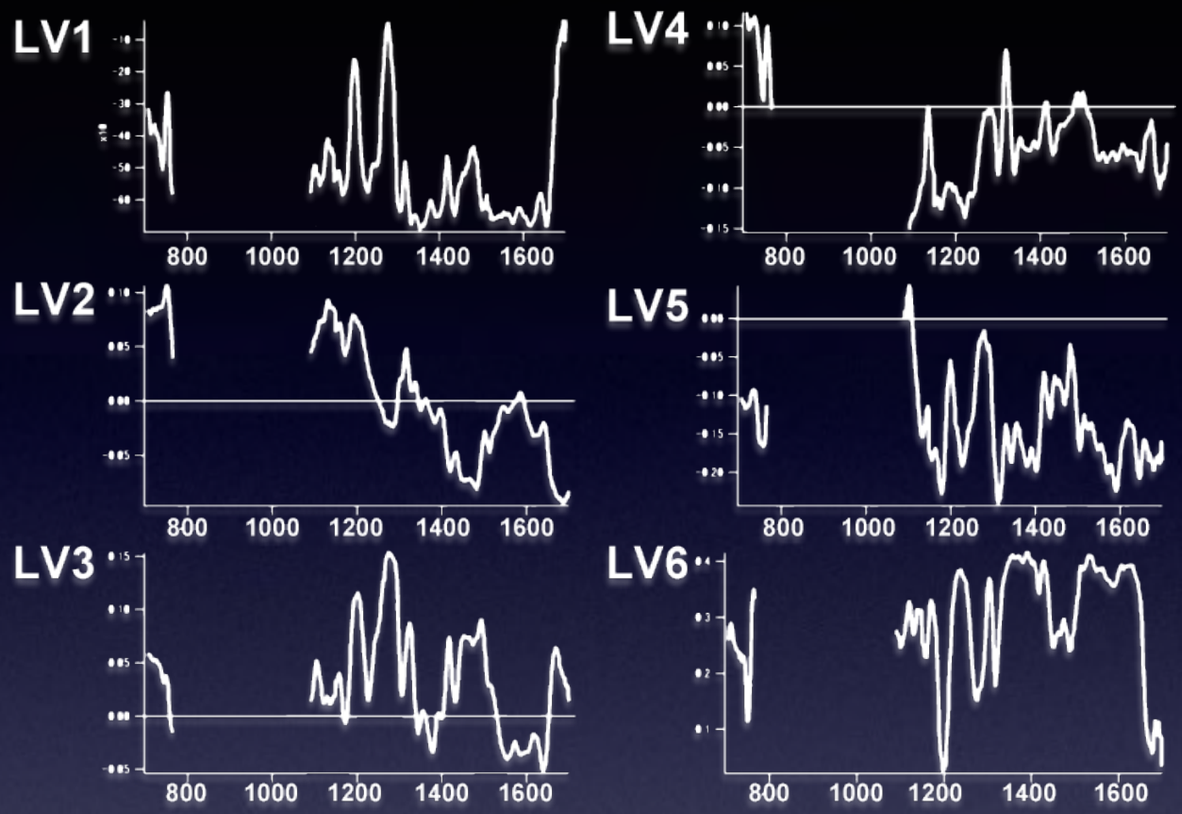


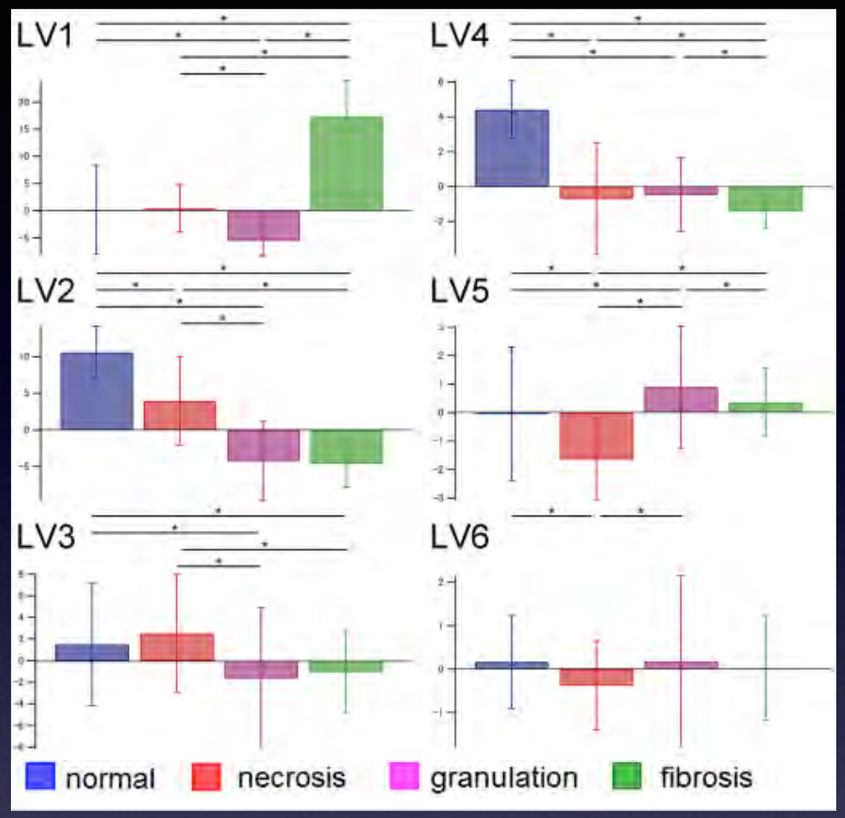
Spectral features of non-cardiomyocyte components



[1] N.N Muranishi et al., Anal. Chem, (2014).

Prediction accuracy of PLS-DA





Raman prediction	Histology				Result	
	Normal	Necrosis	Granulation	Fibrosis	Sensitivity	Specificity
Normal	149	3	2	0	99.3	99.4
Necrosis	0	286	4	0	95.3	99.5
Granulation	1	7	482	13	96.4	96.5
Fibrosis	0	4	12	137	91.3	98.3

[1] N.N Muranishi et al., Anal. Chem, (2014).

Raman imaging of MI using PLS-DA

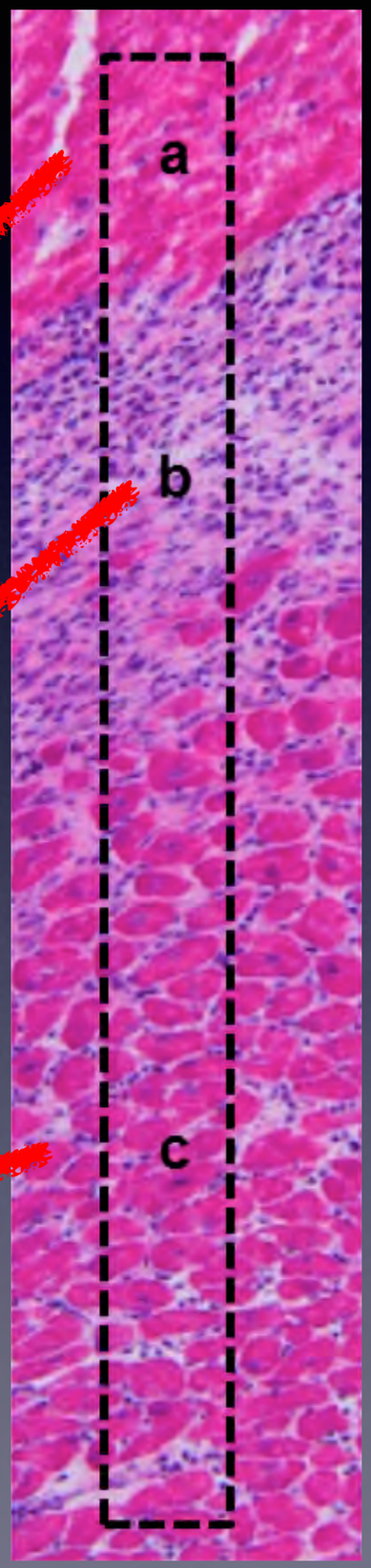
Histology

Necrosis

Granulation

Normal

HE



[1] N.N Muranishi et al., Anal. Chem, (2014).

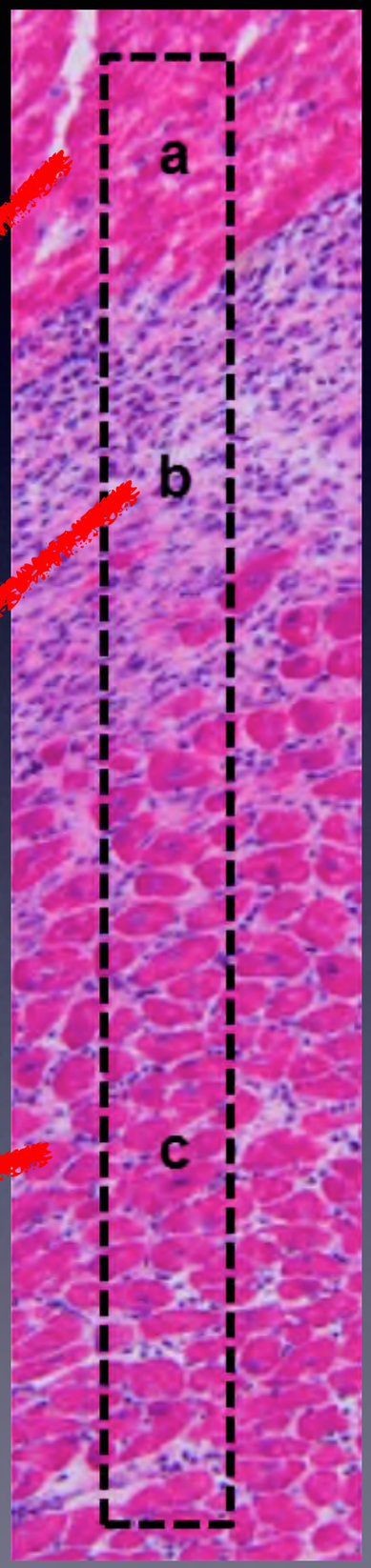
Raman imaging of MI using PLS-DA

Histology

Necrosis

Granulation

Normal



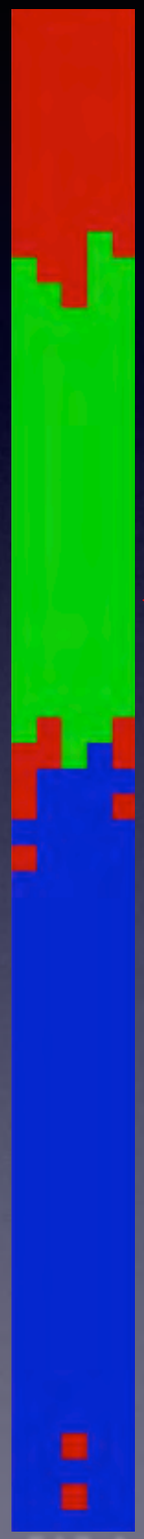
HE

Prediction

Necrosis

Granulation

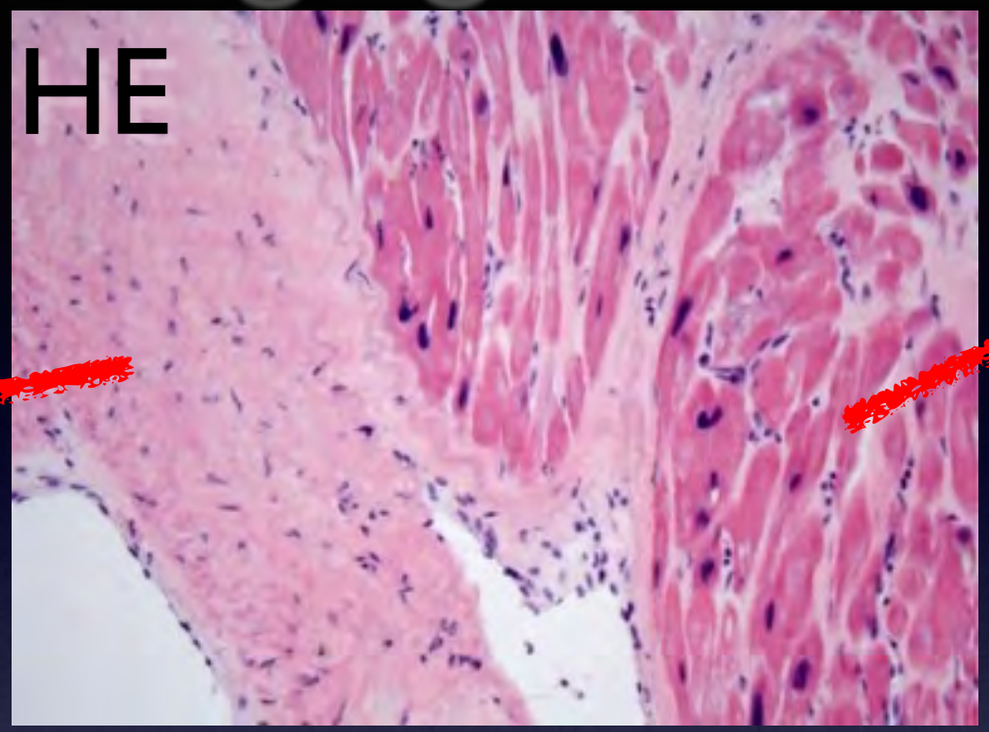
Normal



Raman

[1] N.N Muranishi et al., Anal. Chem, (2014).

Raman imaging of human OMI



Fibrosis
(FT)

Cardiomyocytes
(CM)

Raman

[1] T. Yamamoto, T. Minamikawa et al., (in preparation).

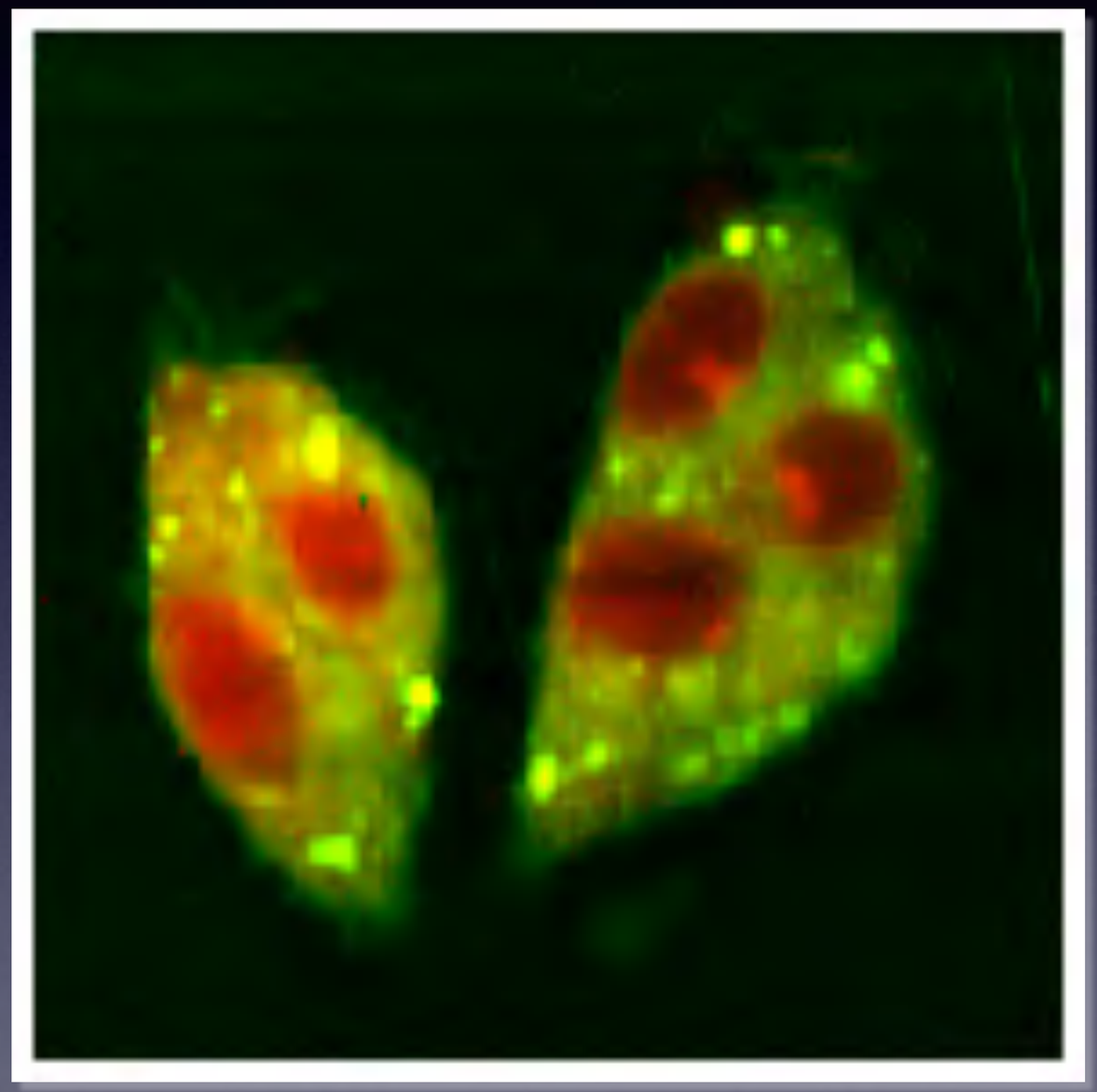
But,

Spontaneous Raman signal is

Pros and Cons of Raman spectroscopy

- ○ Label-free
- ○ Molecular selectivity via molecular vibration
- ○ in vivo capability

- ✗ Low sensitivity
- ✗ Long exposure

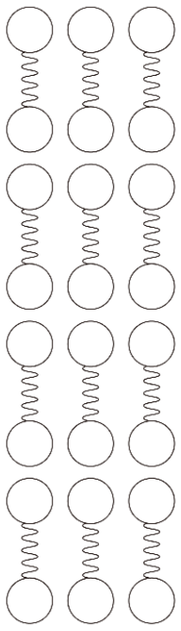


Exposure time
648 s/image

A nonlinear Raman spectroscopy
～Coherent anti-Stokes Raman scattering～

Frequency ω_1

Frequency ω_2



Molecular vibration

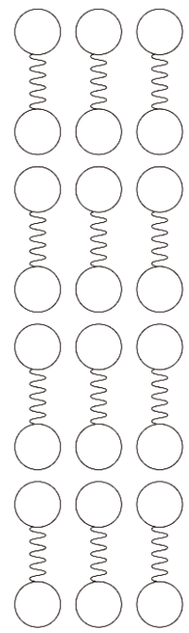
$\Omega=\omega_1-\omega_2$

A nonlinear Raman spectroscopy
～Coherent anti-Stokes Raman scattering～

Frequency ω_3

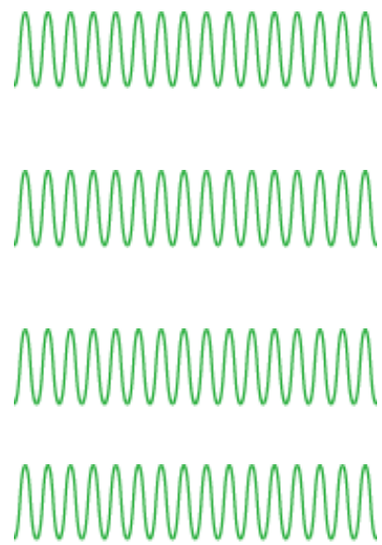
Frequency ω_1

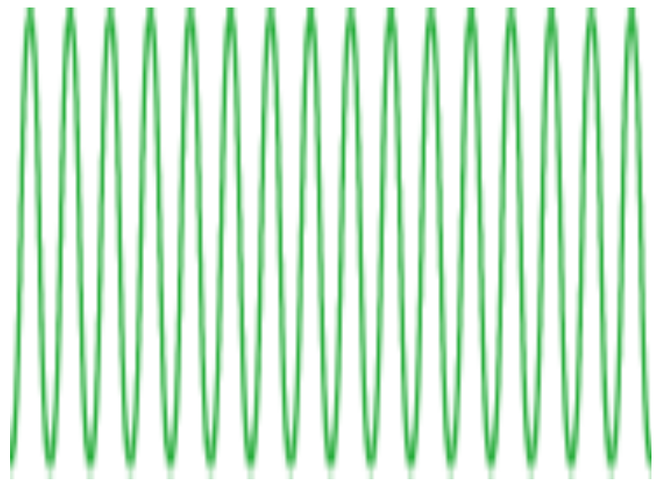
Frequency ω_2



CARS

$\omega_{CARS} = \omega_1 - \omega_2 + \omega_3$





Molecular vibration

$\Omega = \omega_1 - \omega_2$

Constructive interference!!

Towards better molecular imaging in biology and medicine

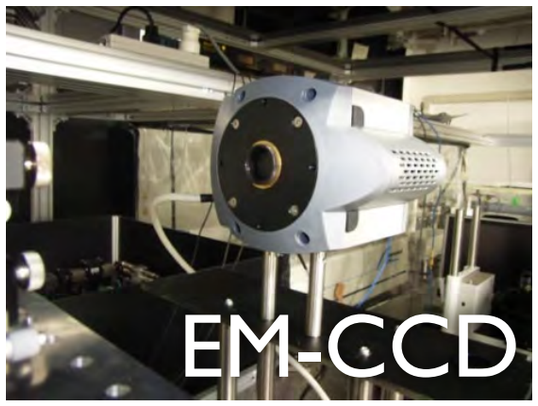
- ✓ 速く撮りたい！
- ✓ 分子の選択性を高めたい！
- ✓ 分子分布以外の分子情報を得たい！

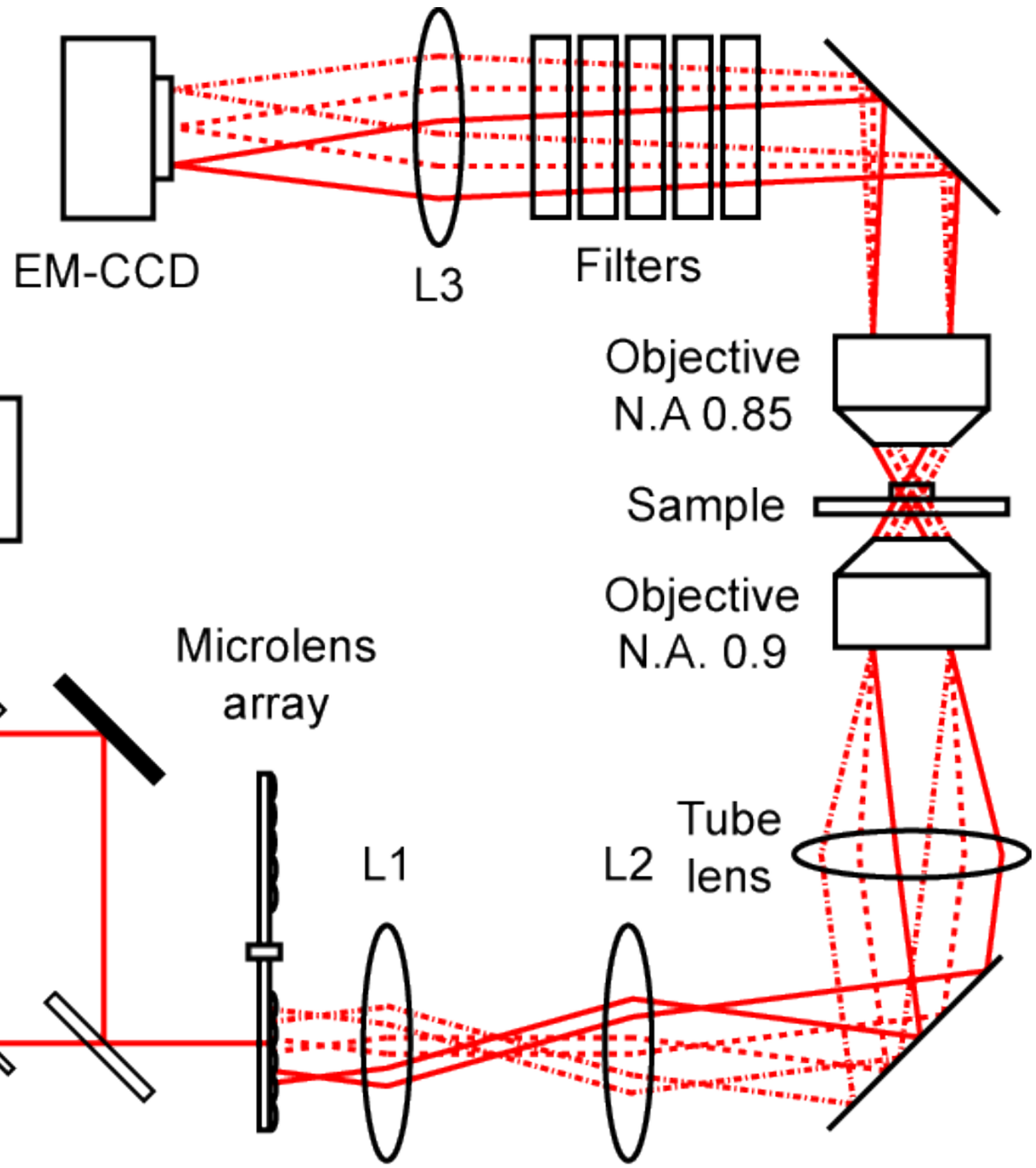
顕微ラマン散乱分光法＋非線形光学

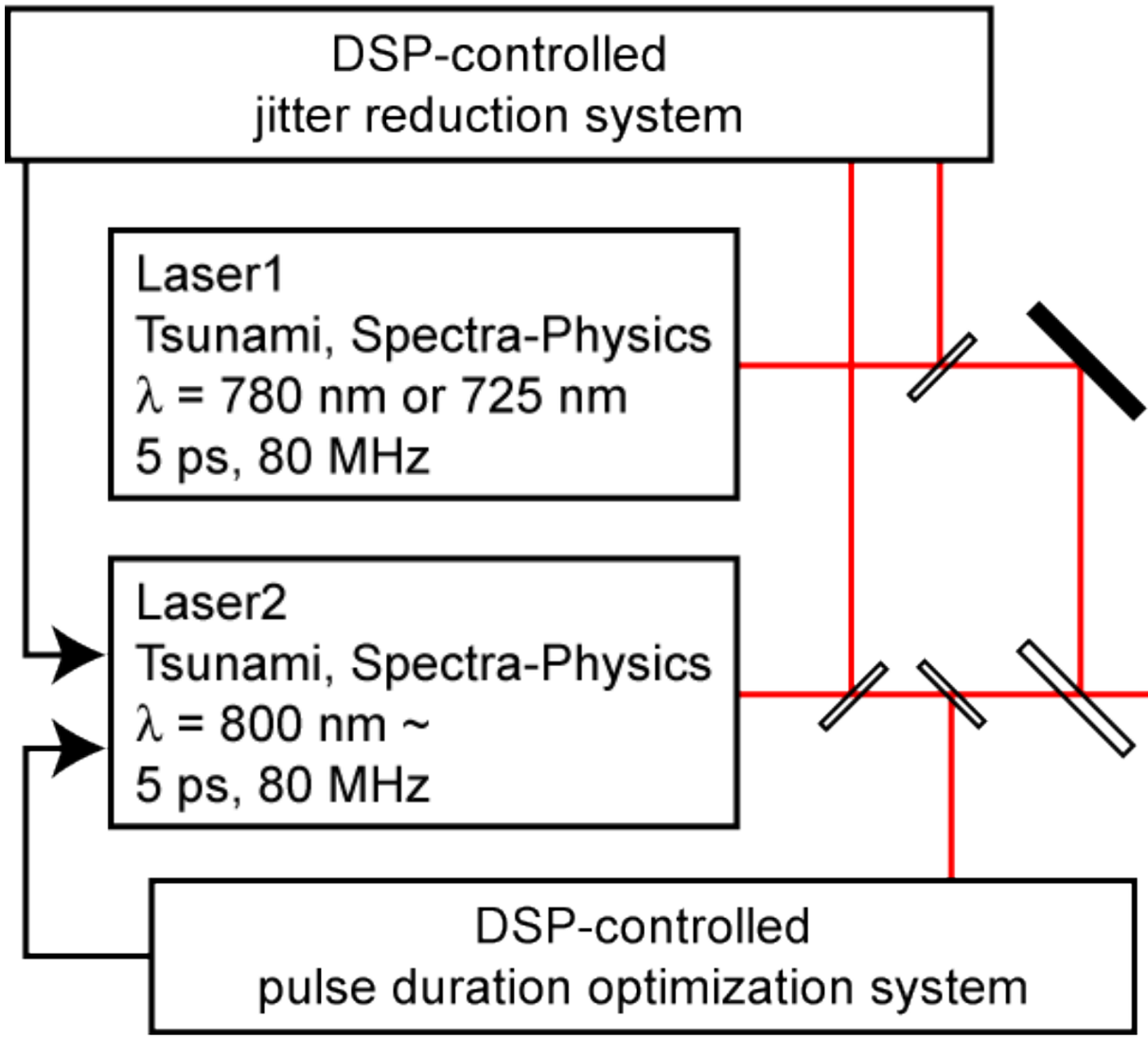
非線形顕微ラマン散乱分光法

(CARS: Coherent anti-Stokes Raman scattering)

Multi-focus CARS microscope







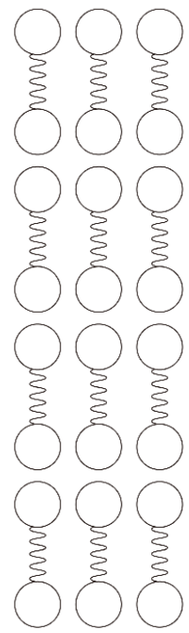
[ref] T. Minamikawa et al., Opt. Express, 17, 9526 (2009)

A nonlinear Raman spectroscopy
～Coherent anti-Stokes Raman scattering～

Frequency ω_3

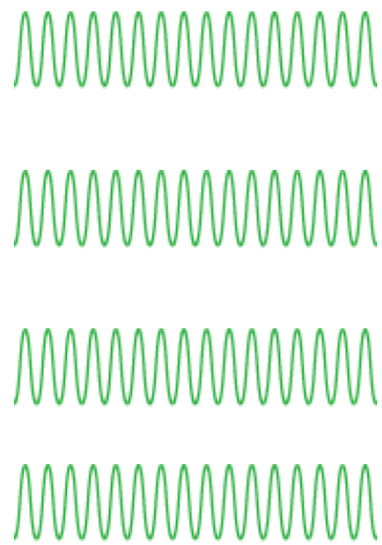
Frequency ω_1

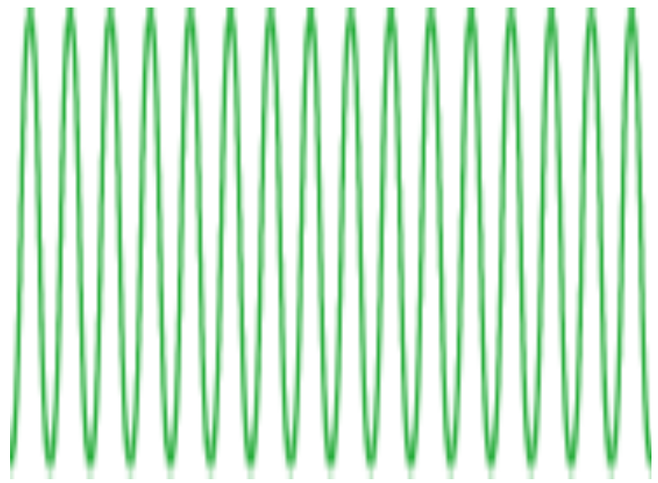
Frequency ω_2



CARS

$\omega_{CARS} = \omega_1 - \omega_2 + \omega_3$



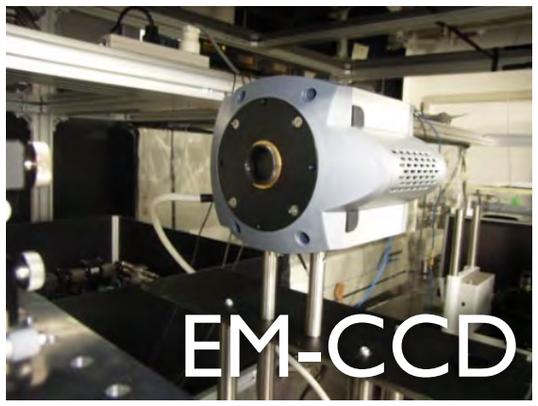


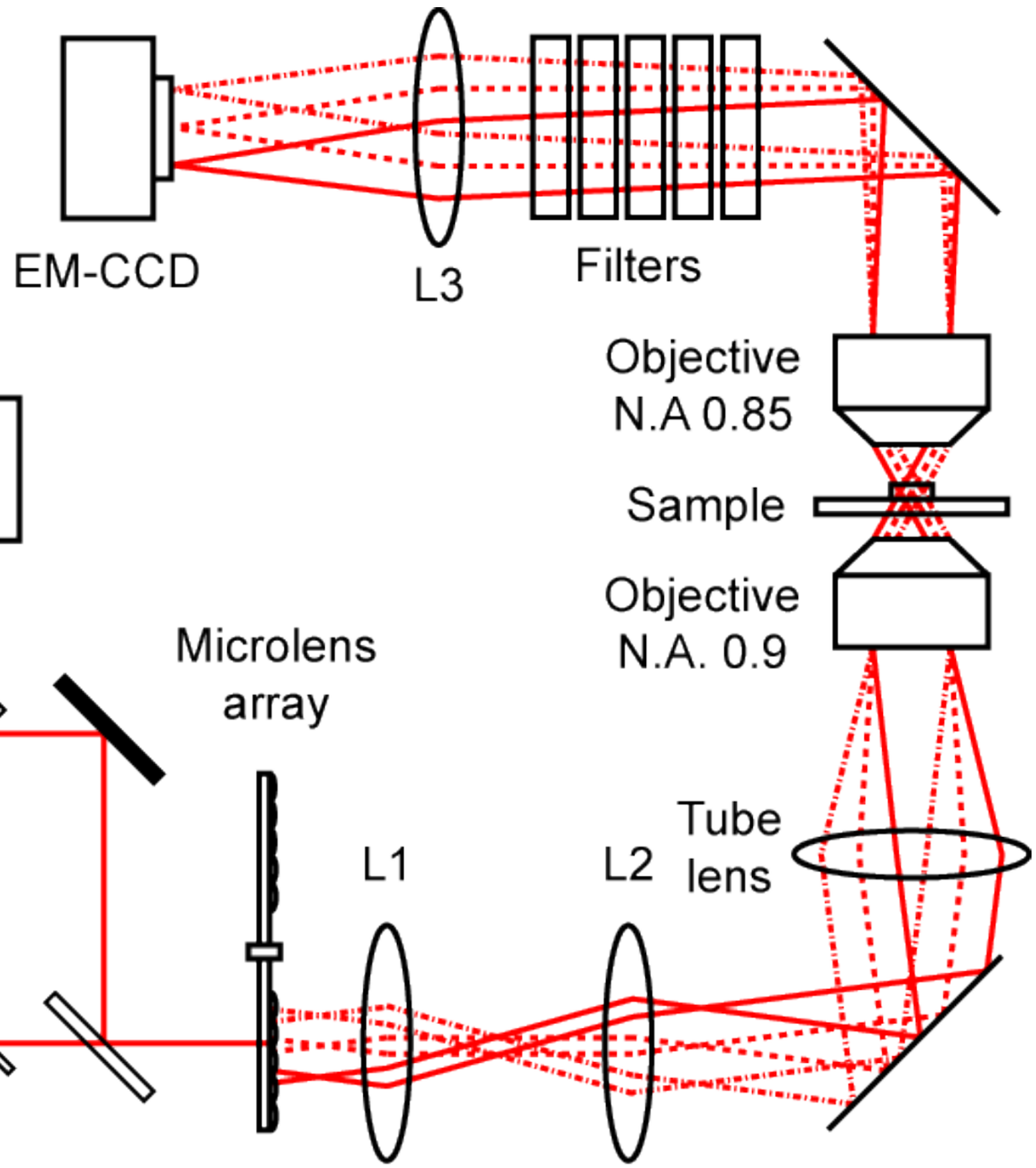
Molecular vibration

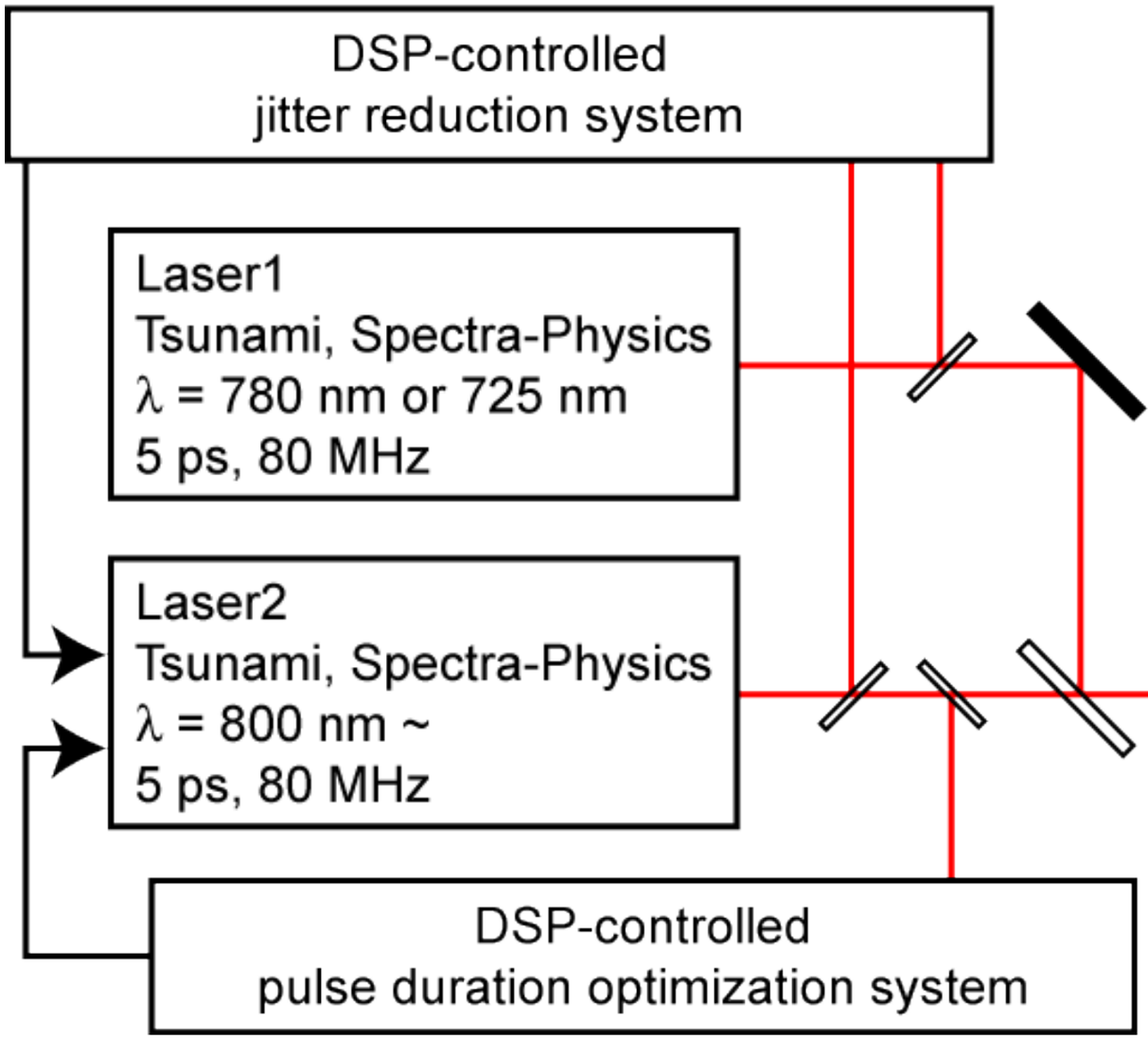
$\Omega = \omega_1 - \omega_2$

Constructive interference!!

Multi-focus CARS microscope

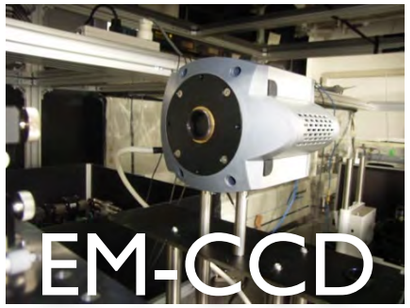


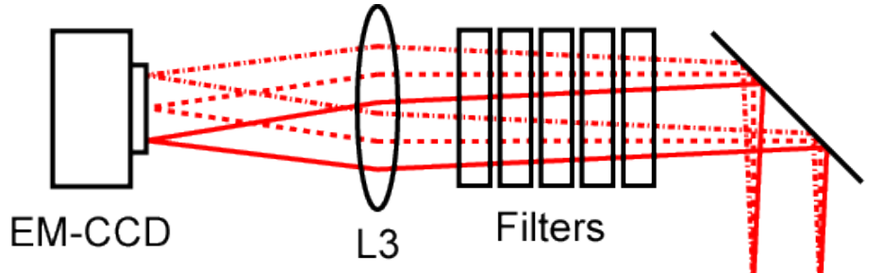


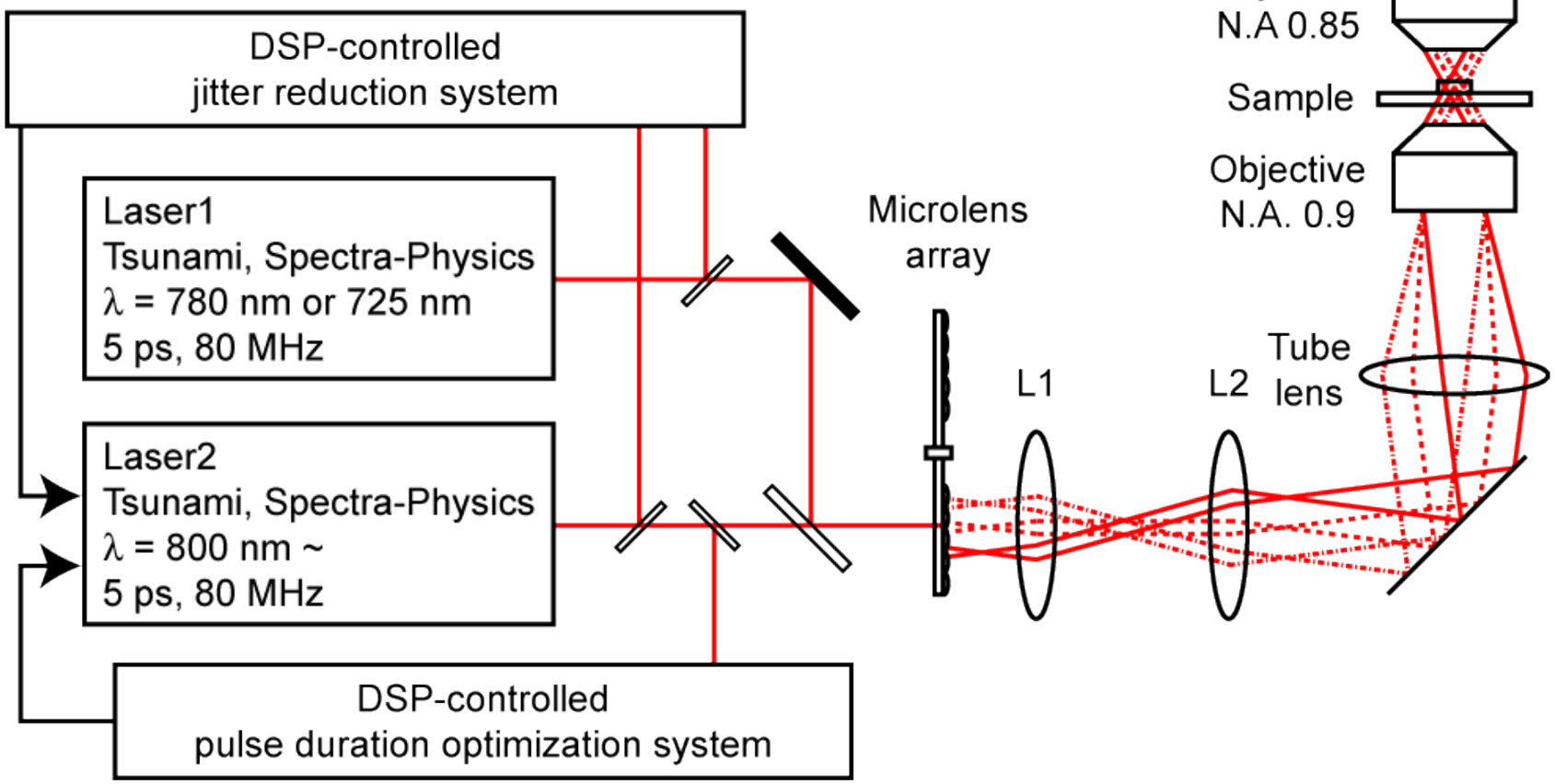


[ref] T. Minamikawa et al., Opt. Express, 17, 9526 (2009)

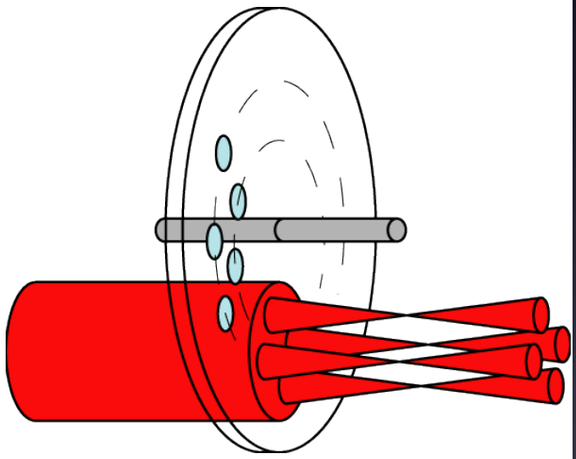
Multi-focus CARS microscope





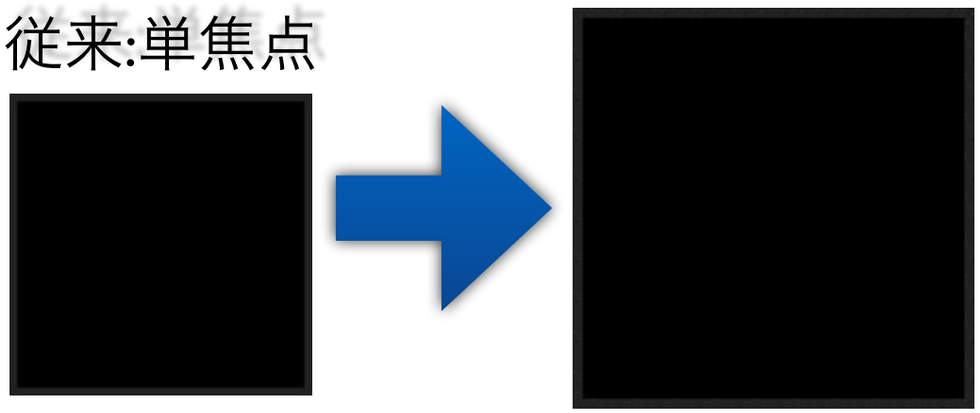


マイクロレンズアレイによる多焦点化



多焦点！

従来:単焦点

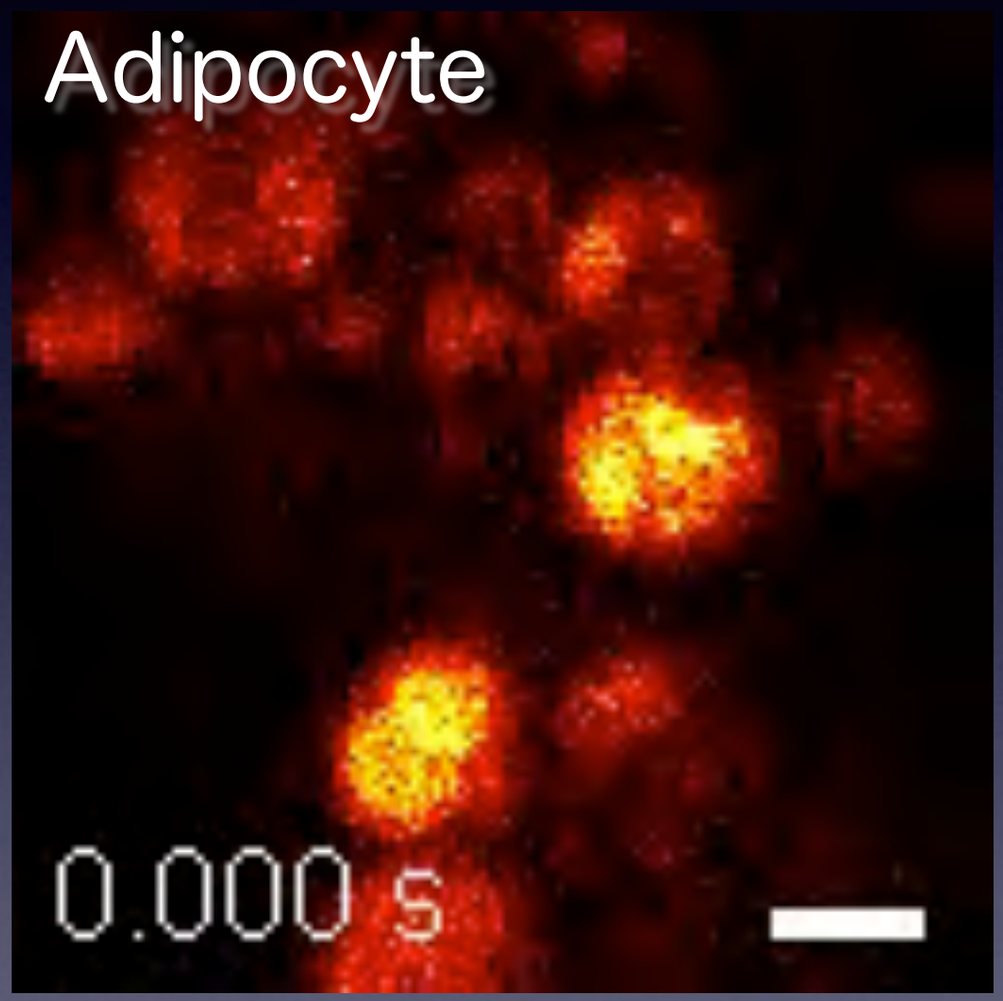


[ref] T. Minamikawa et al., Opt. Express, 17, 9526 (2009)

High speed CARS imaging

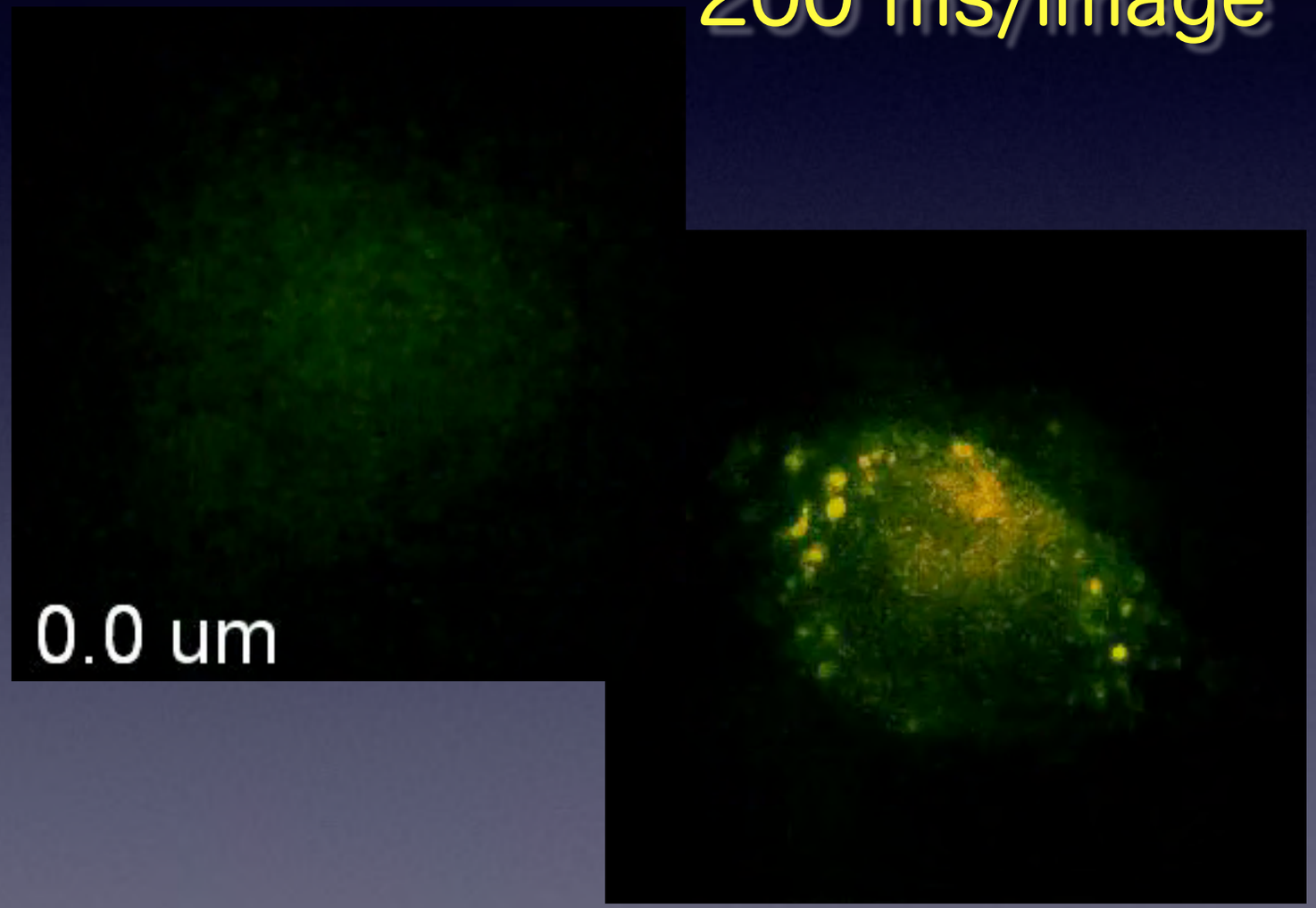
Lipid imaging （2850 cm^{-1}）

Adipocyte



10 ms/image

HeLa cell

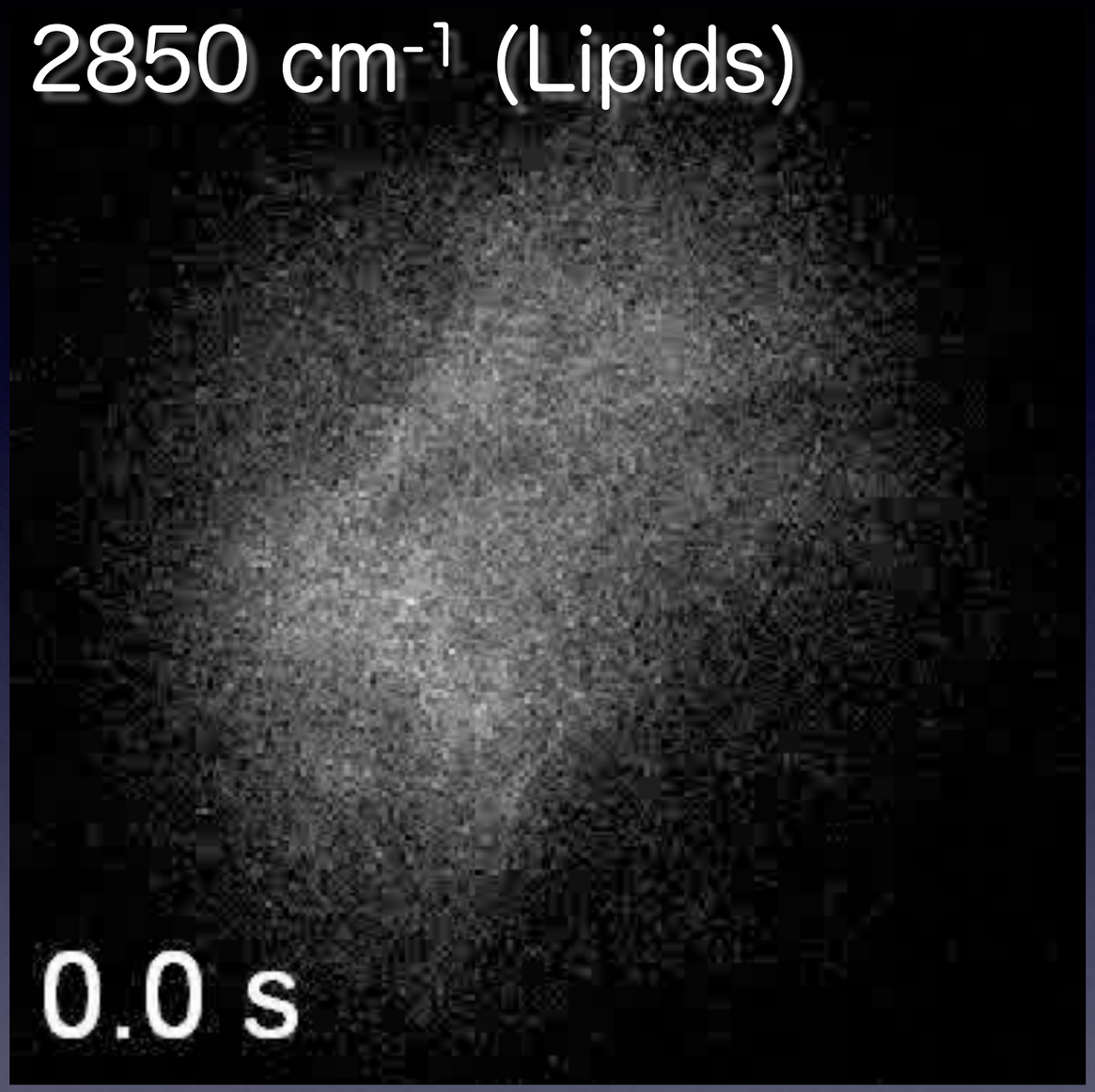


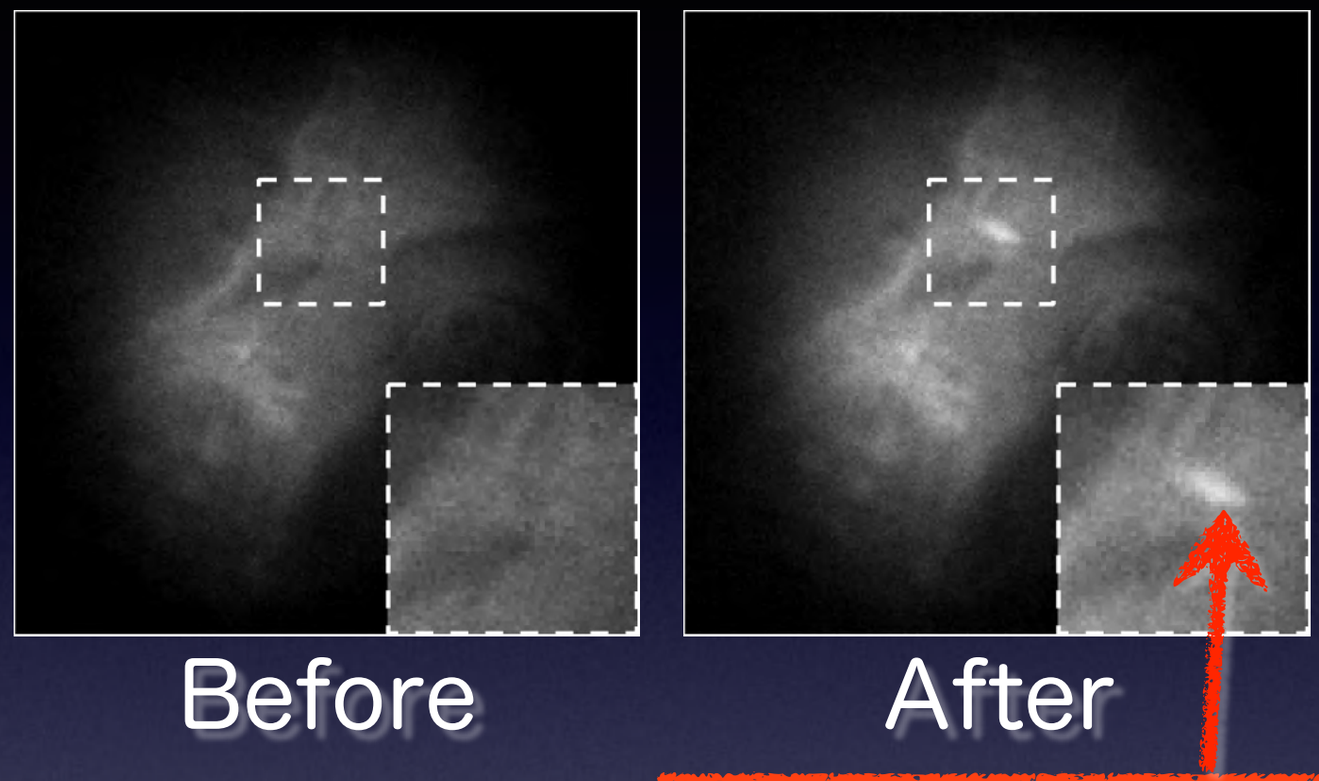
z-scan
200 ms/image

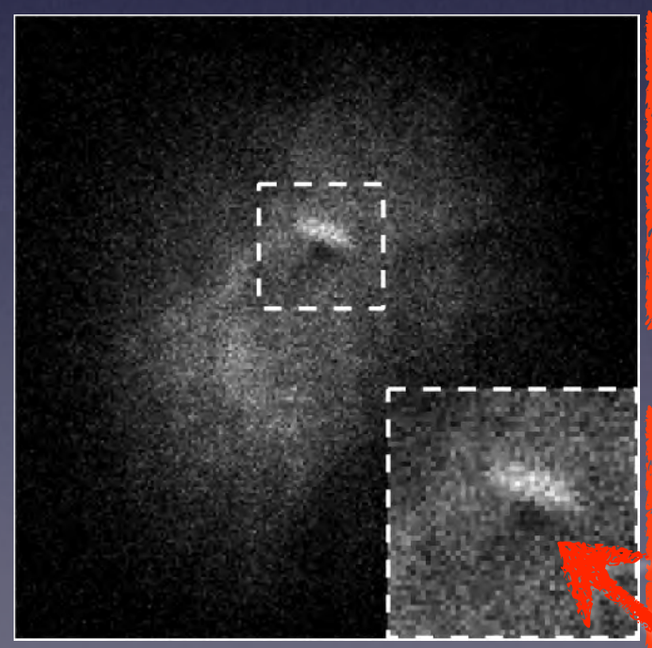
17 s/volume
(85 slices)

[ref] H. Cahyadi, J. Biomed. Opt. 18, 96009 (2013), T. Minamikawa et al., Opt. Express, 17, 9526 (2009)

Membrane disruption and response







信号強
→　脂質：多い！

アブレーションによる膜破壊
→　後に修復

Laser ablation

Laser intensity:	63.9 mW @ 709 nm
Pulse duration:	5 ps
Exposure time:	0.1 s

[ref] T. Minamikawa et al., J. Biomed. Opt., 16, 021111 (2011)

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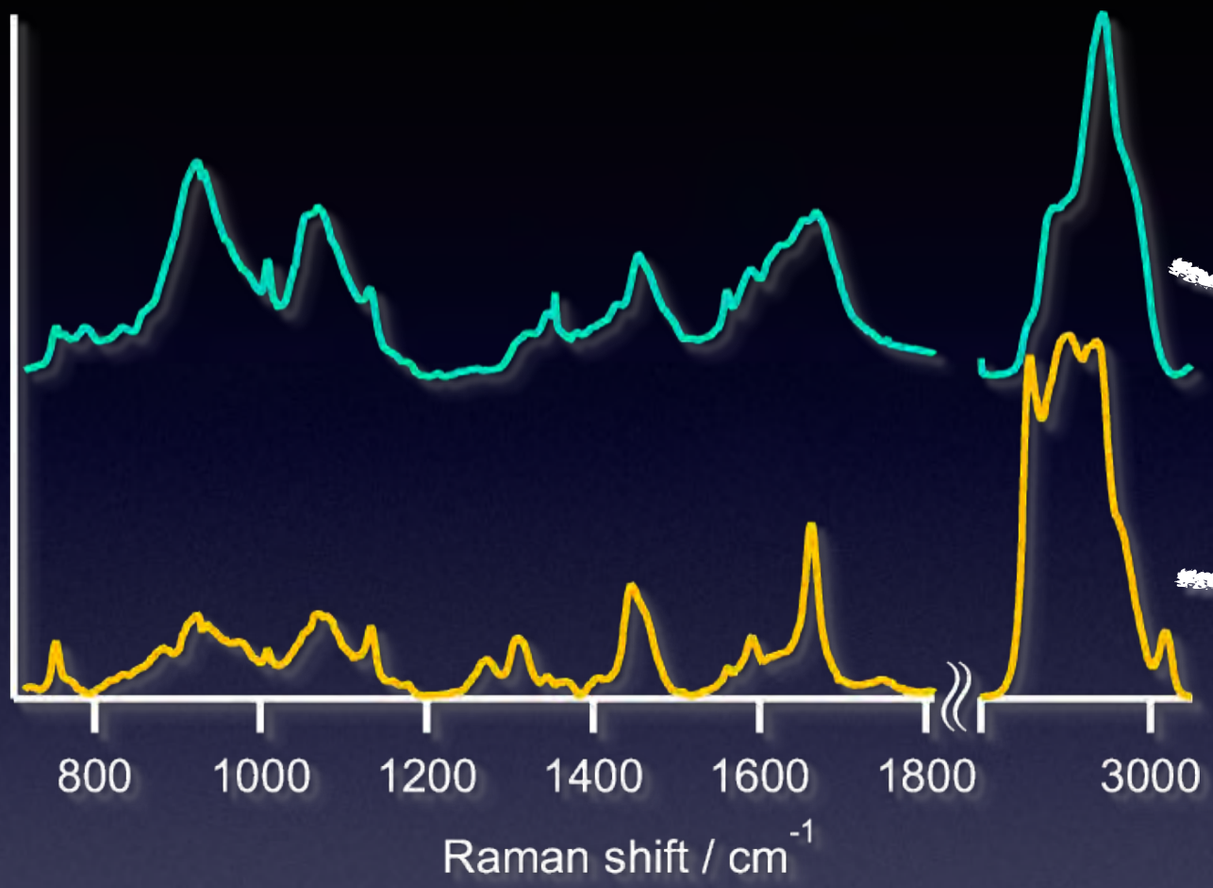
- ✓ 速く撮りたい！
- ✓ 分子の選択性を高めたい！
- ✓ 分子分布以外の分子情報を得たい！

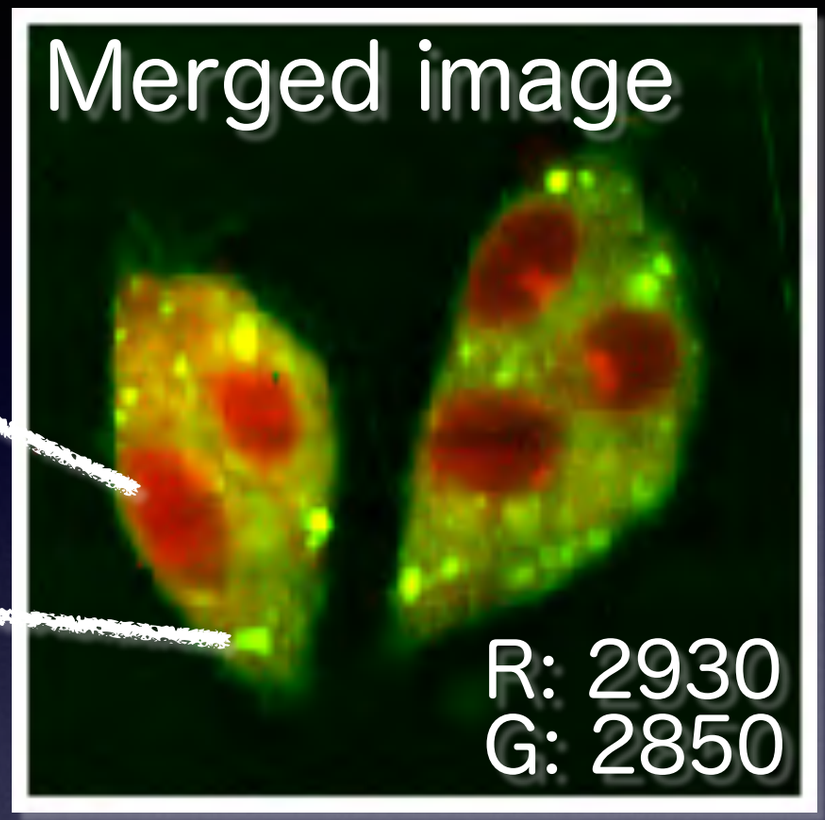
顕微ラマン散乱分光法＋非線形光学

非線形顕微ラマン散乱分光法

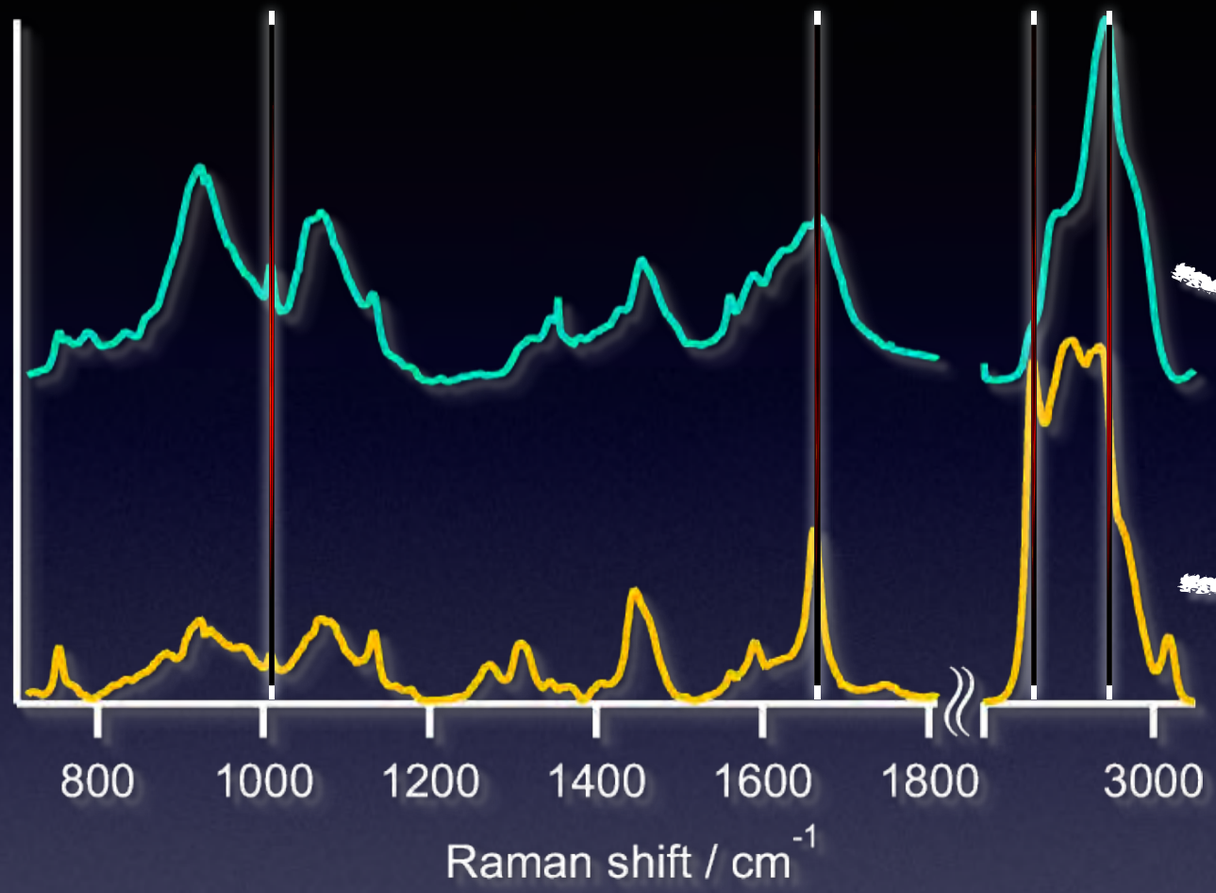
(CARS: Coherent anti-Stokes Raman scattering)

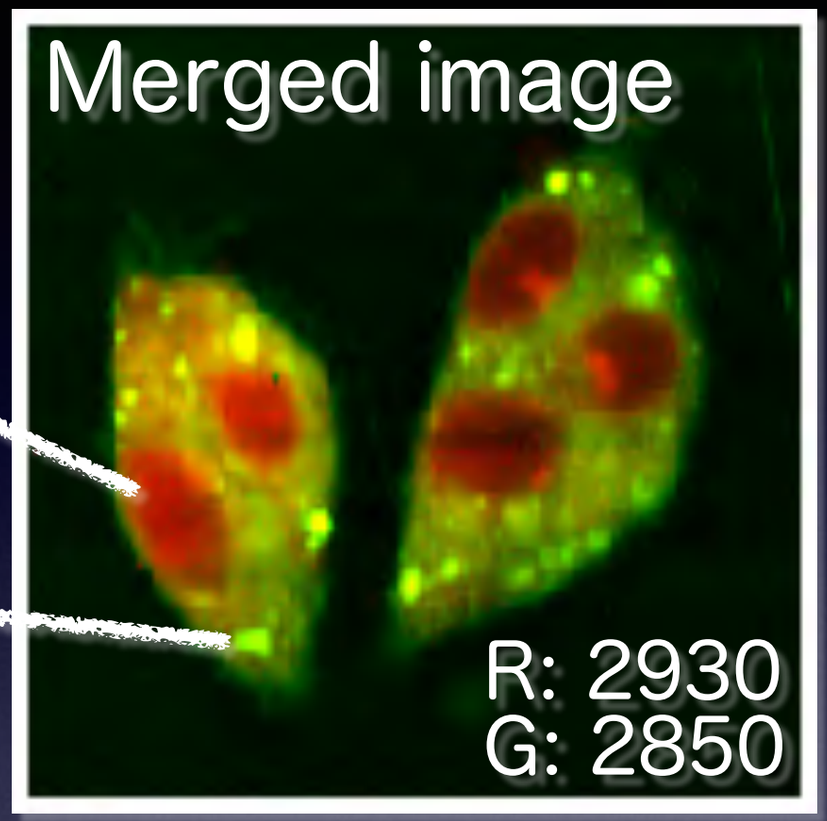
Spectral Raman imaging





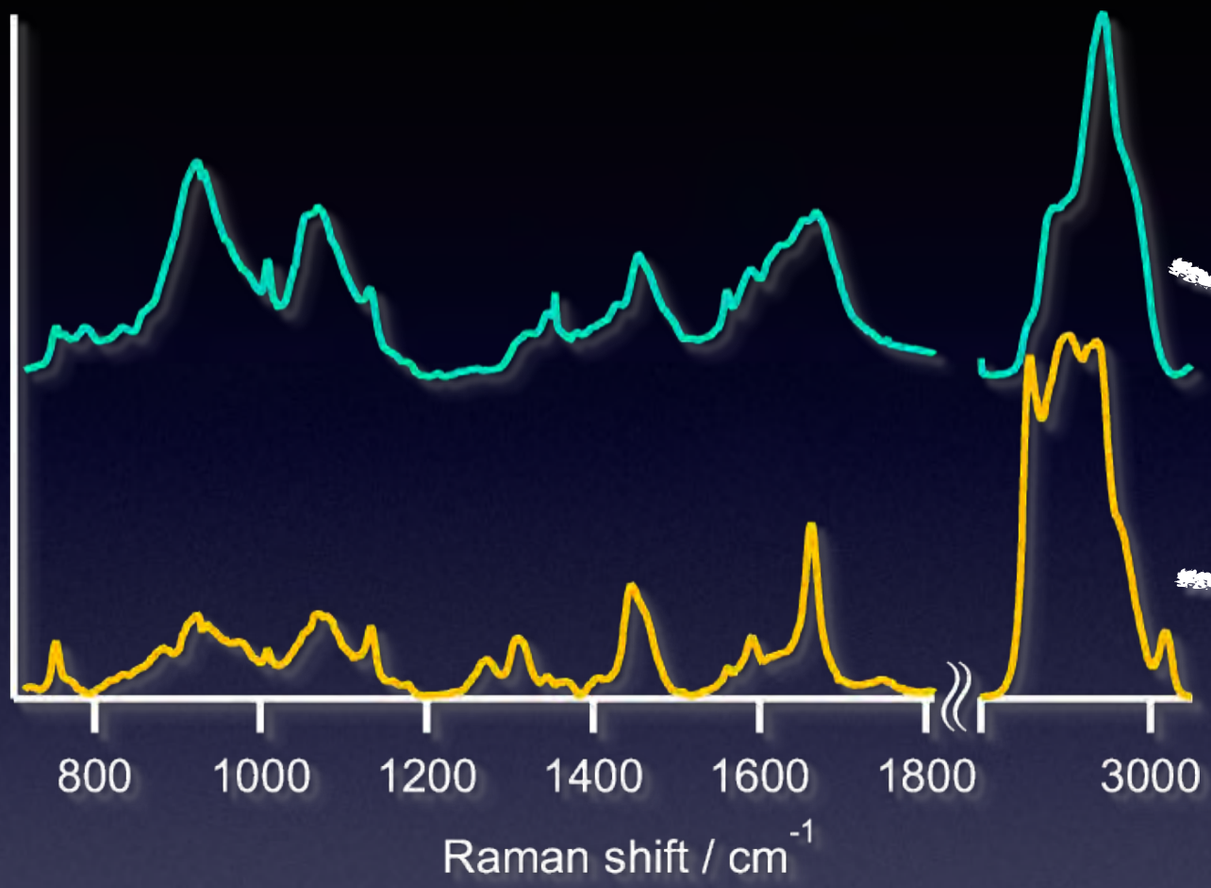
Spectral Raman imaging

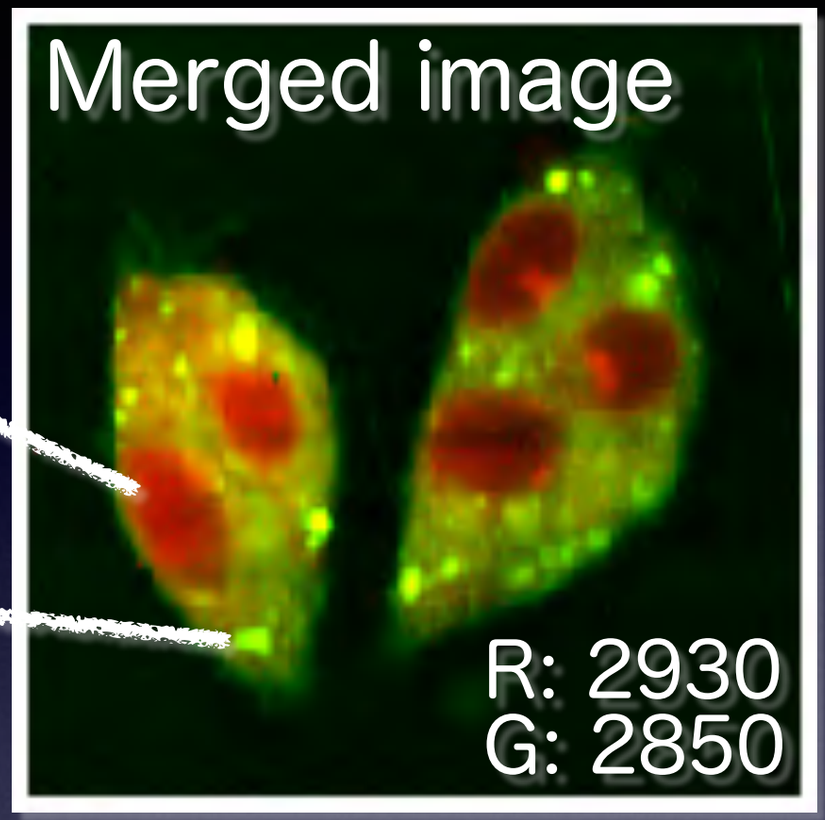


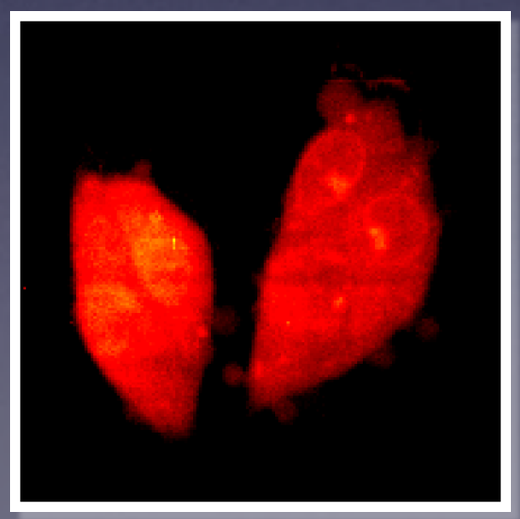


1000 cm^{-1} 1650 cm^{-1} 2850 cm^{-1} 2930 cm^{-1}

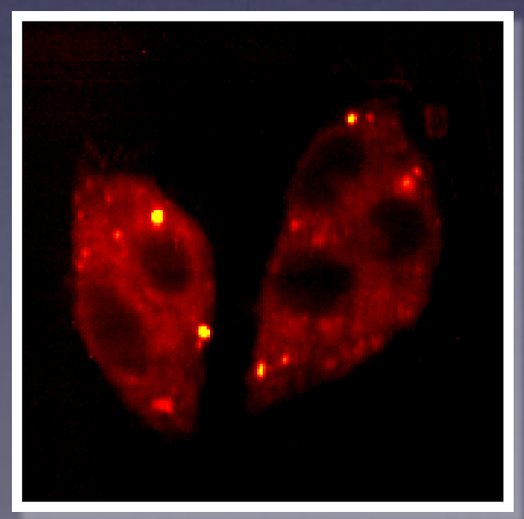
Spectral Raman imaging



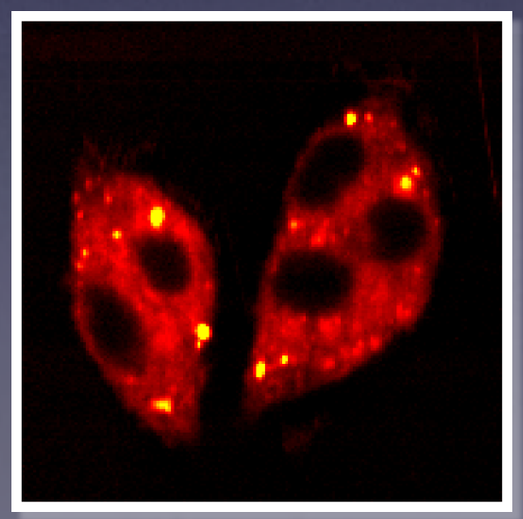




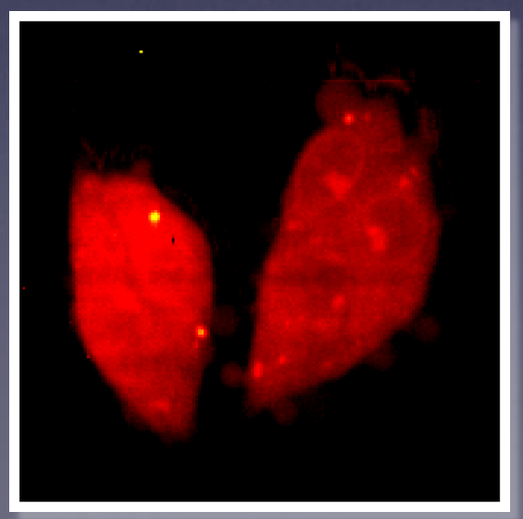
1000 cm^{-1}



1650 cm^{-1}



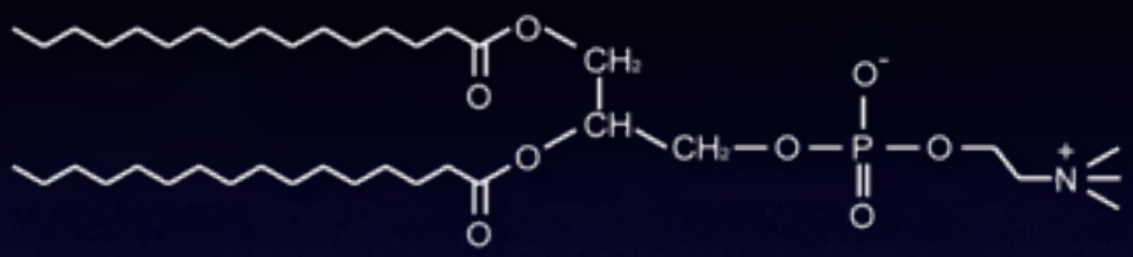
2850 cm^{-1}



2930 cm^{-1}

Estimation of molecular structures

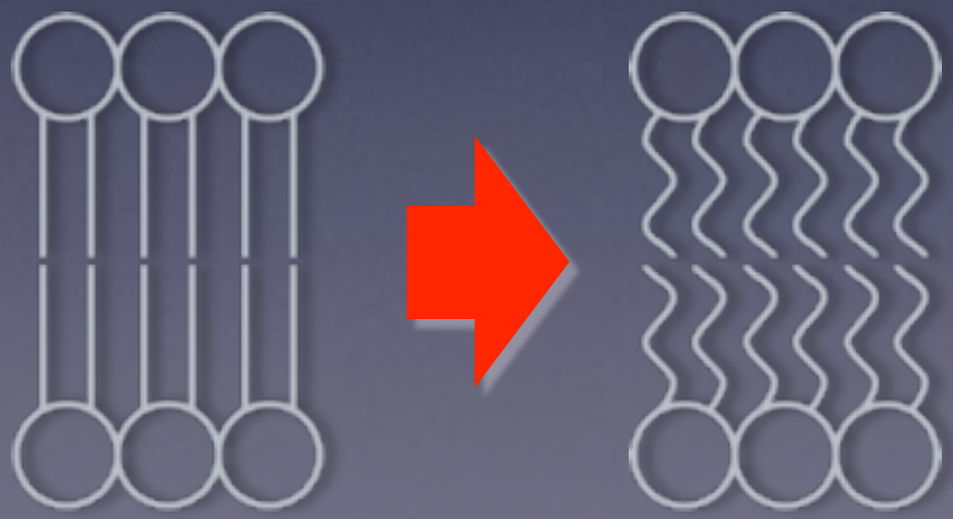
リン脂質(DPPC)

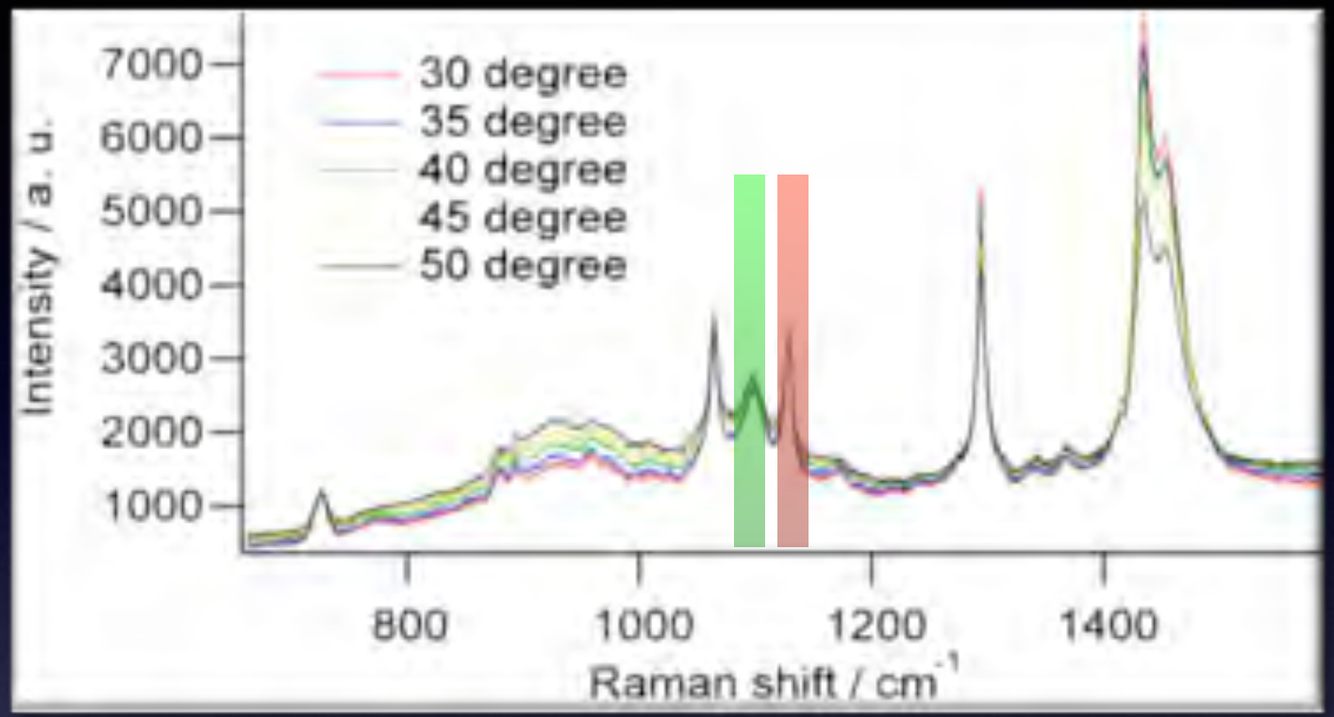


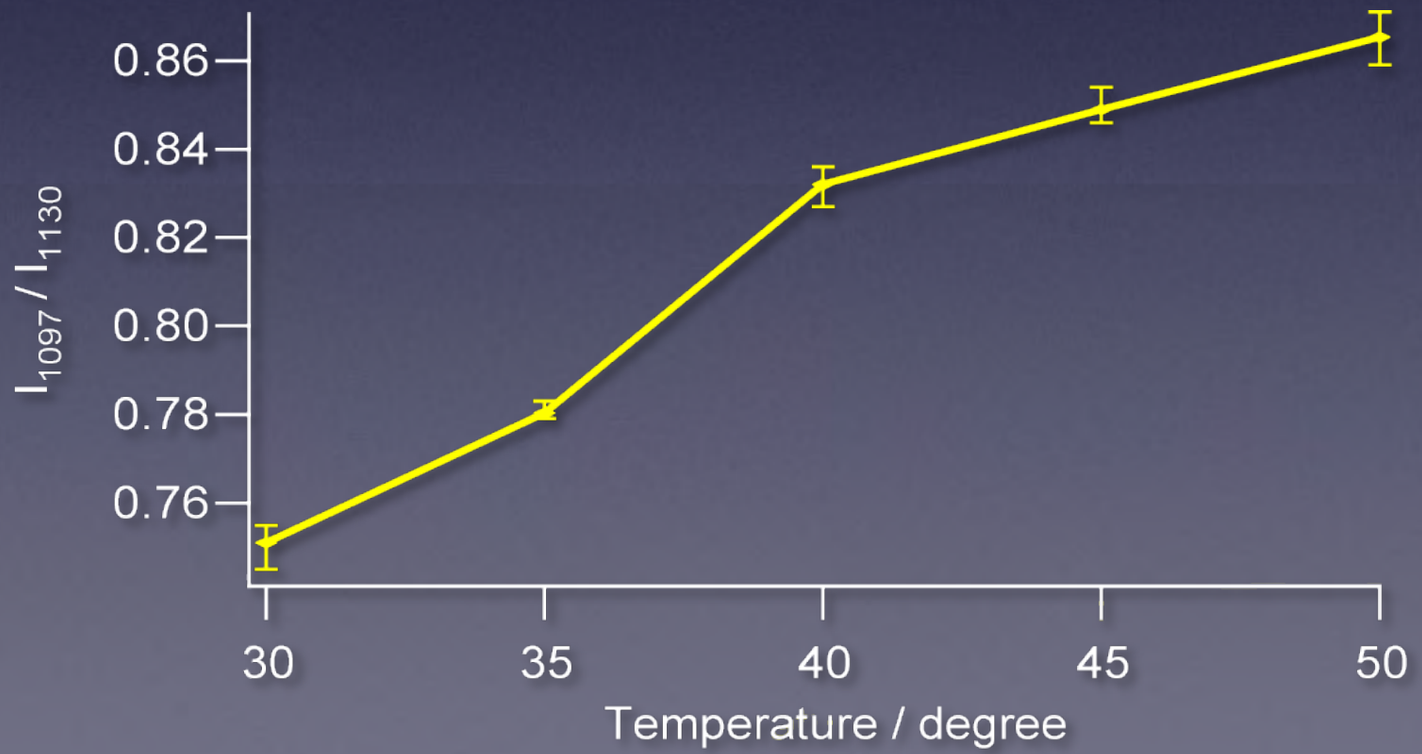
温度上昇：

DPPC脂質のAcyl鎖

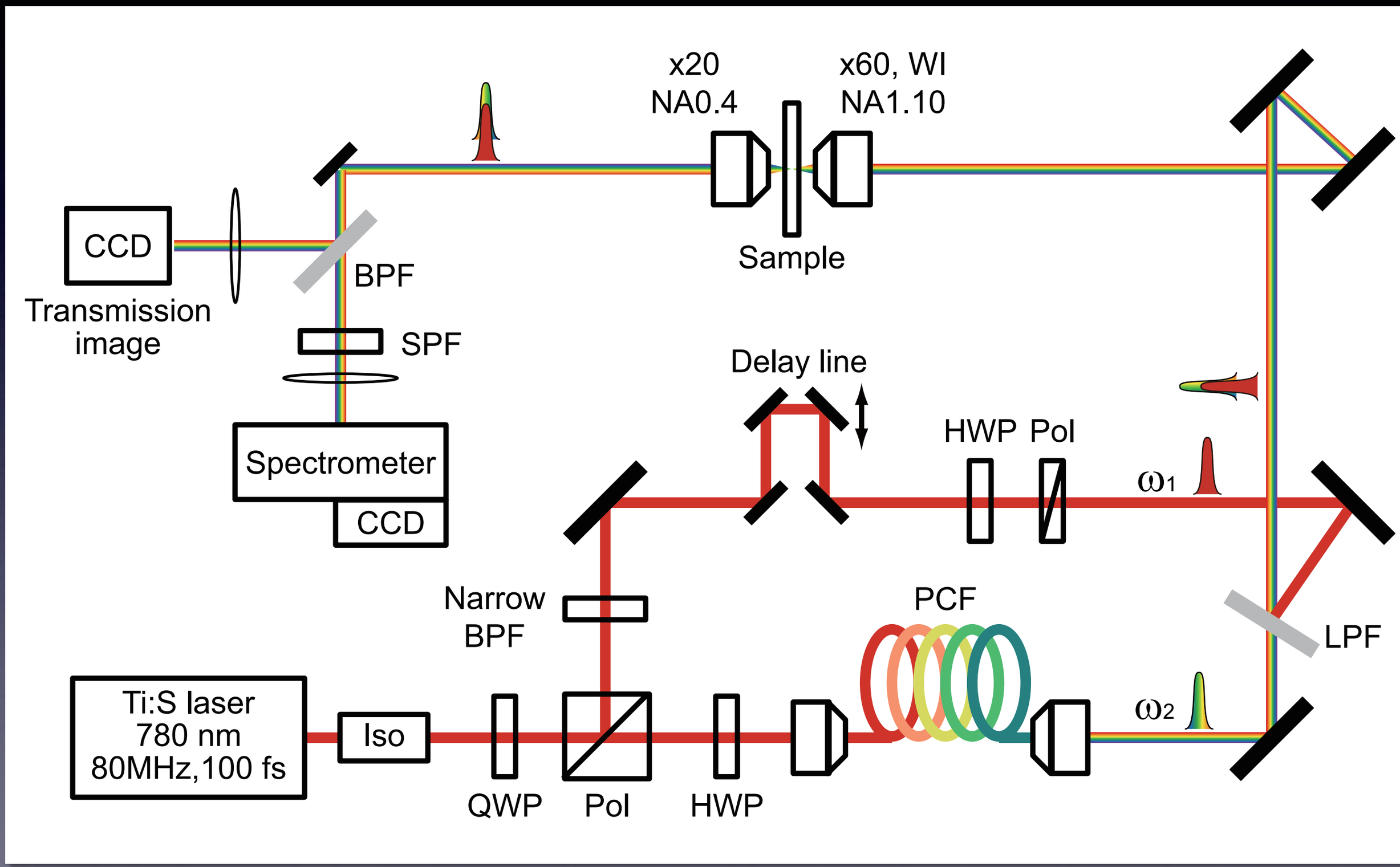
trans → gauche



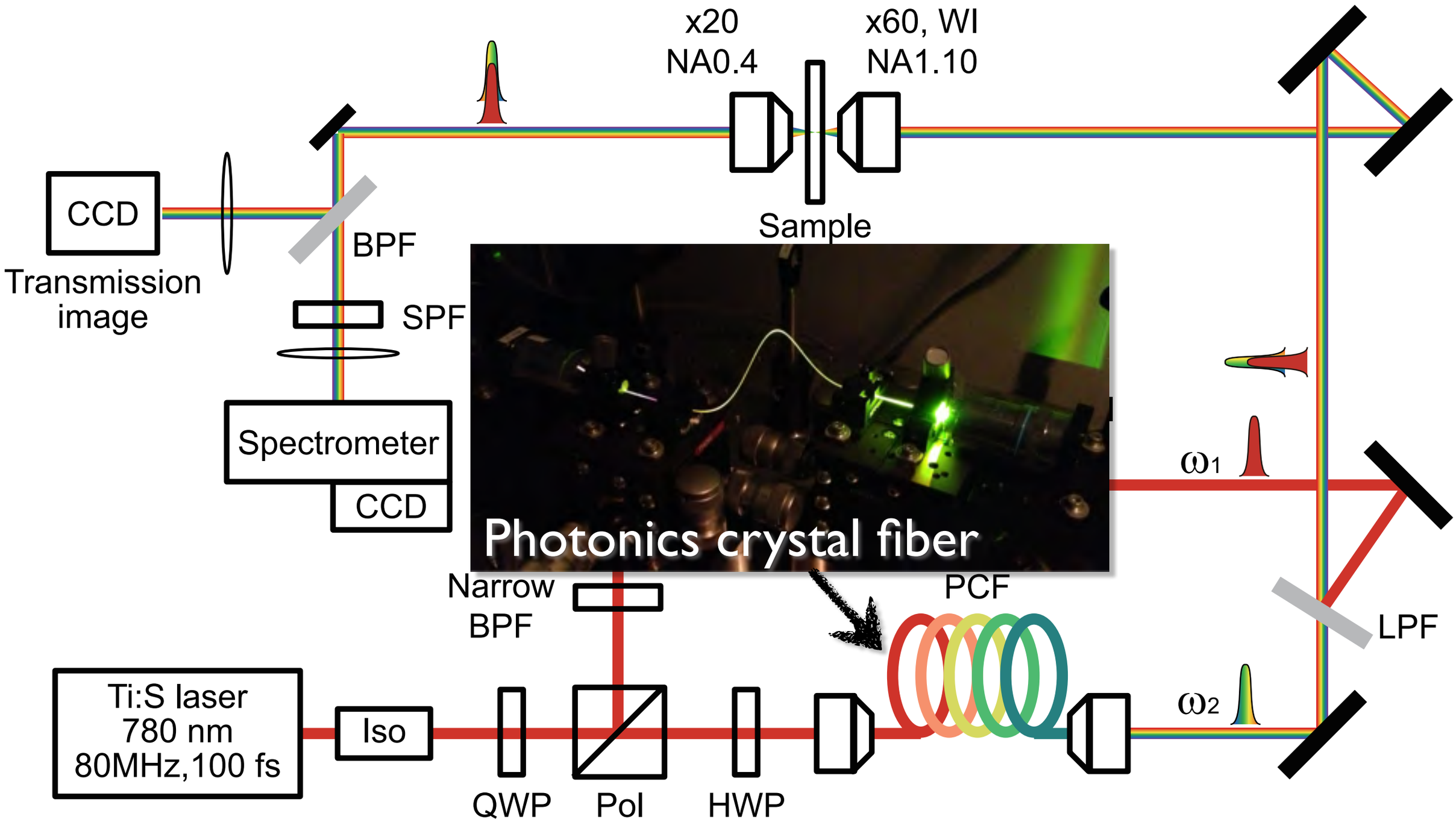




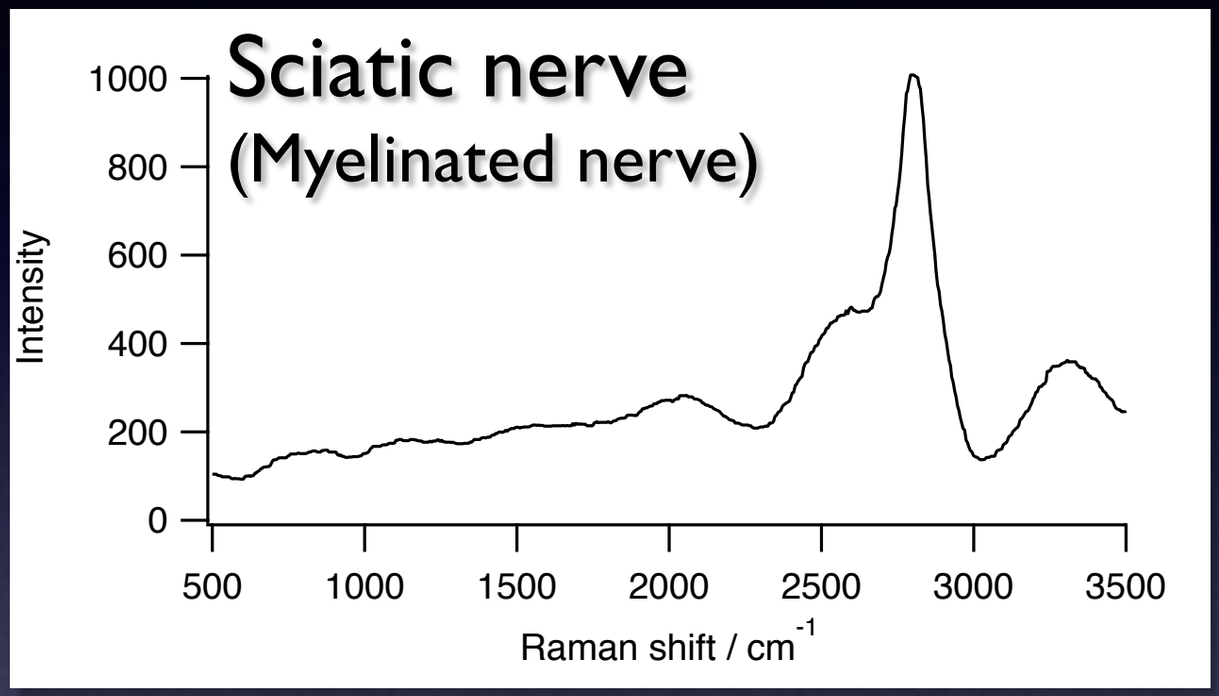
Optical setup - Multiplex CARS -

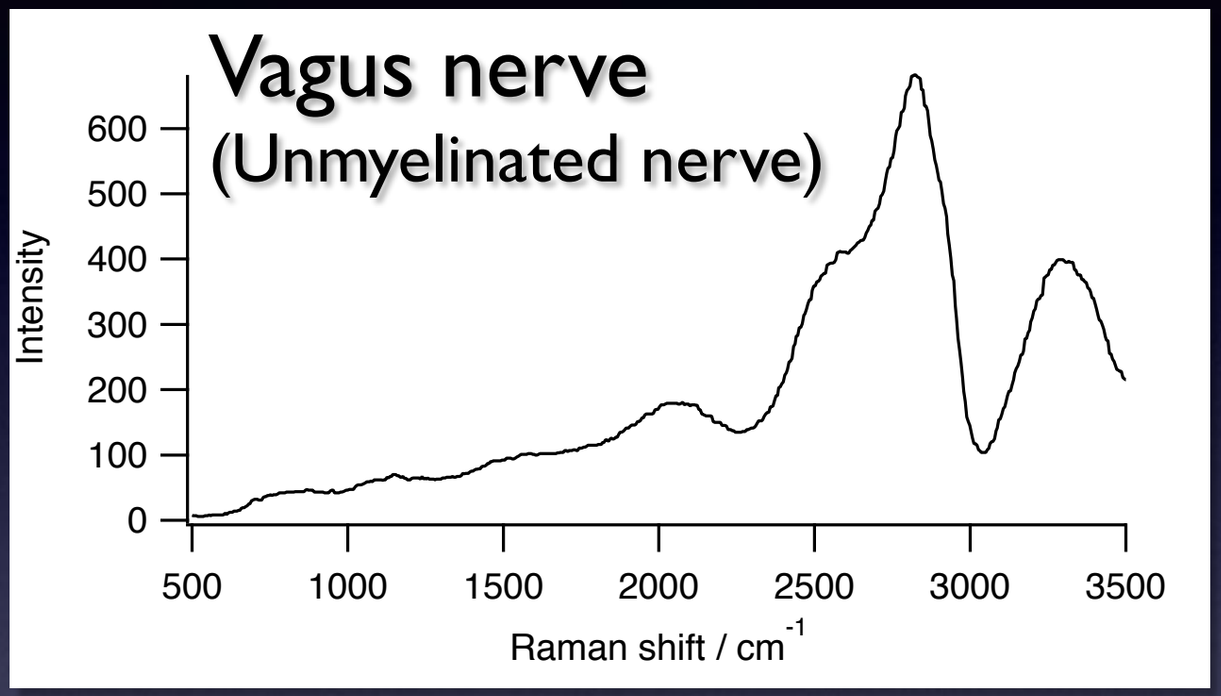


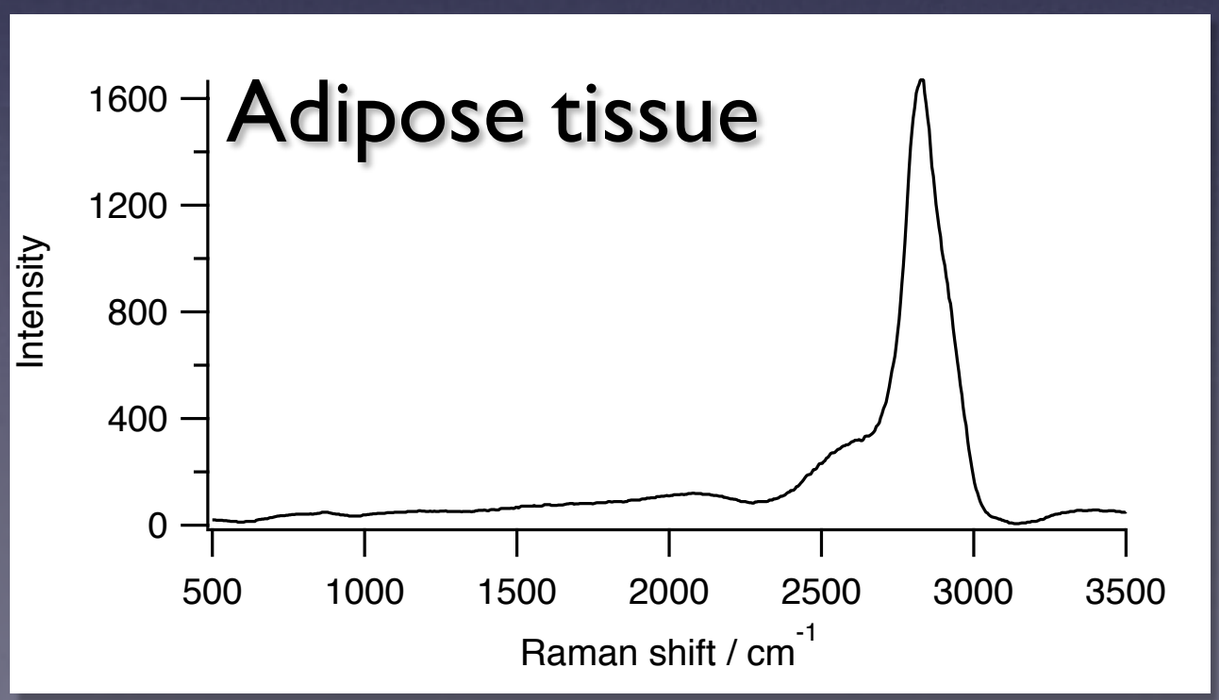
Optical setup - Multiplex CARS -

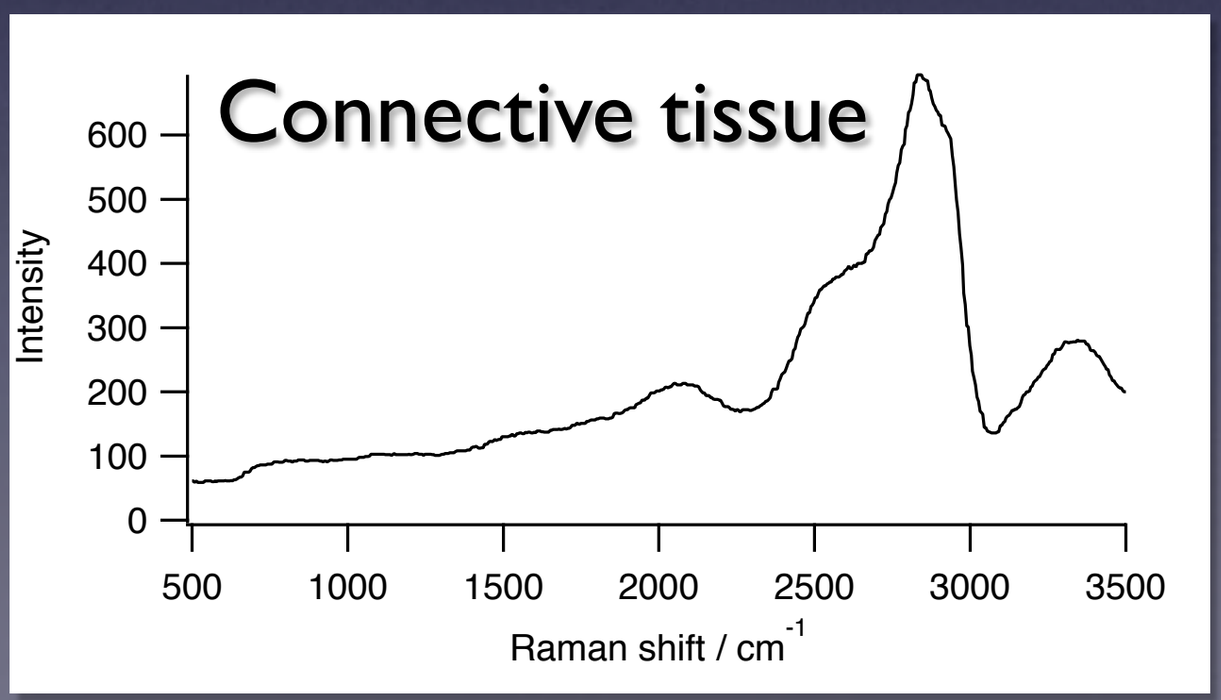


Broadband CARS spectra of peripheral nerves

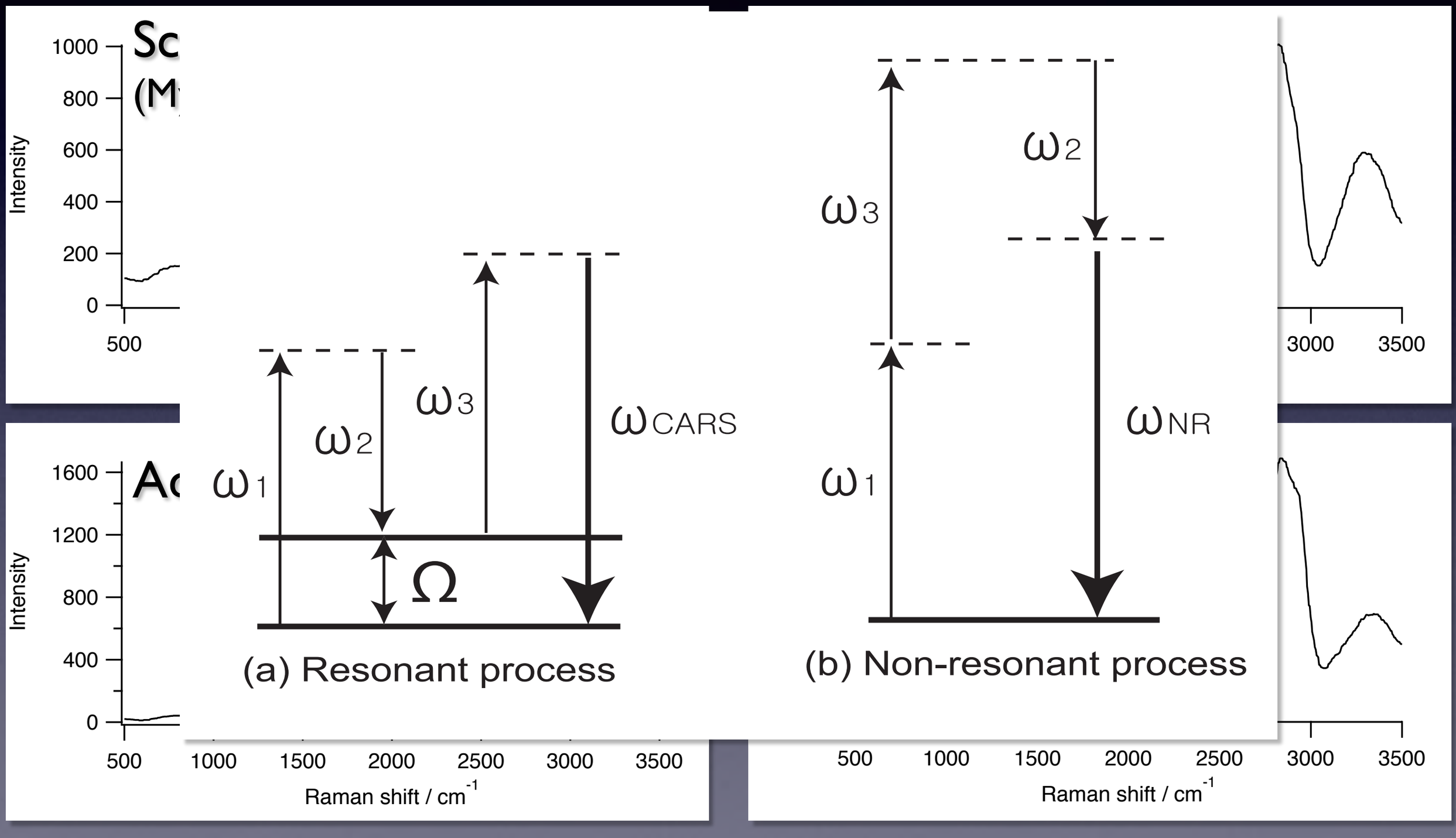




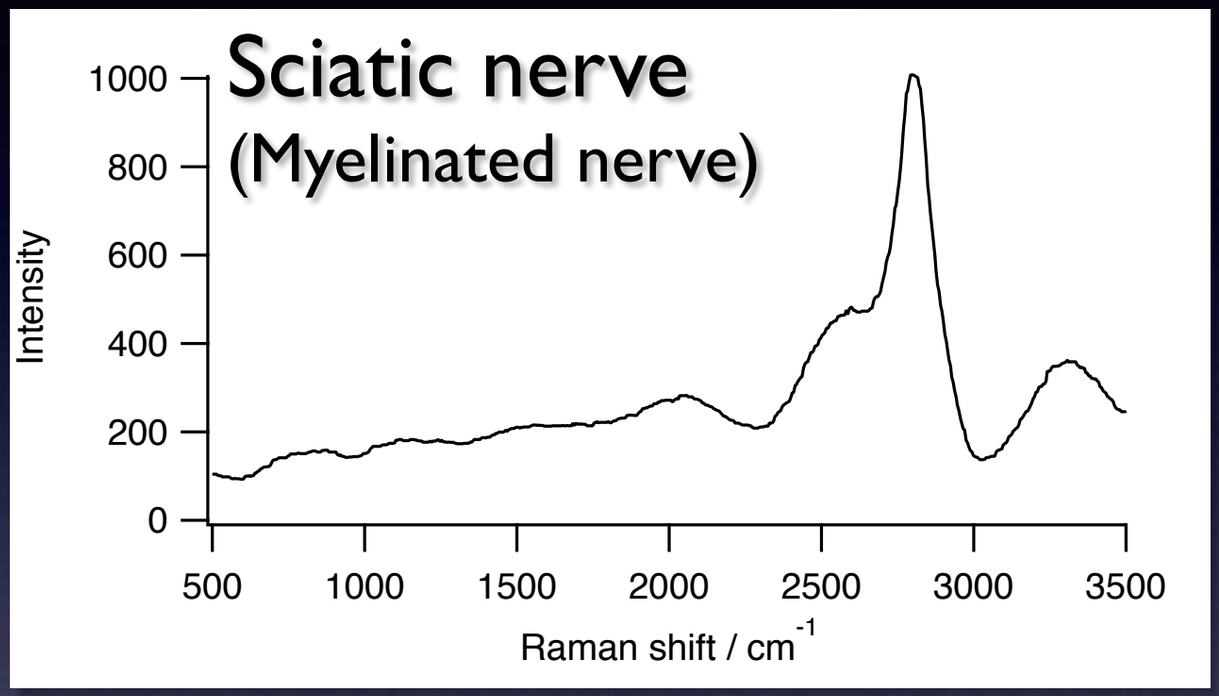


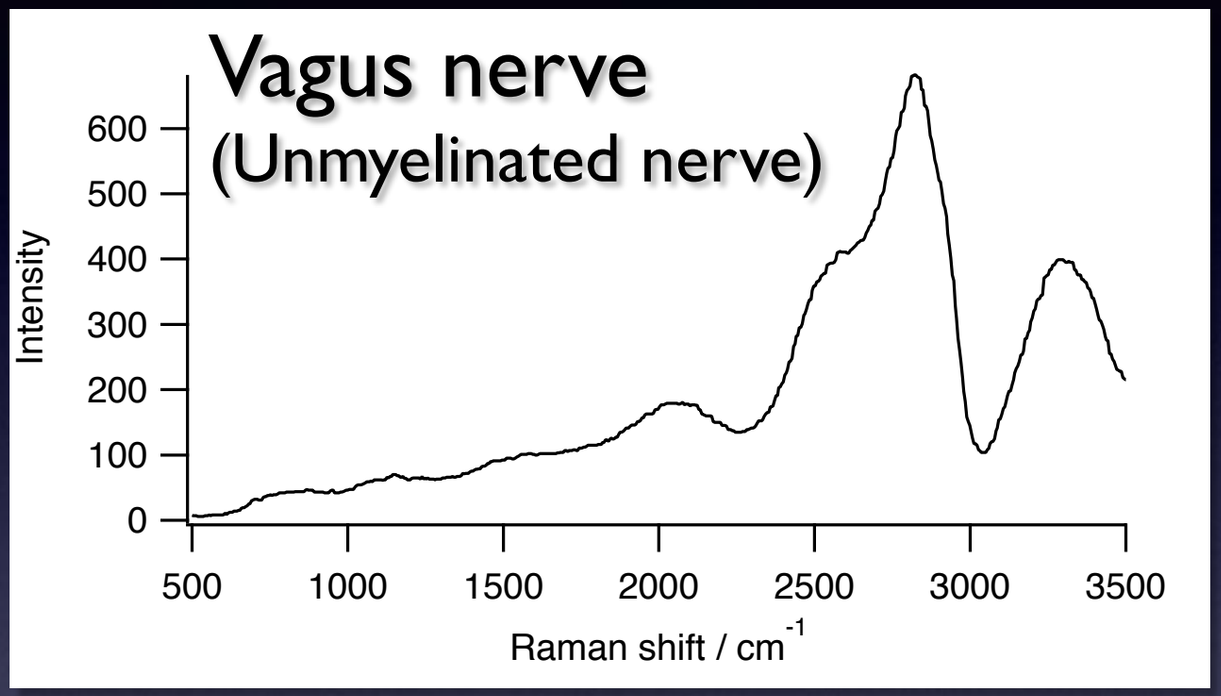


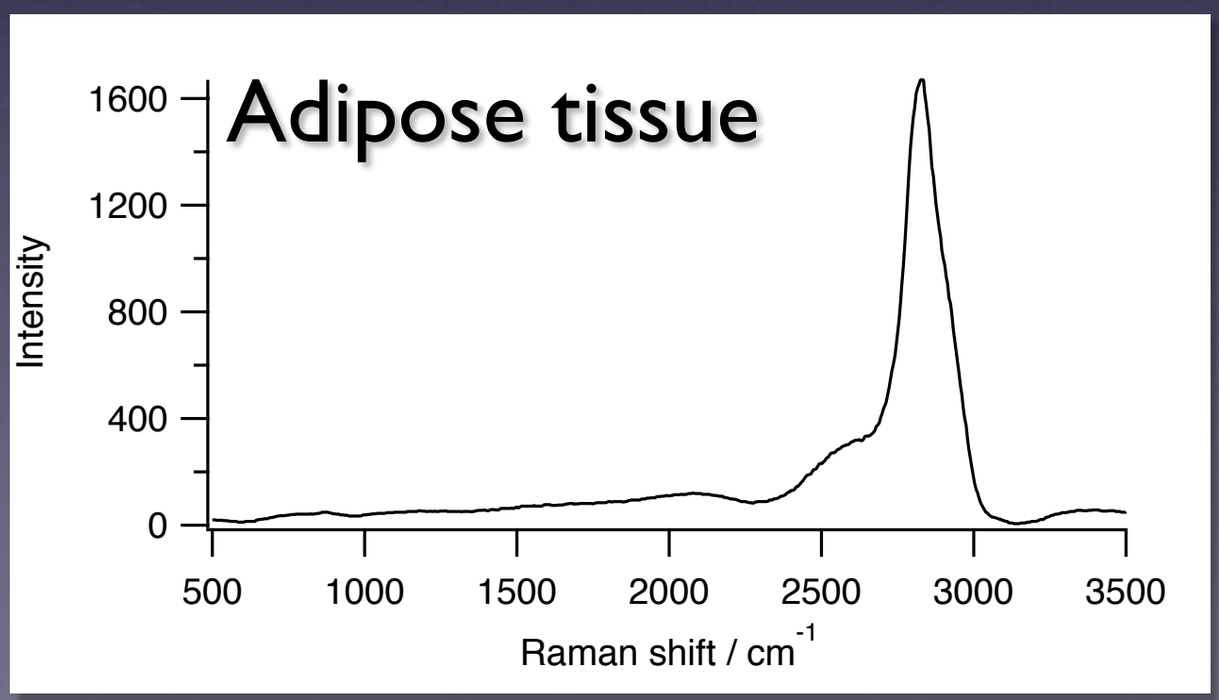
Broadband CARS spectra of peripheral nerves

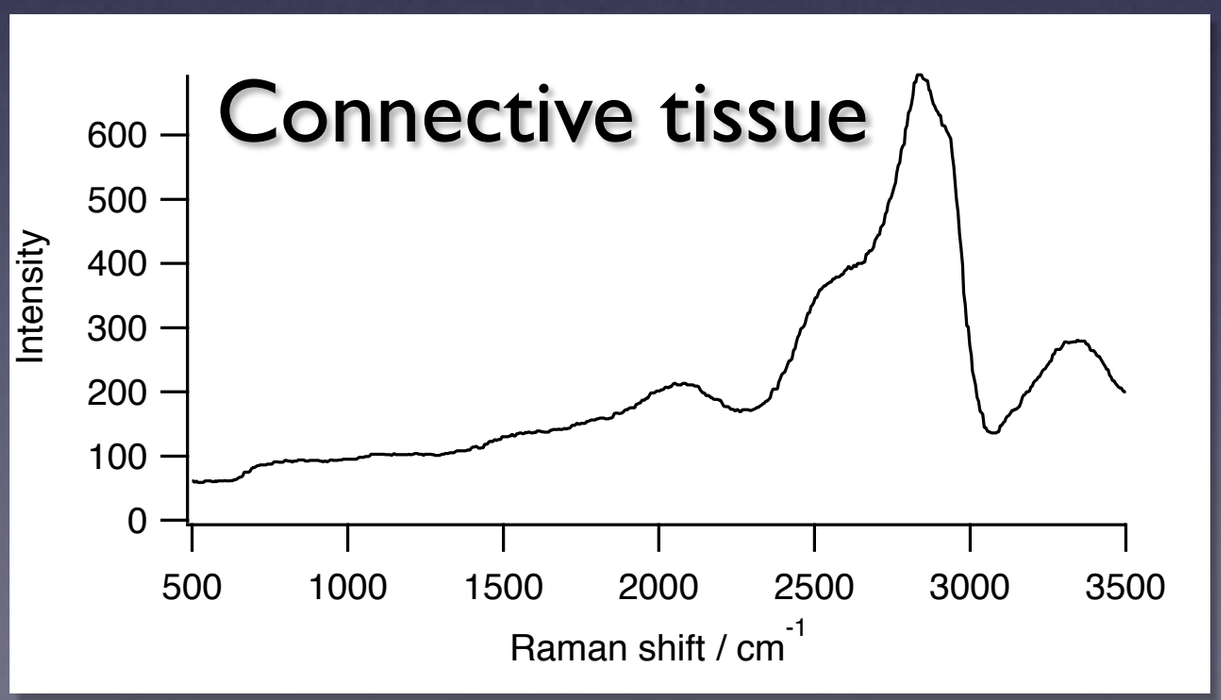


Broadband CARS spectra of peripheral nerves



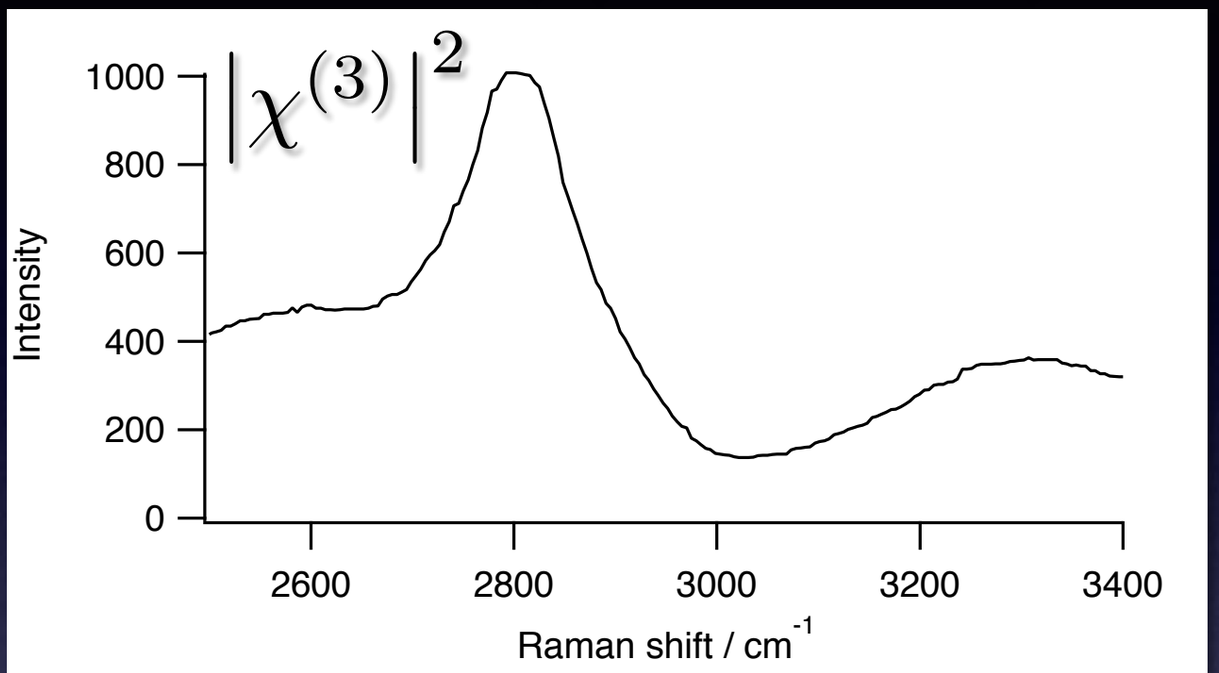




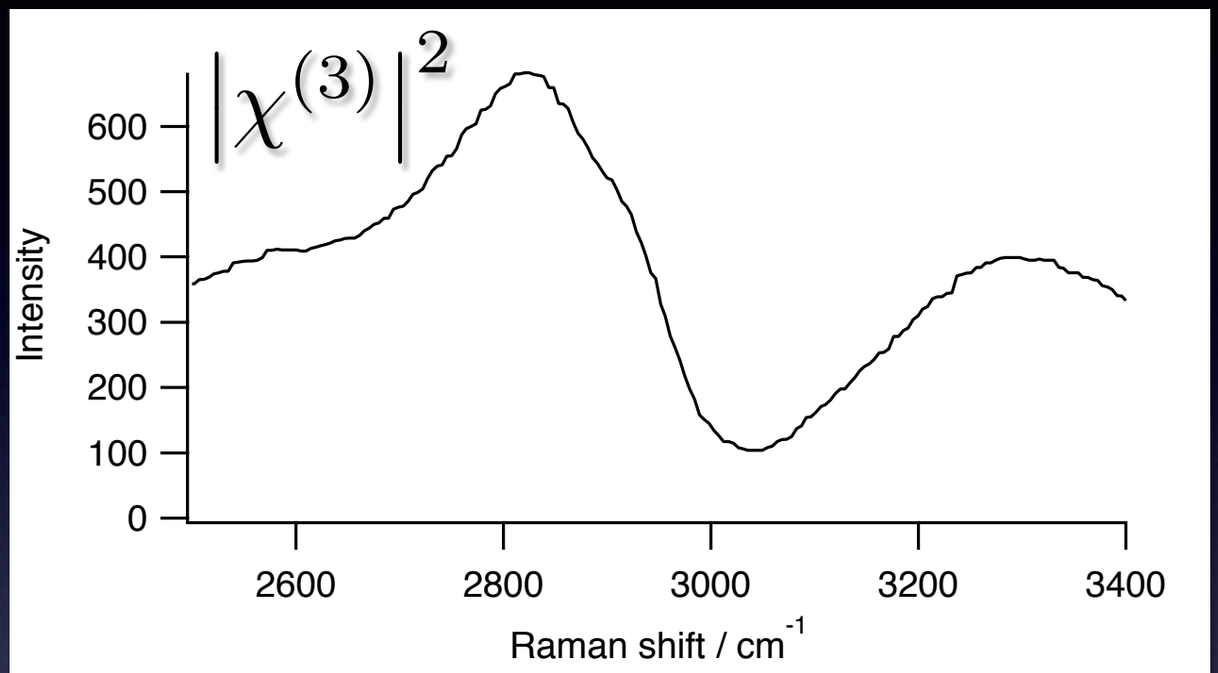


CARS spectra at CH region

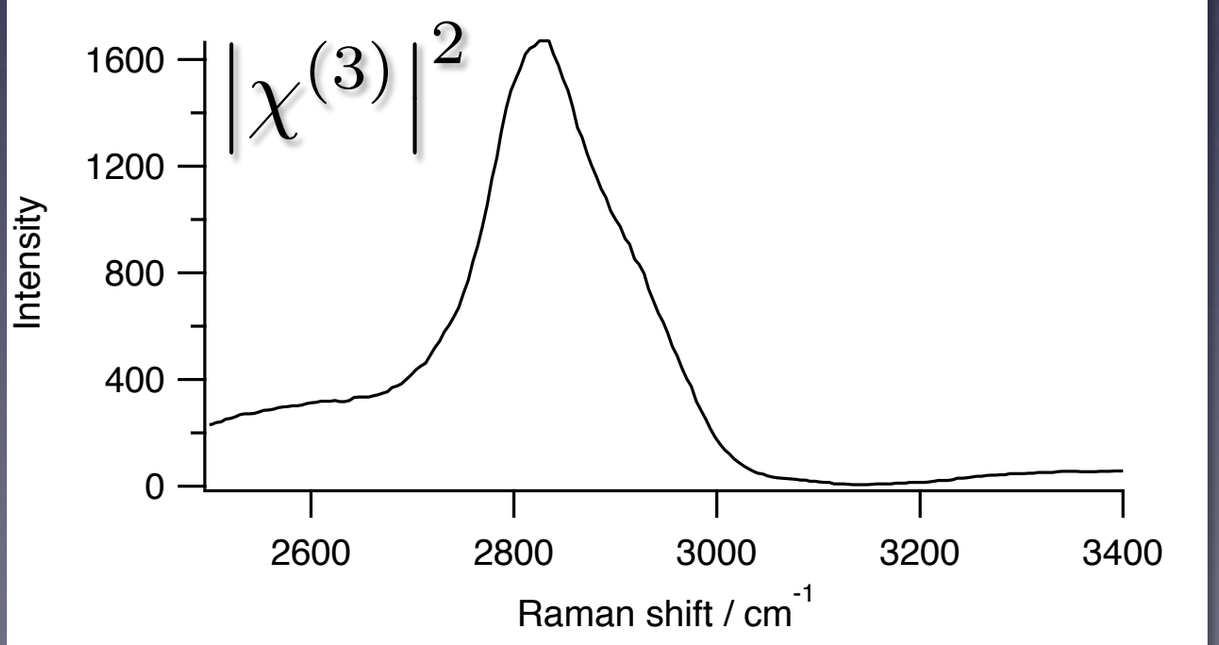
Sciatic nerve (Myelinated nerve)



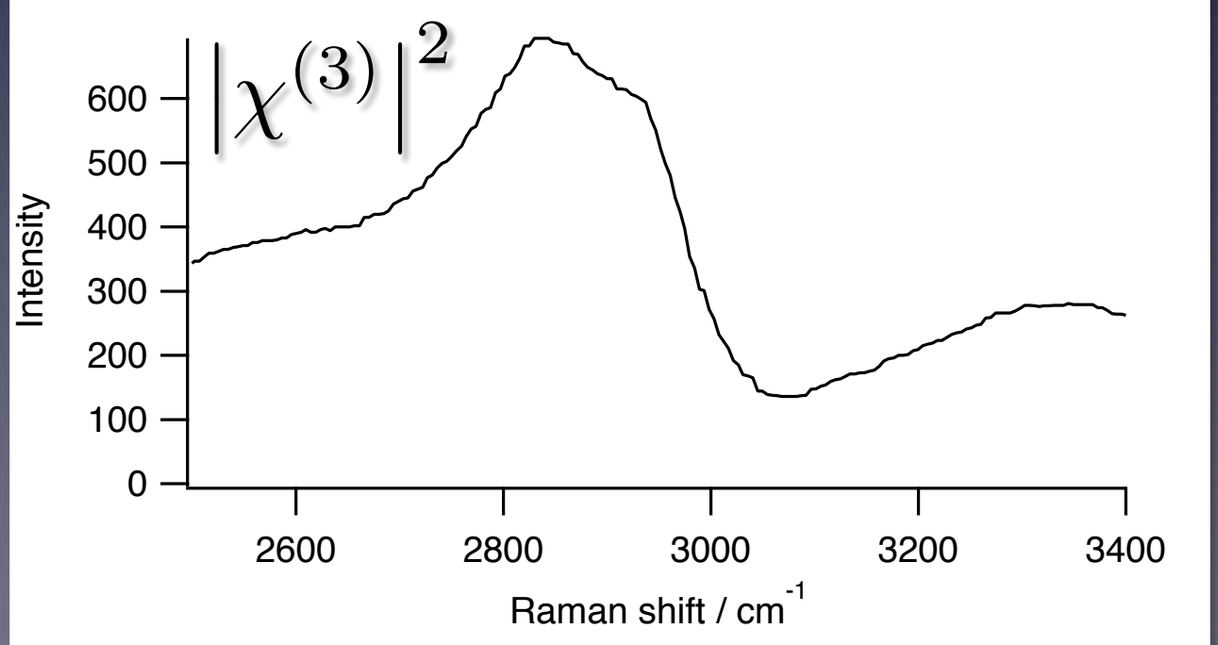
Vagus nerve (Unmyelinated nerve)



Adipose tissue

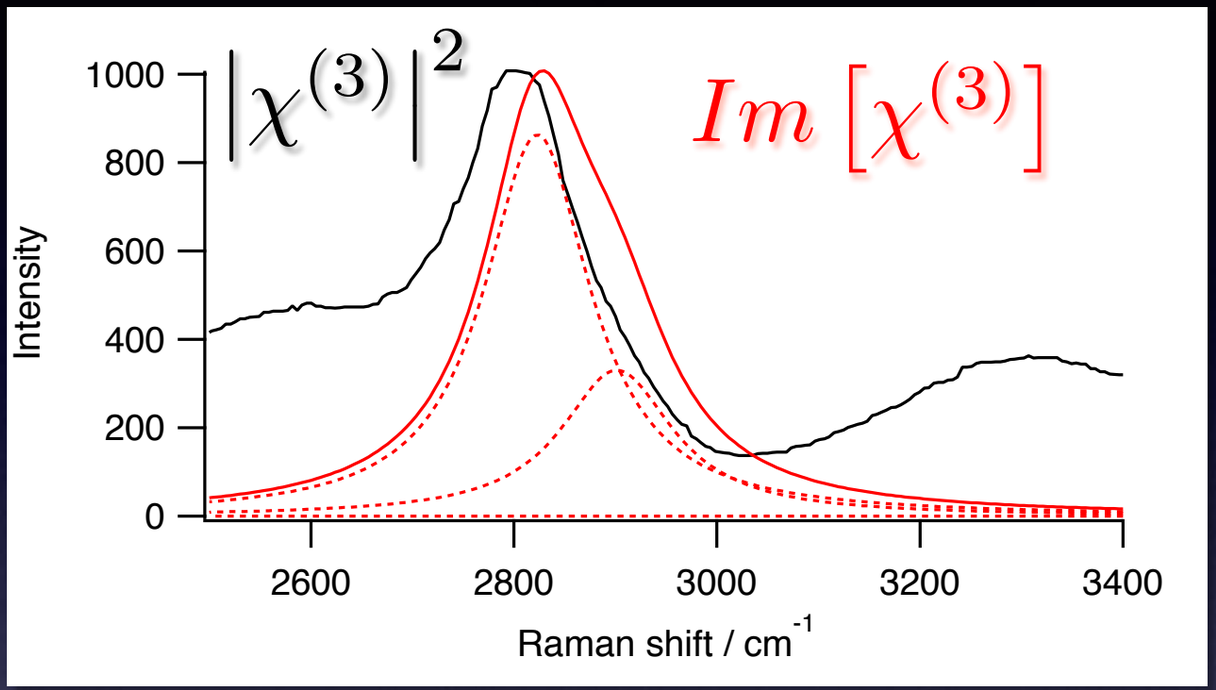


Connective tissue

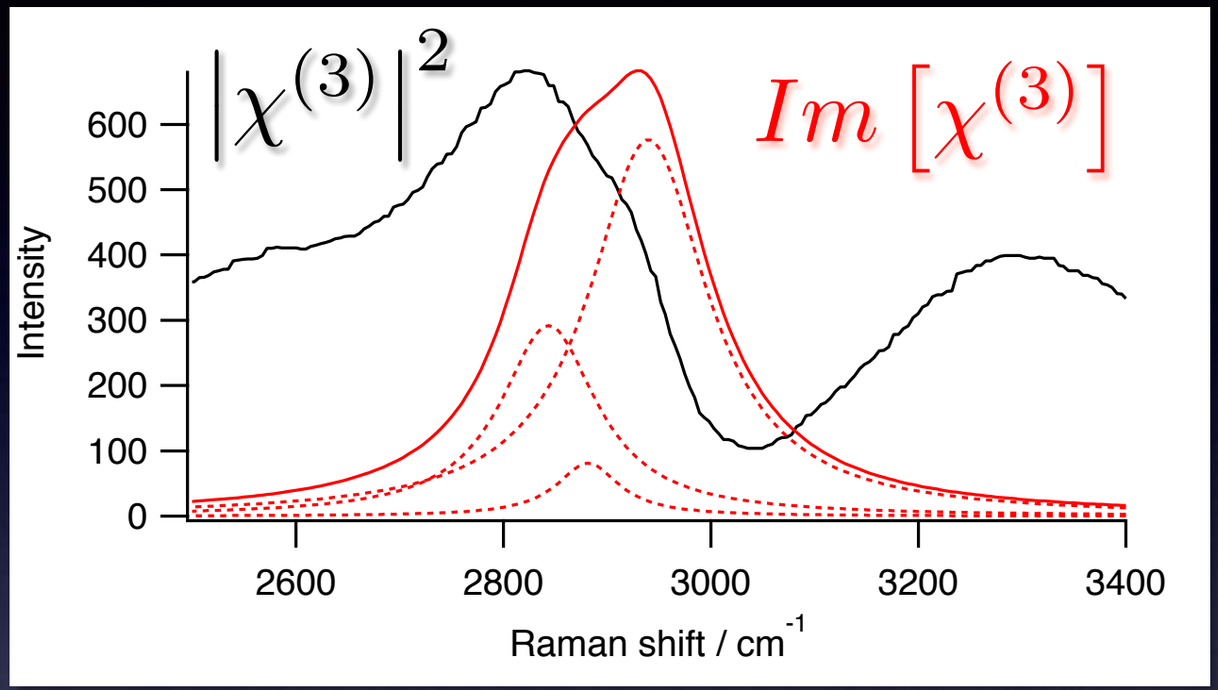


CARS spectra at CH region

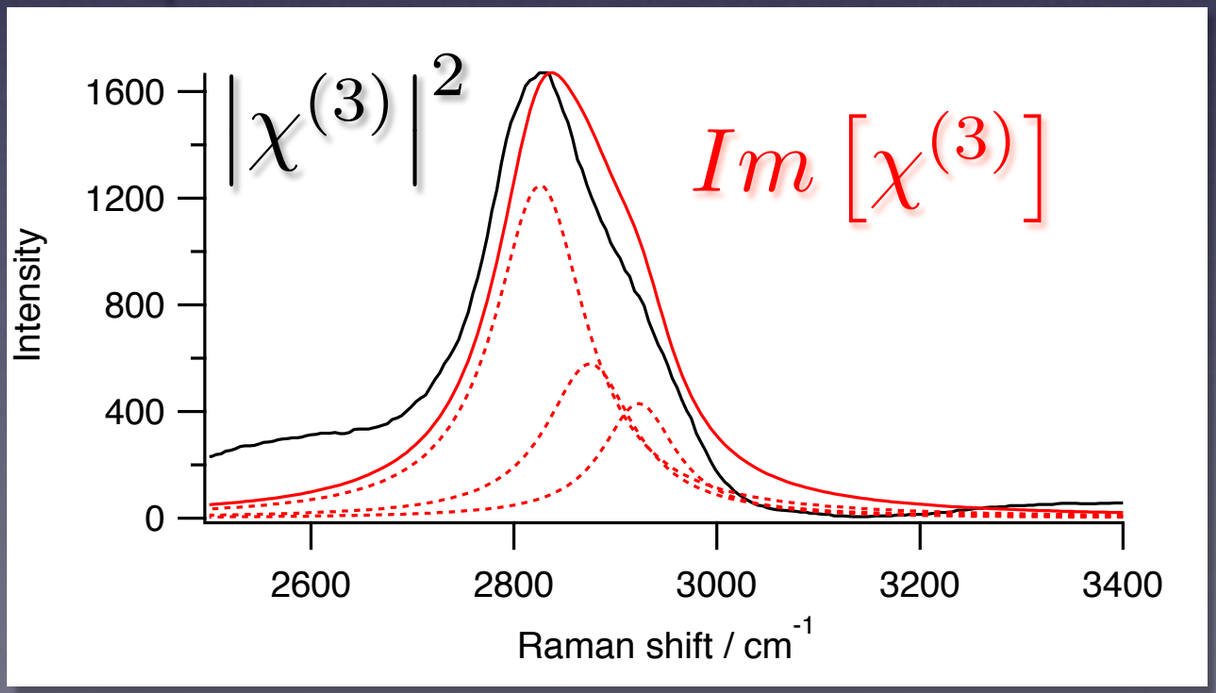
Sciatic nerve (Myelinated nerve)



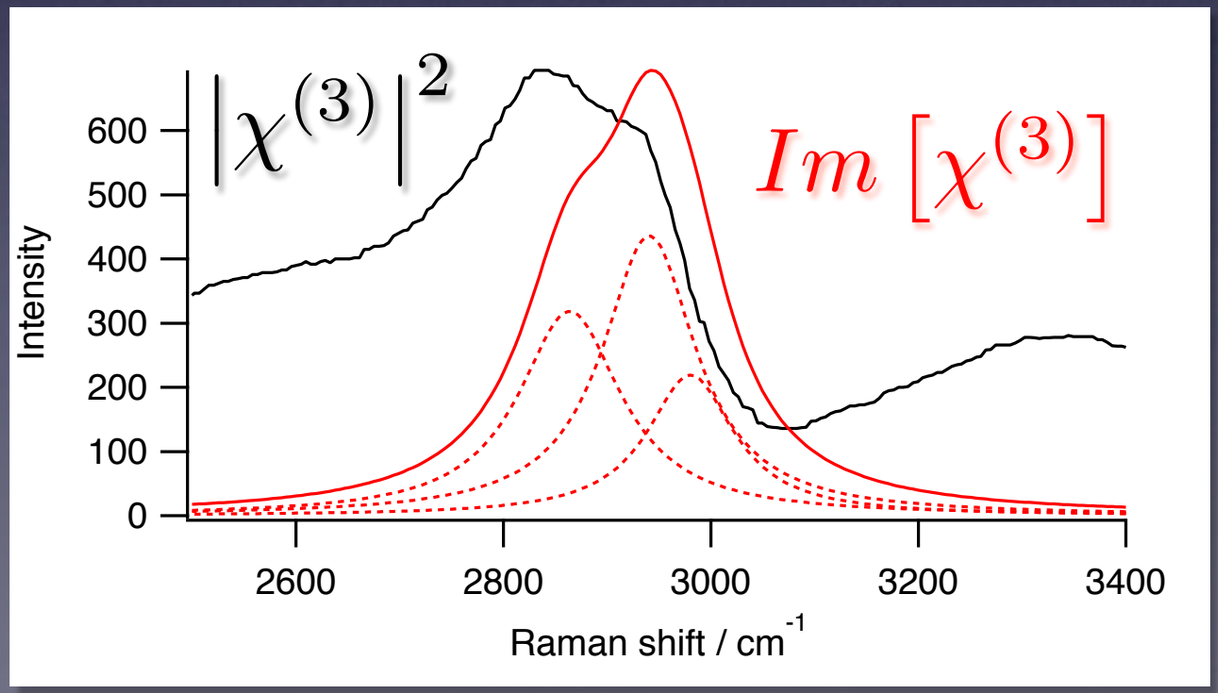
Vagus nerve (Unmyelinated nerve)



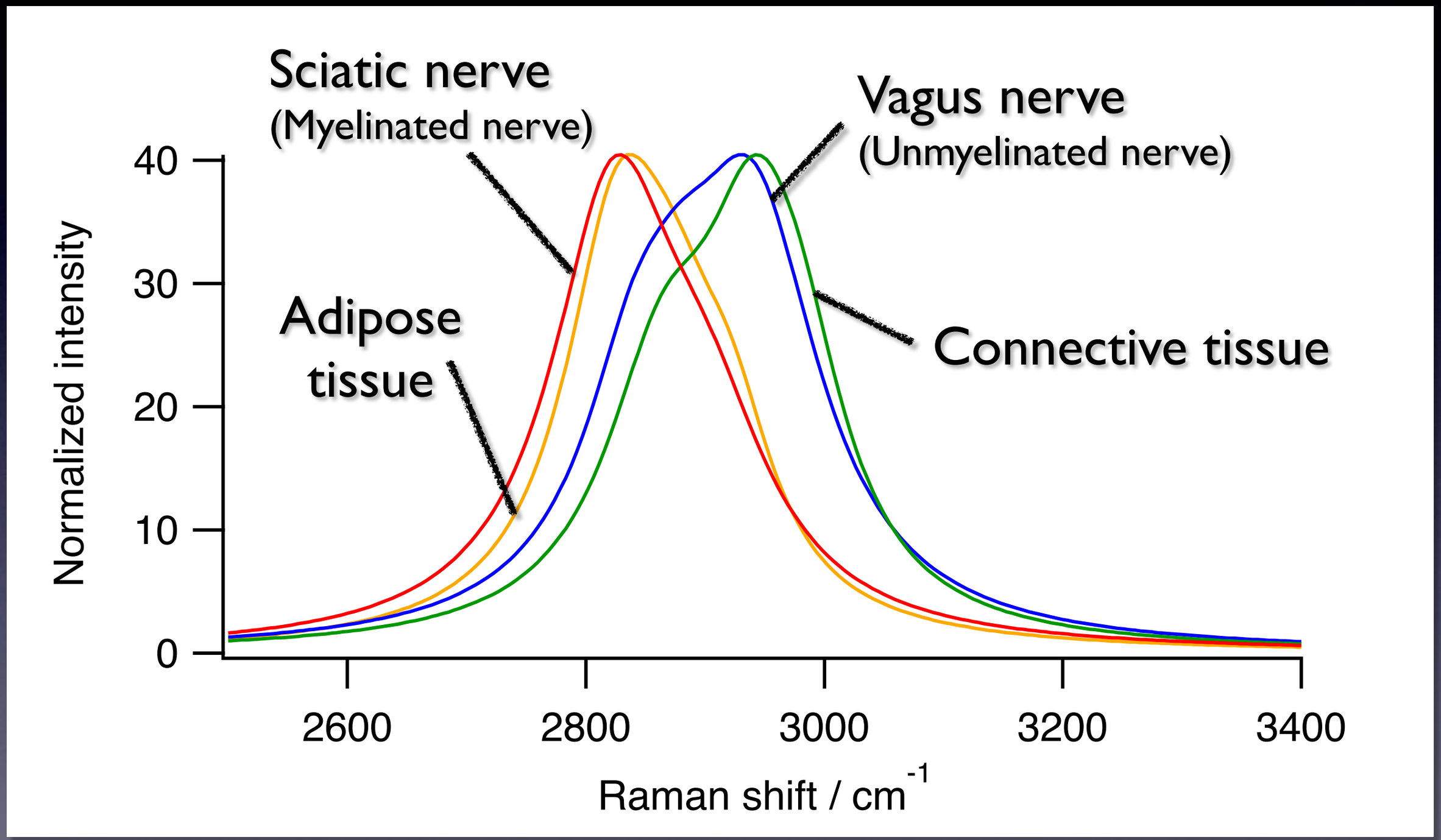
Adipose tissue



Connective tissue

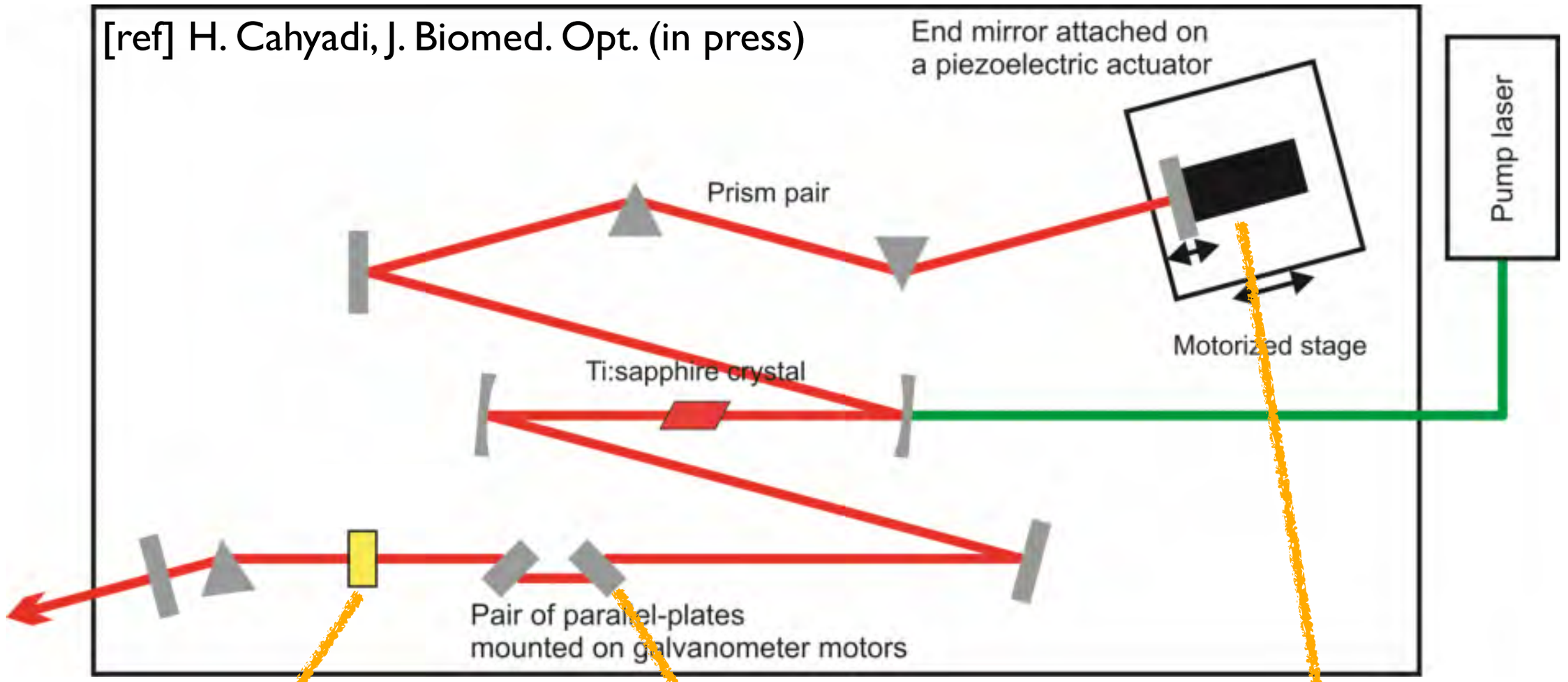


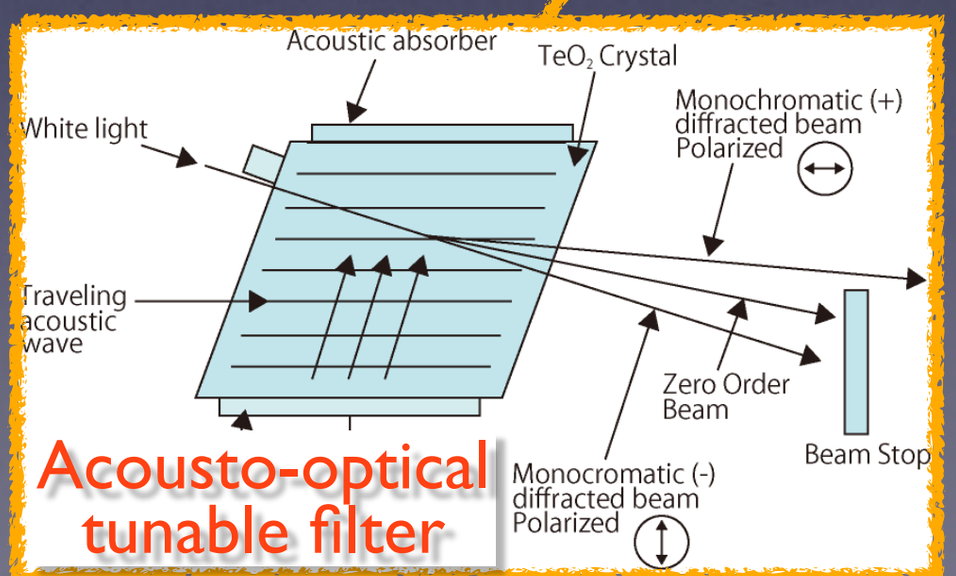
CARS spectra at CH region



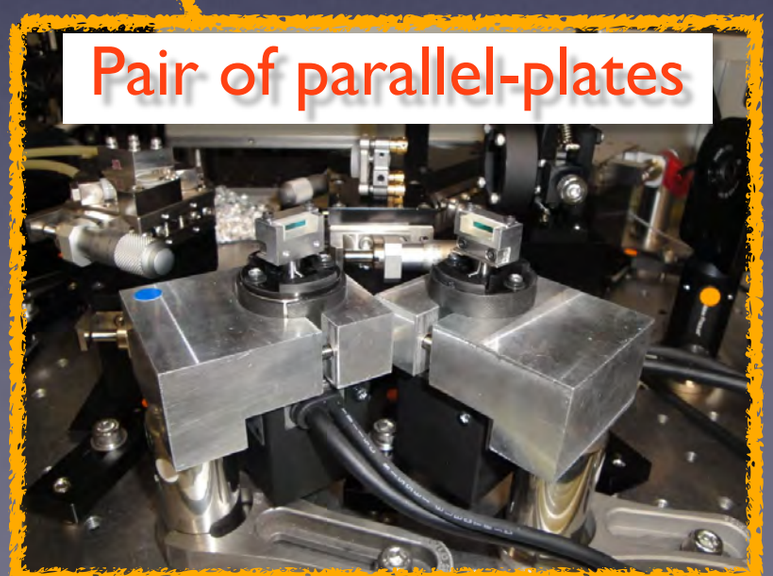
Fast spectral CARS imaging

[ref] H. Cahyadi, J. Biomed. Opt. (in press)

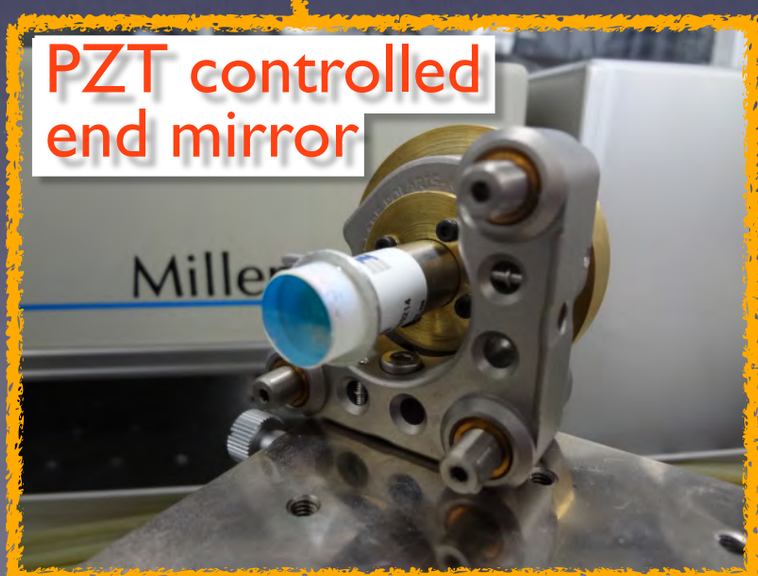




Acousto-optical tunable filter



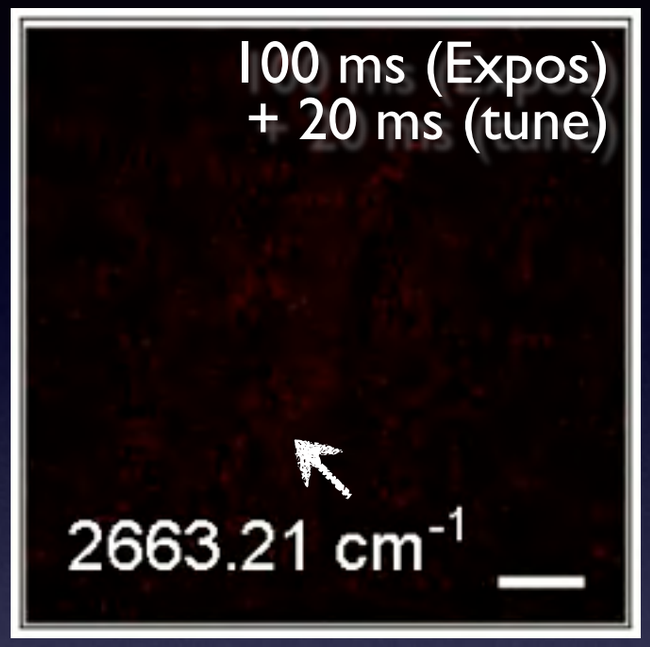
Pair of parallel-plates



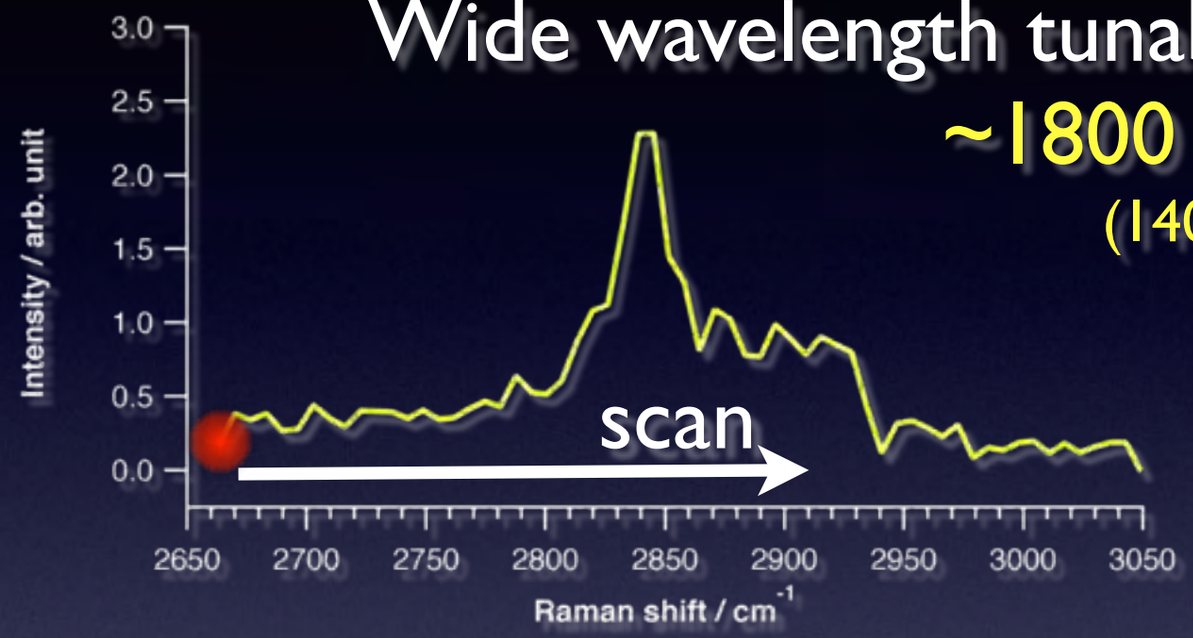
PZT controlled end mirror

Fast spectral CARS imaging

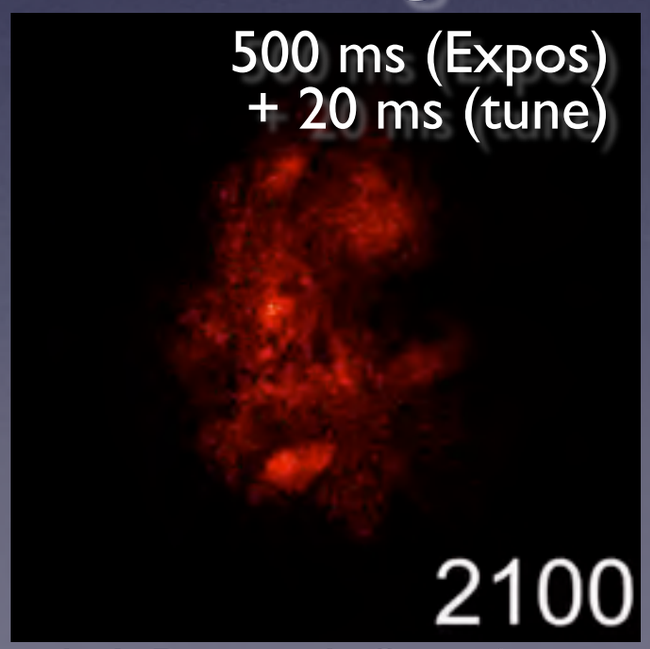
✓ Fast wavelength tuning <20 ms

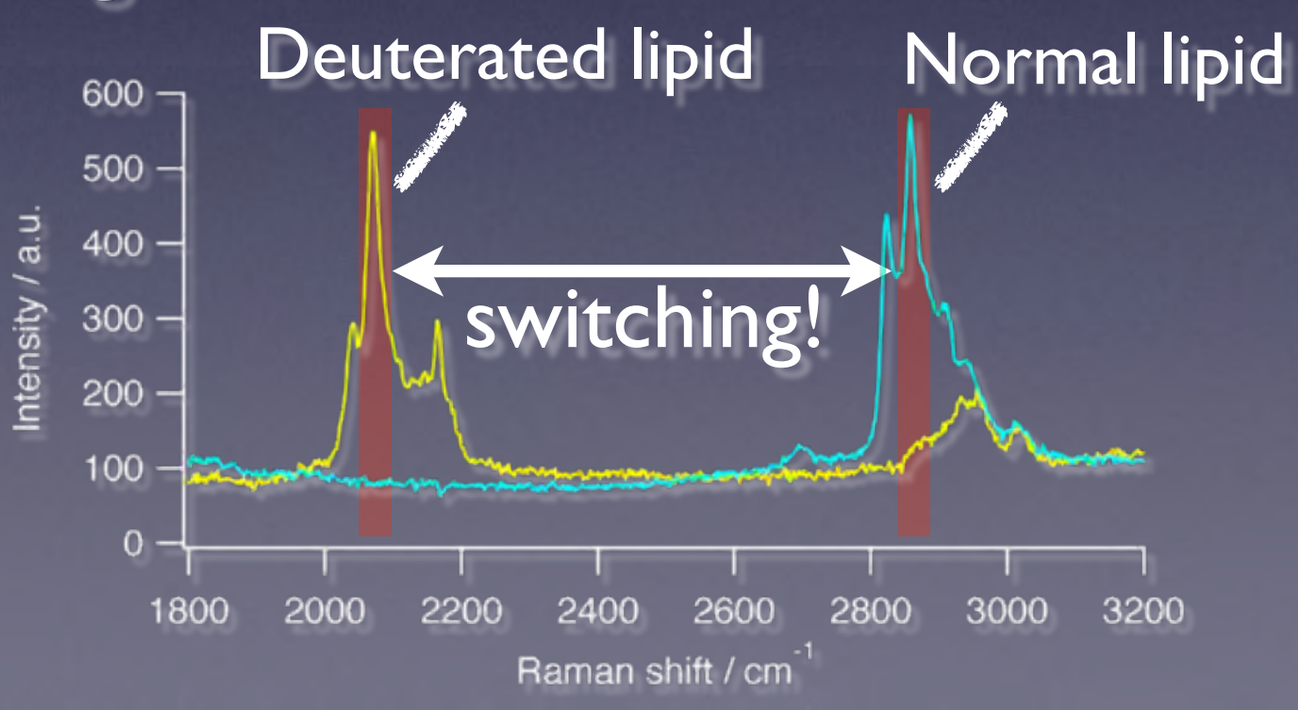


Wide wavelength tunability
~1800 cm-1
(140 nm~)



✓ Fast wavelength switching <20 ms





[ref] H. Cahyadi, J. Biomed. Opt. (in press)

Towards better molecular imaging in biology and medicine

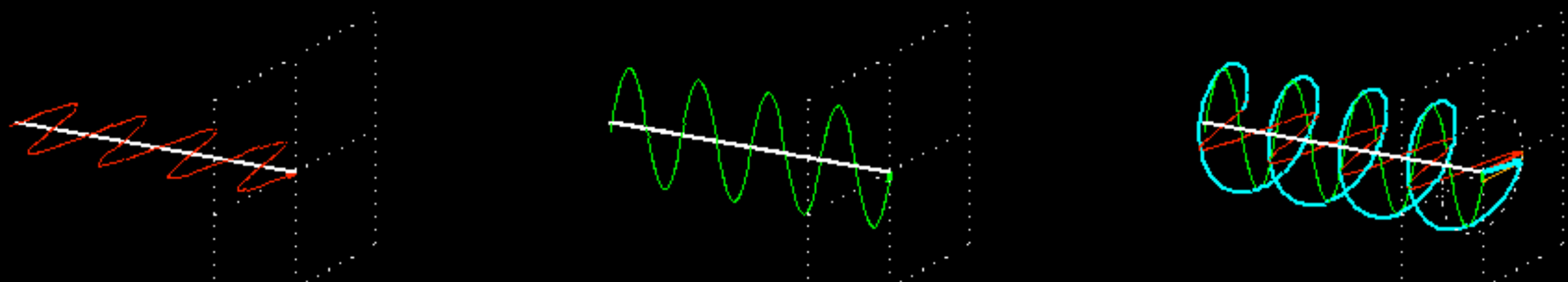
- ✓ 速く撮りたい！
- ✓ 分子の選択性を高めたい！
- ✓ 分子分布以外の分子情報を得たい！

顕微ラマン散乱分光法＋非線形光学

非線形顕微ラマン散乱分光法

(CARS: Coherent anti-Stokes Raman scattering)

Orientation measurement by light polarization



直線偏光 (水平)　　直線偏光 (垂直)　　円偏光

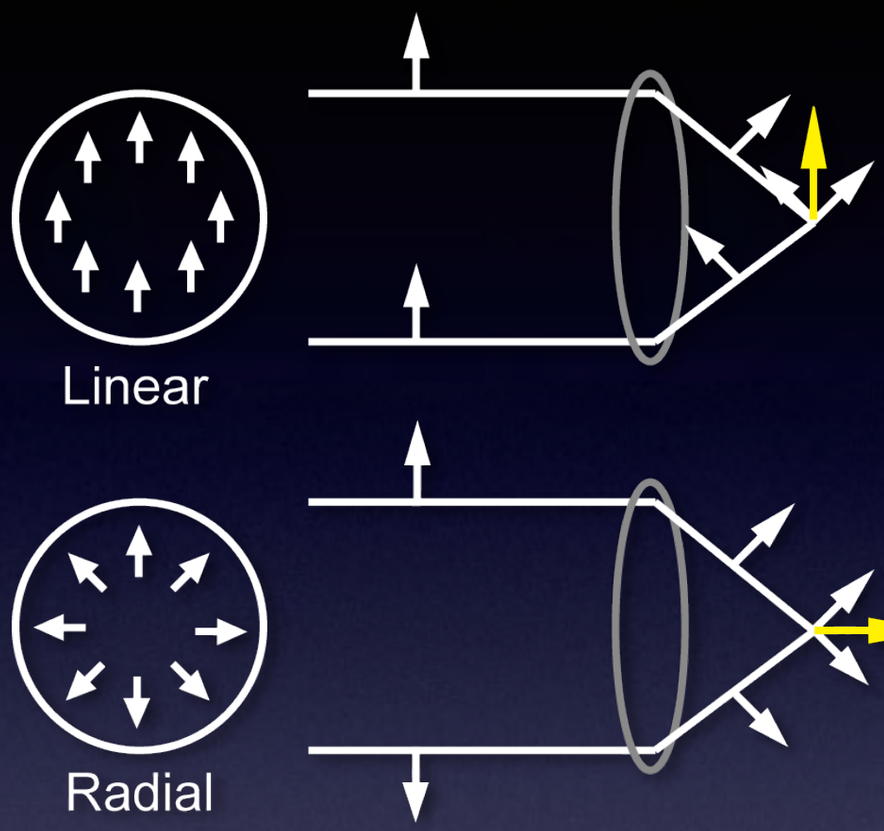
光電場の
方向

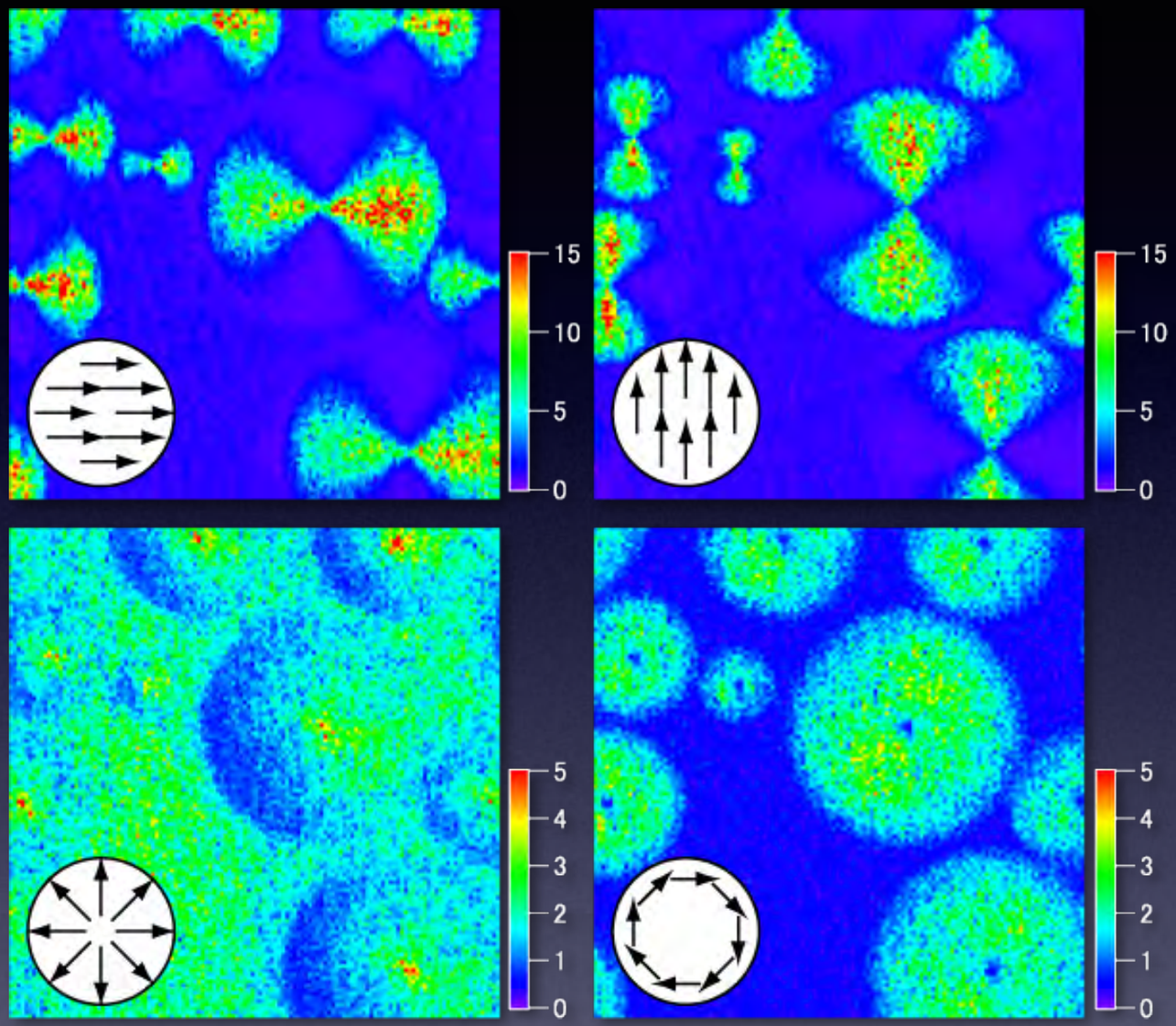
光電場の
大きさ

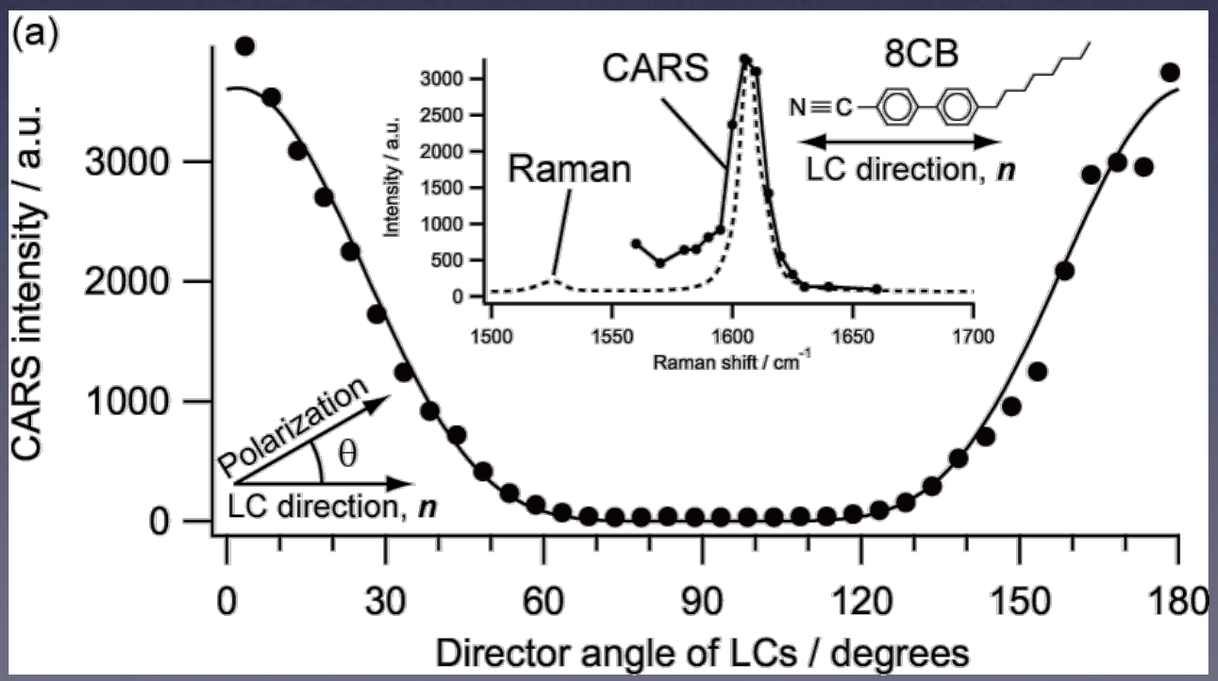
分子振動の偏光依存性

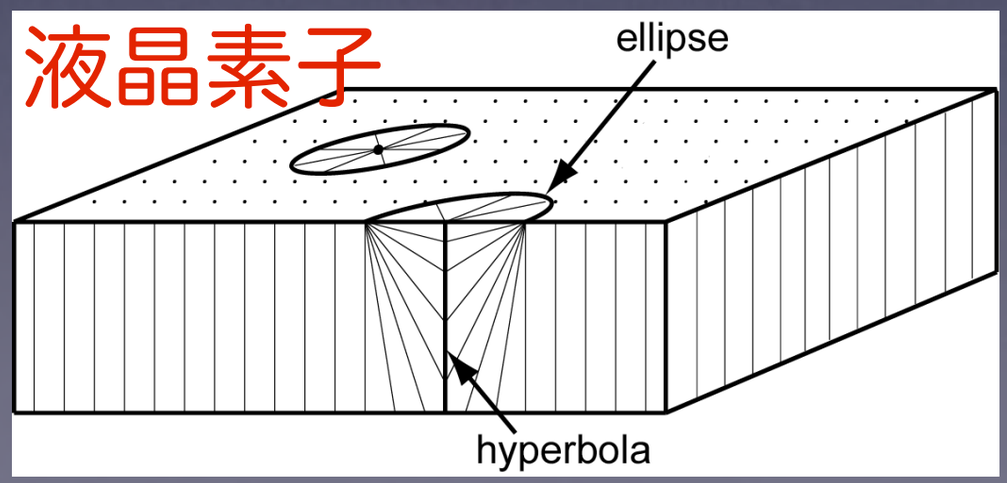
偏光制御ラマンイメージング

3D orientation measurement









[ref] T Minamikawa et al. Appl. Phys. Express (2013)

ラマン散乱分光法の文化財への応用

～浮世絵の彫摺技術復元を目指して～

プロジェクトの目的

- **浮世絵**
  - 江戸時代に発展した多色摺木版画であり、現在では日本を代表する伝統美術

葛飾北斎、富嶽三十六景 凱風快晴

喜多川歌麿、ポッピンを吹く女

浮世絵技法の復元による、
伝統文化の次世代への継承・新たな価値の創造

浮世絵技法の復元の難しさ

版下

色版

桃遷、生田敦盛

主版　立命館ARC所蔵 GNo:arcMD01-0015

版画　立命館ARC所蔵 arcUP4142

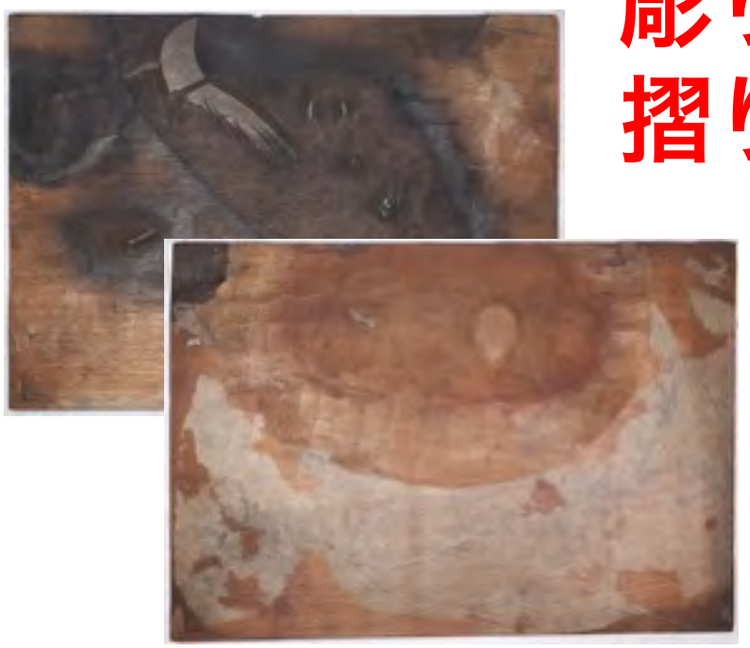
歴史的遺産であるため、破壊的分析が出来ない

浮世絵技法の復元の難しさ

版下

色材？
版木？
用紙？

色版



彫りの技術？
摺りの技術？

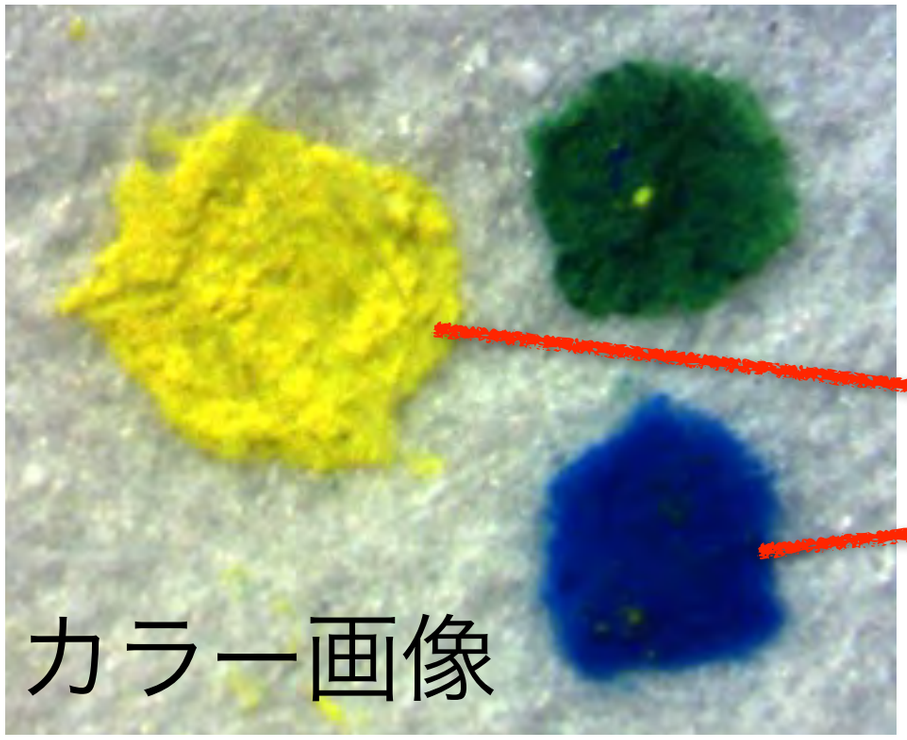
桃遷、生田敦盛

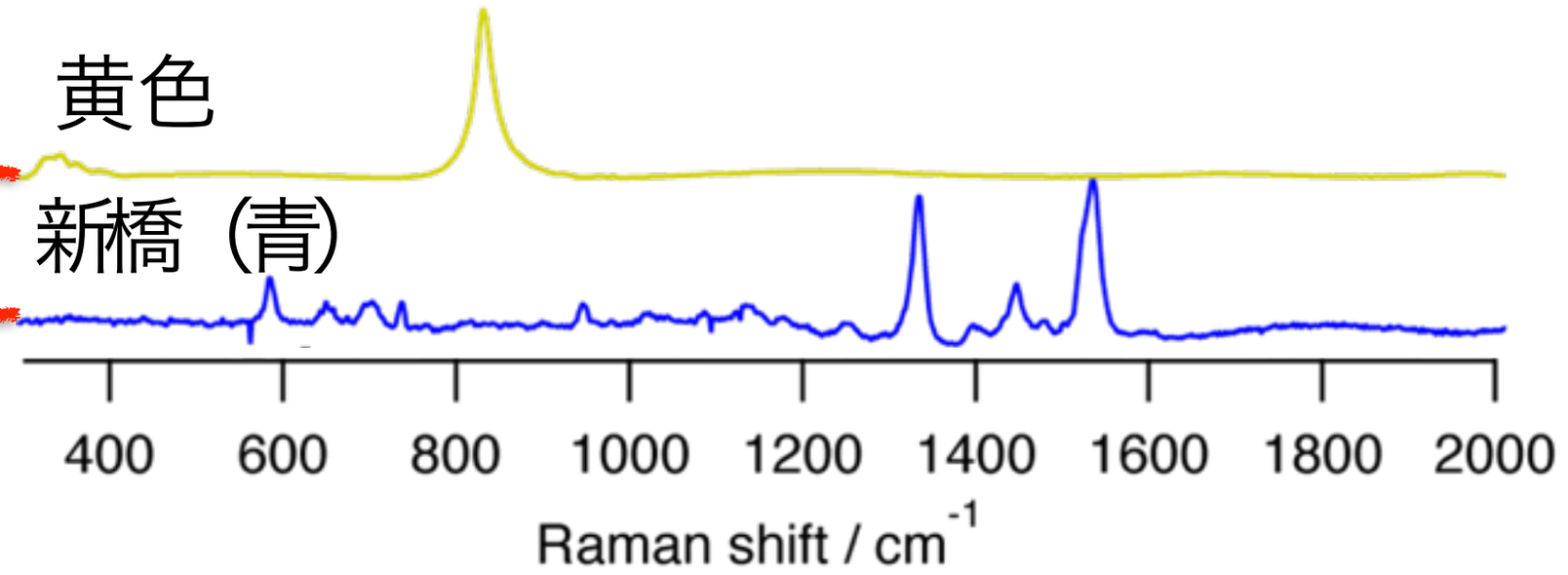
主版　立命館ARC所蔵 GNo:arcMD01-0015

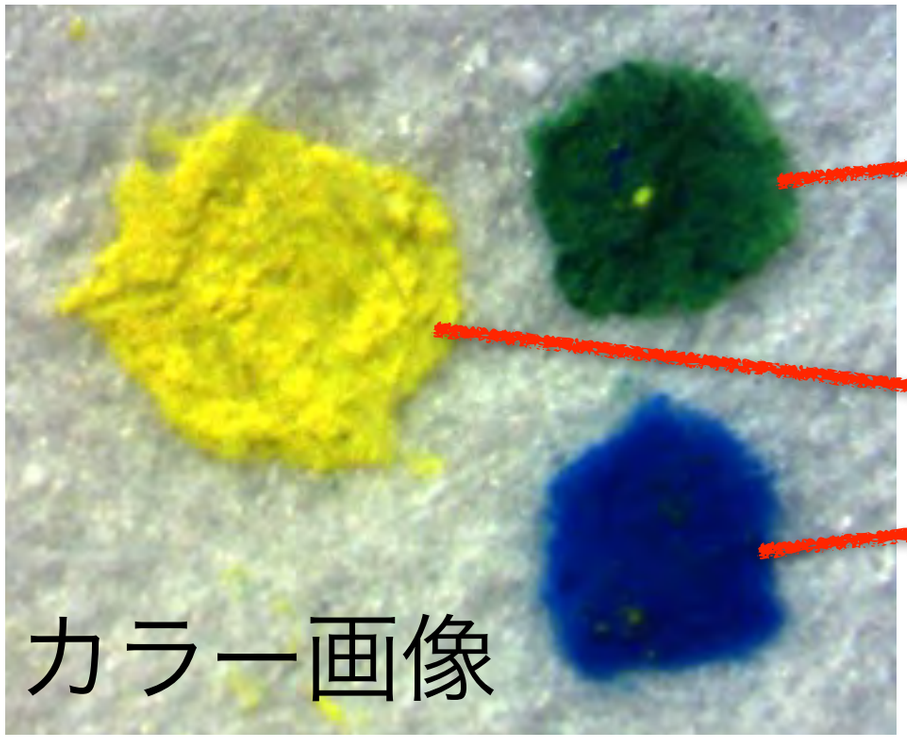
版画　立命館ARC所蔵 arcUP4142

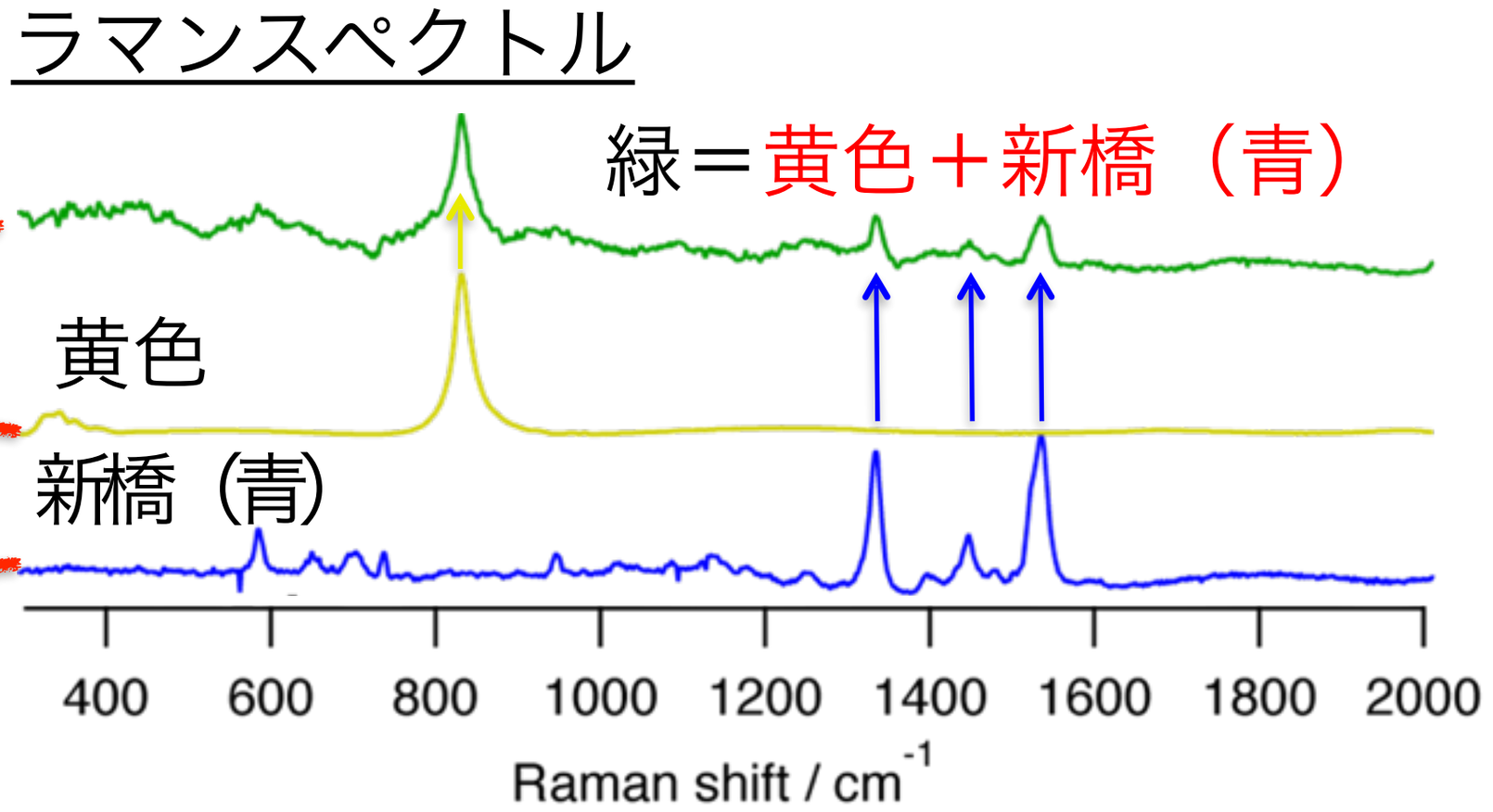
歴史的遺産であるため、破壊的分析が出来ない

ラマンスペクトル

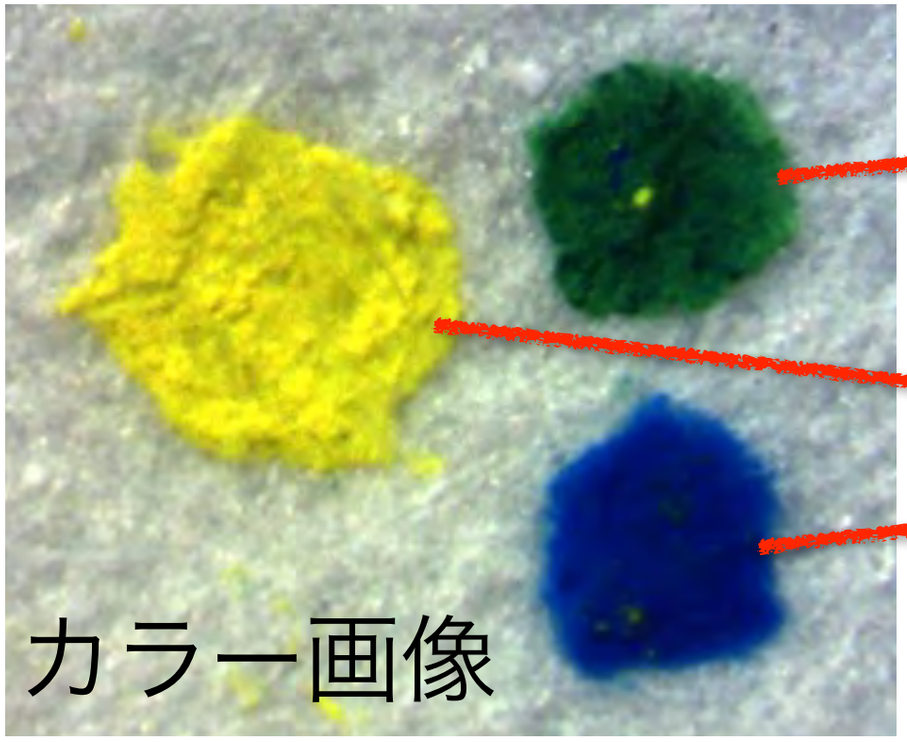

カラー画像

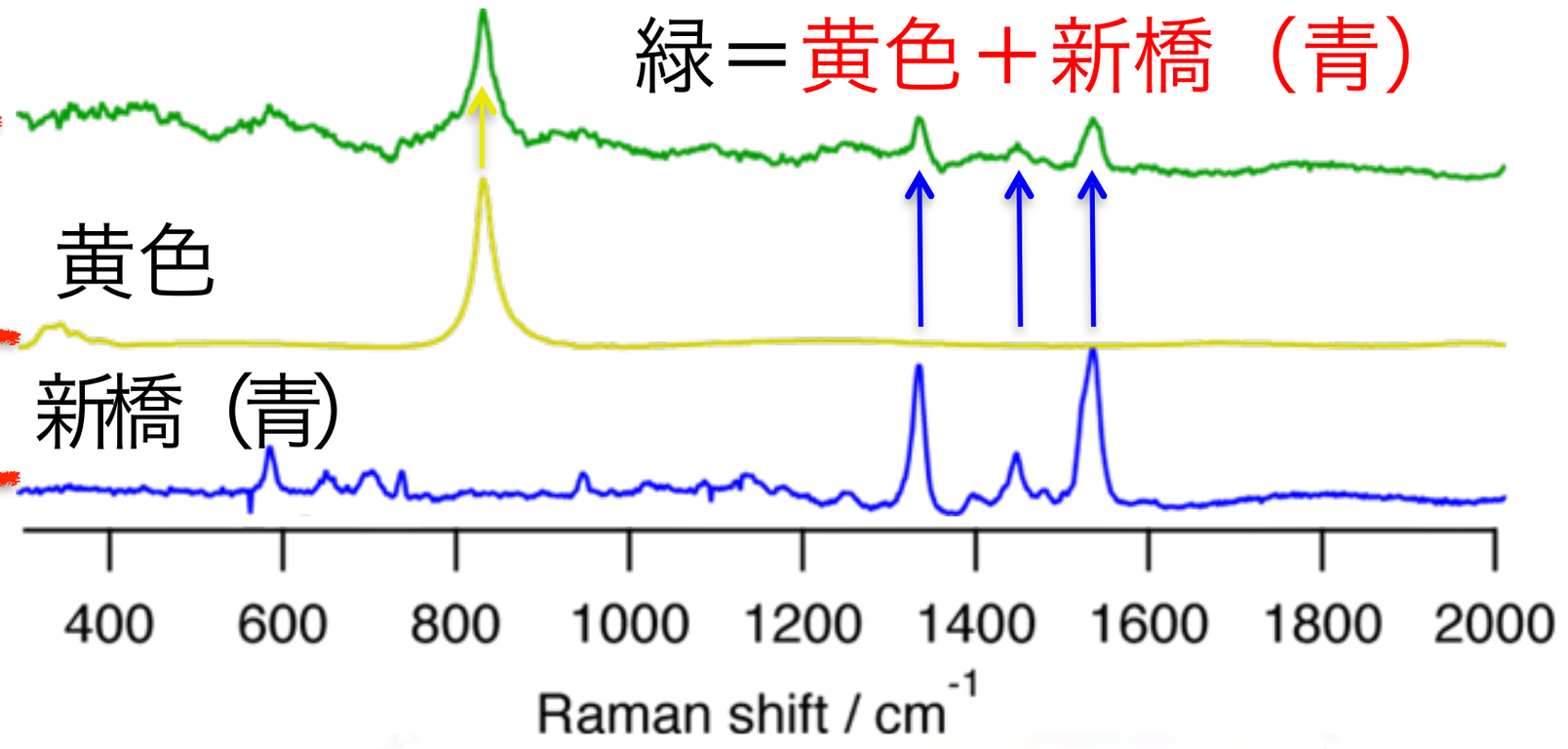

黄色
新橋（青）
400
600
800
1000
1200
1400
1600
1800
2000
Raman shift / cm-1

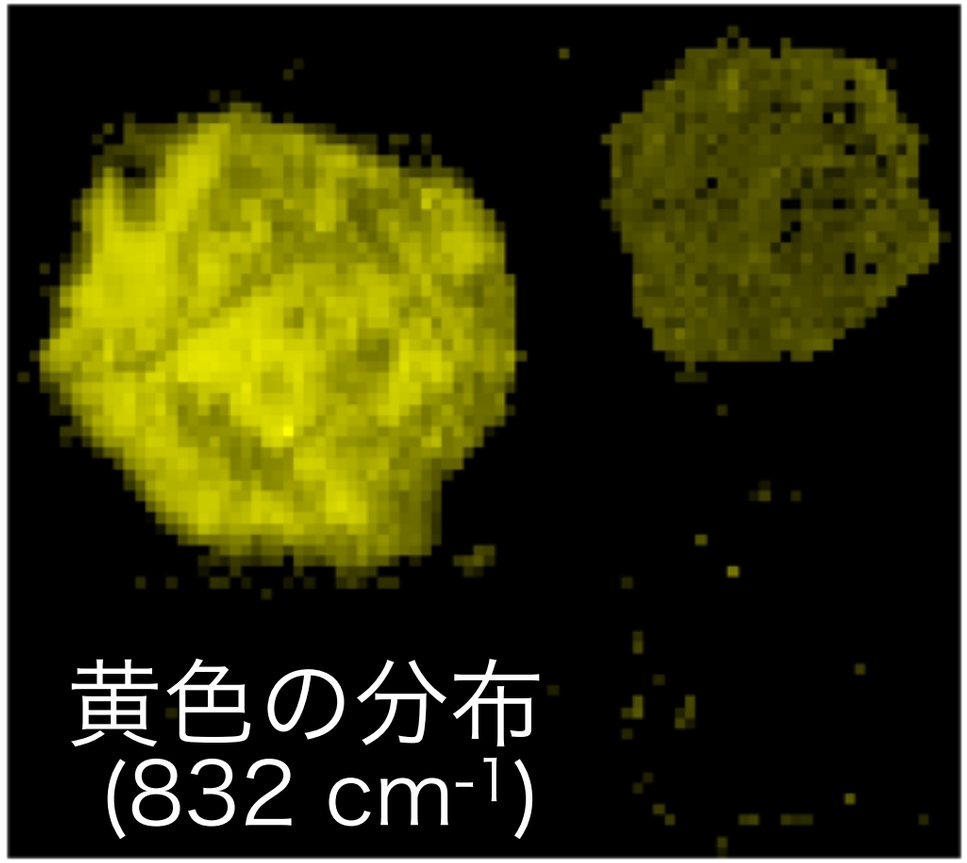

カラー画像

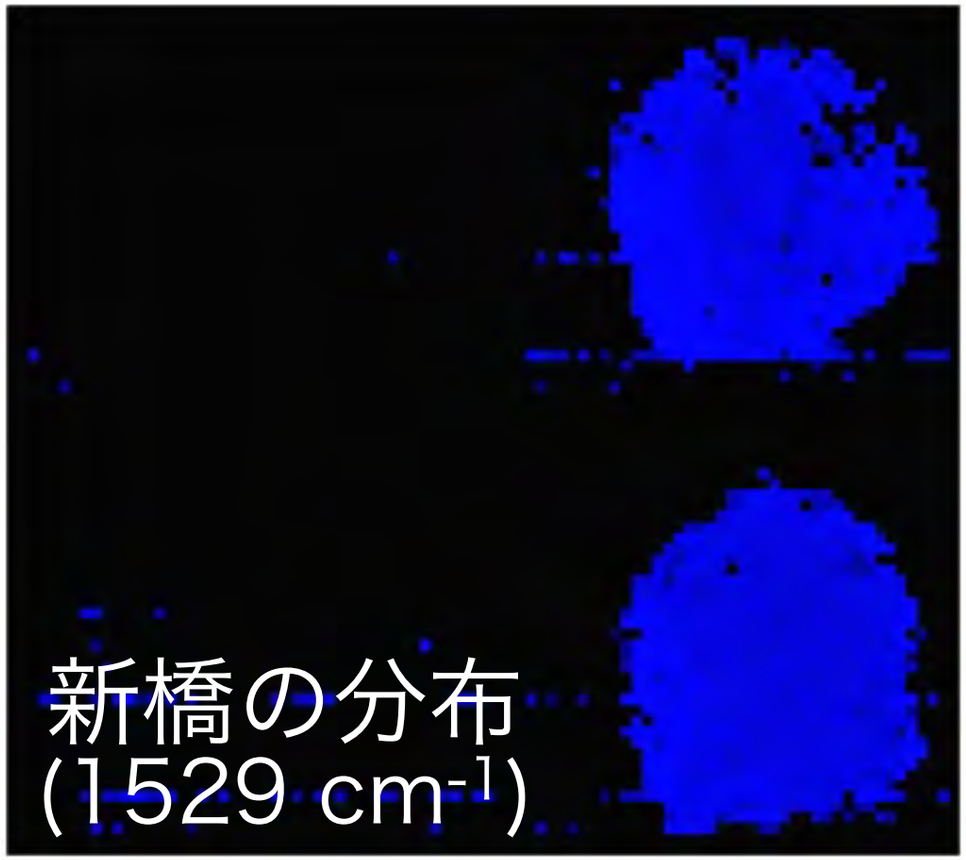

ラマンスペクトル
緑＝黄色＋新橋（青）
黄色
新橋（青）
400
600
800
1000
1200
1400
1600
1800
2000
Raman shift / cm-1

ラマンスペクトル







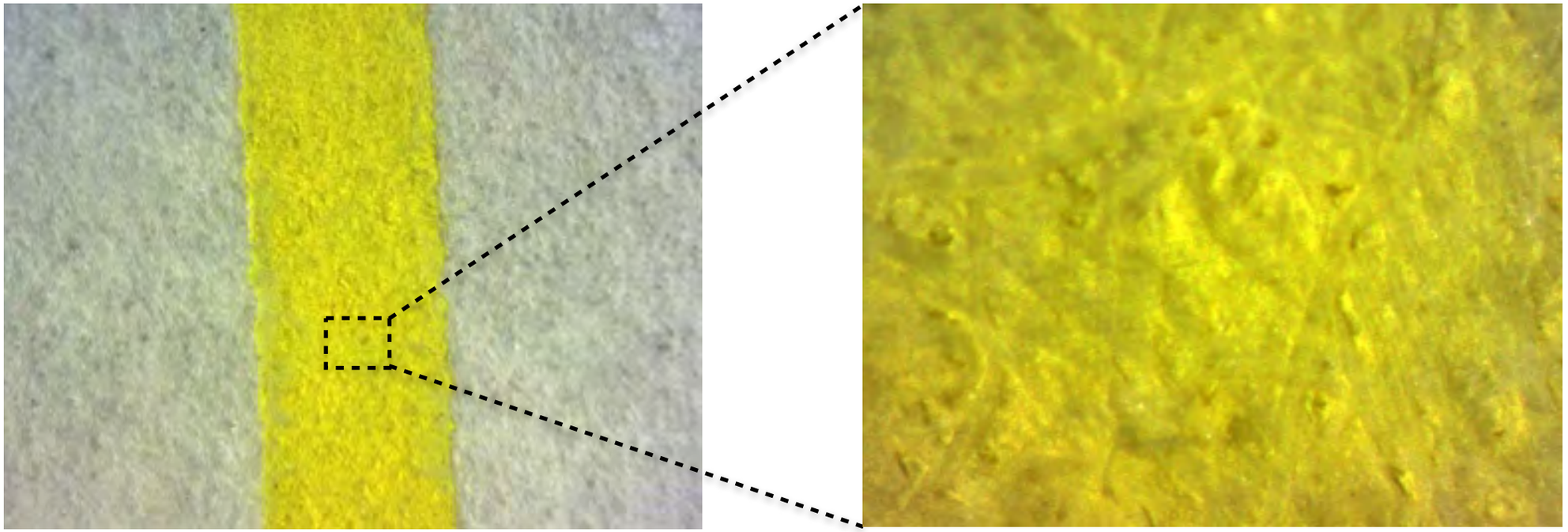


ラマン散乱分光法で「分子構造・分子分布分析」が可能

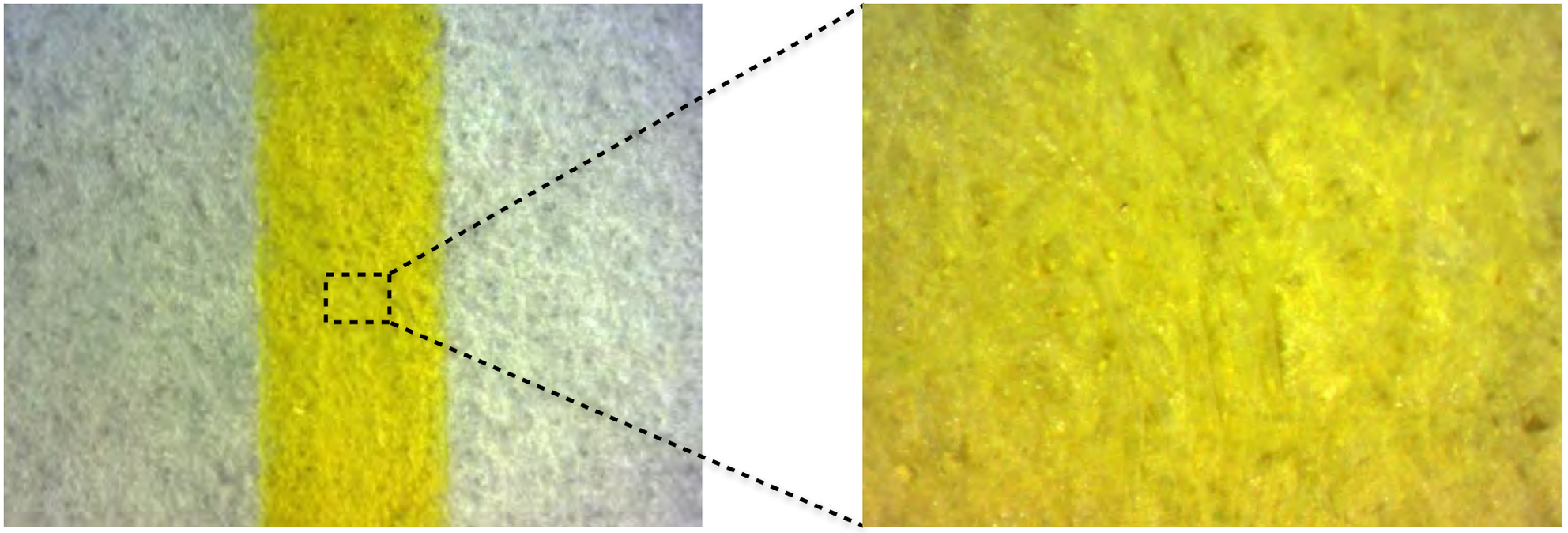
カラー画像による摺り技術の推定？

竹笹堂・摺師　永井大規作

黄色
のみで
摺り



黄色＋膠
で摺り

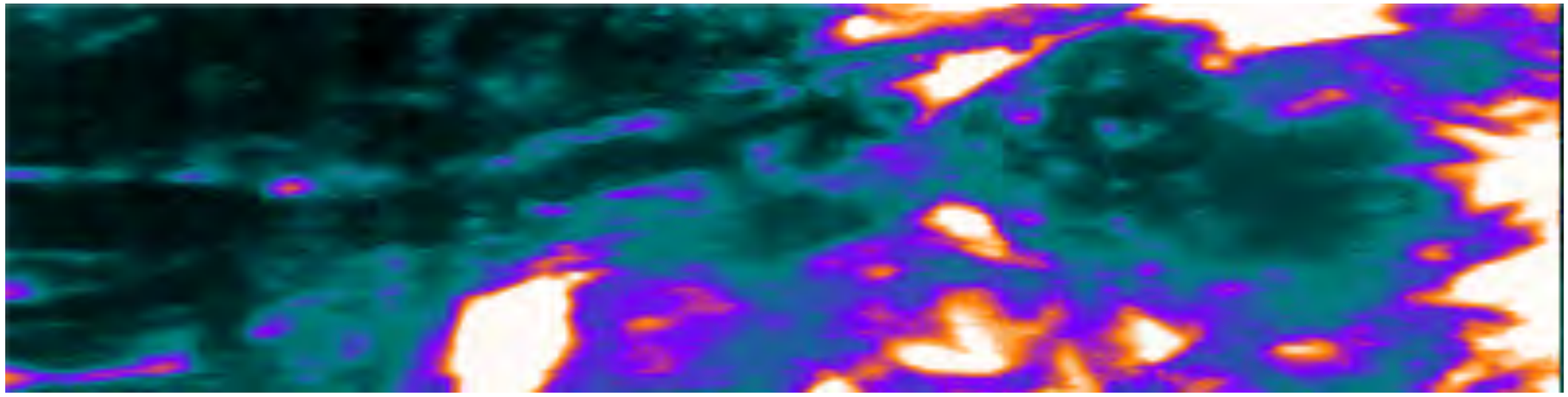


カラー画像のみでは、摺り技術の違いは明確でない

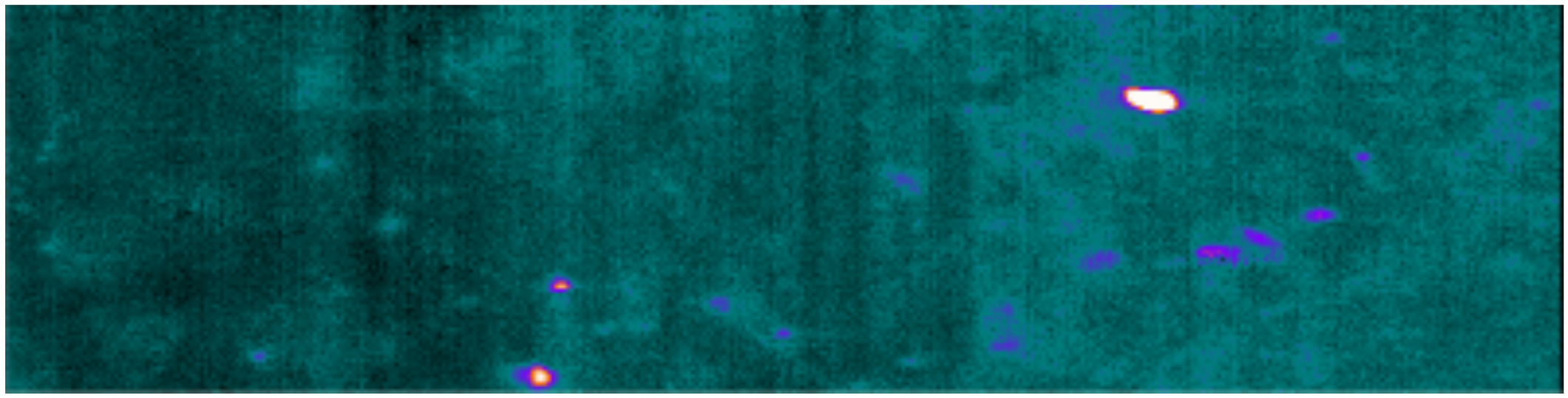
ラマン散乱分光法による摺り技術の推定

観察波数：832 cm^{-1}

竹笹堂・摺師　永井大規作



黄色色材のみで摺り　　→ 色材が**不均一分布**



黄色＋膠で摺り　　→ 色材が**一様分布**

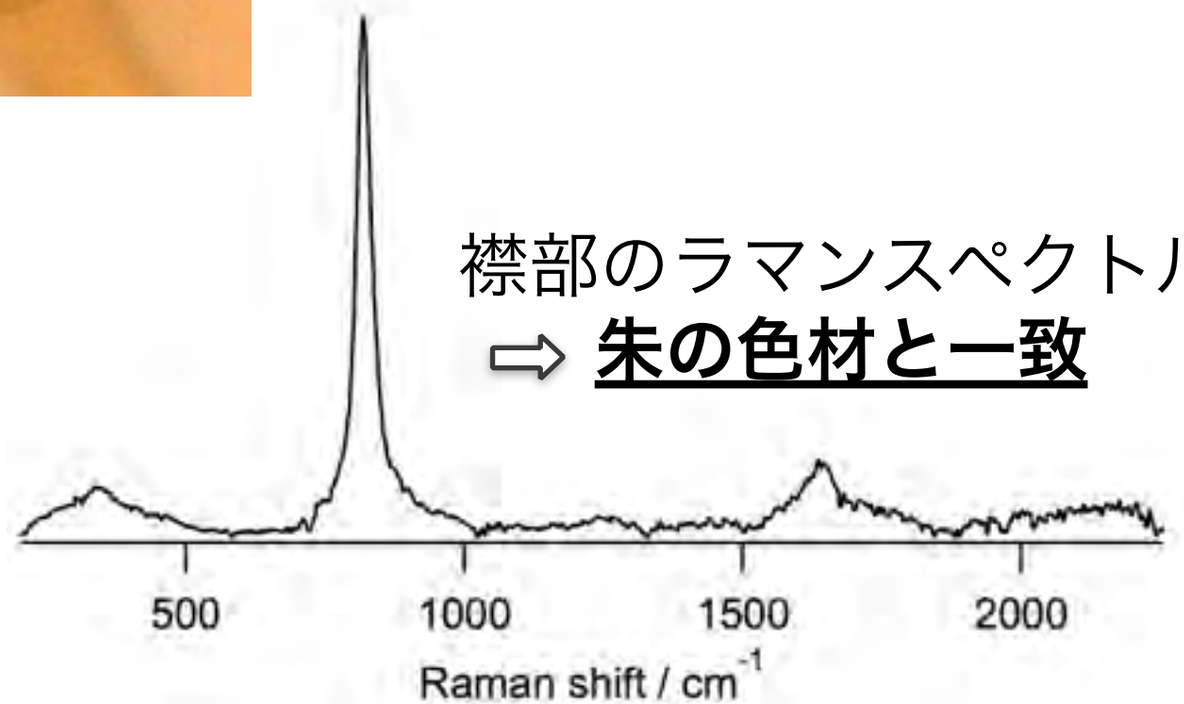
ラマン散乱分光法による色材分布観察

→ 摺りの状態を高いコントラストで観察可能

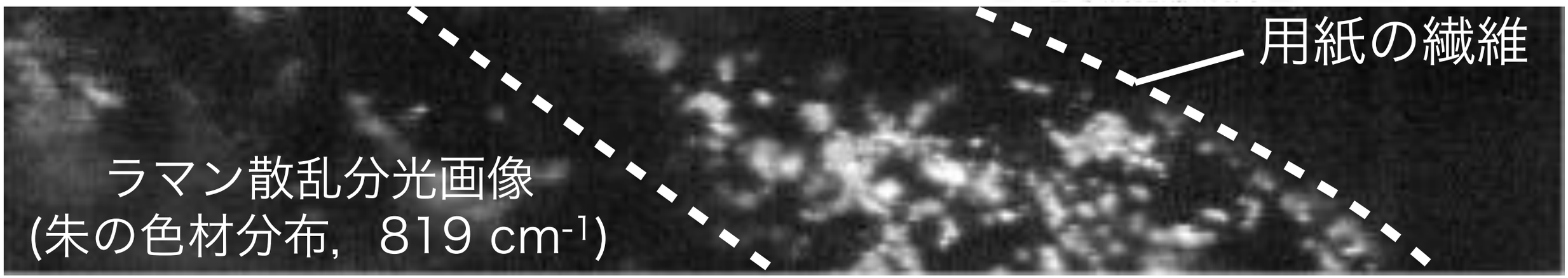
ラマン散乱分光法による摺り技術の推定

団扇をもつおひさ、竹笹堂製作

<u>カラー画像</u>
用紙繊維上の
色材分布は計測困難



襟部のラマンスペクトル
⇨ **<u>朱の色材と一致</u>**



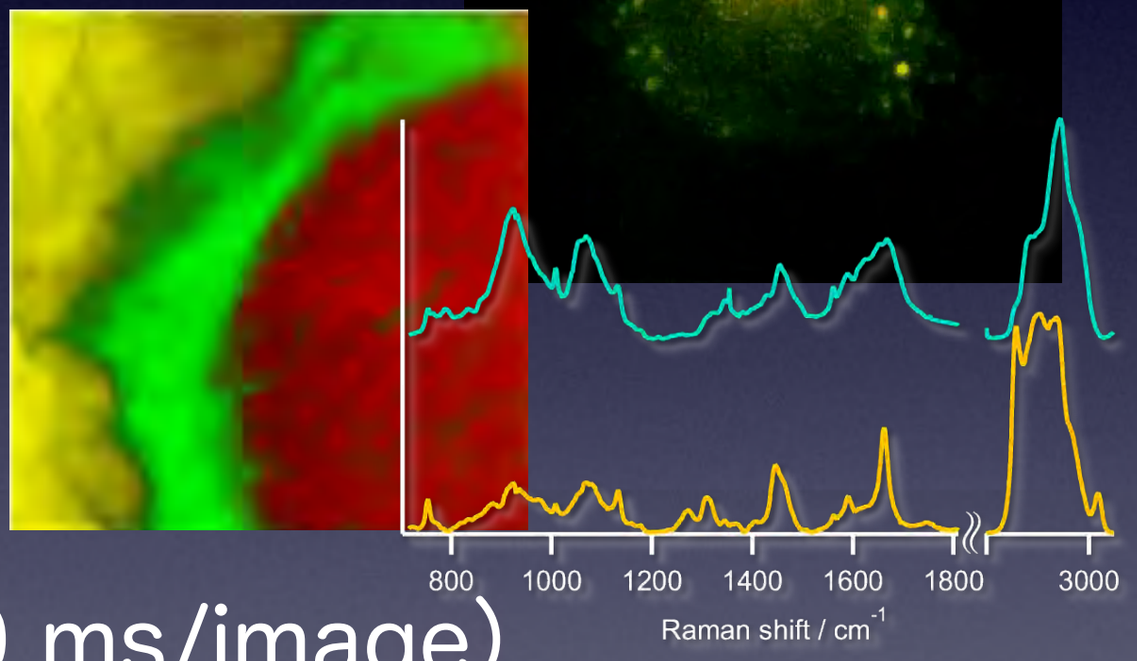
紙の繊維レベルでの色材の分布を観察可能 → 摺り技術を反映？

Summary

顕微ラマン散乱分光法による
分子イメージング法

- ✓ 無染色・無固定に計測可能
- ✓ 分子選択的イメージング
- ✓ 分子構造の推定
- ✓ 分子配向解析
- ✓ ＋非線形光学

→　高速イメージング（10 ms/image）



医学・生物学を中心として
新たな計測ツール

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Division of Cardiovascular and Pediatric Cardiovascular Surgery
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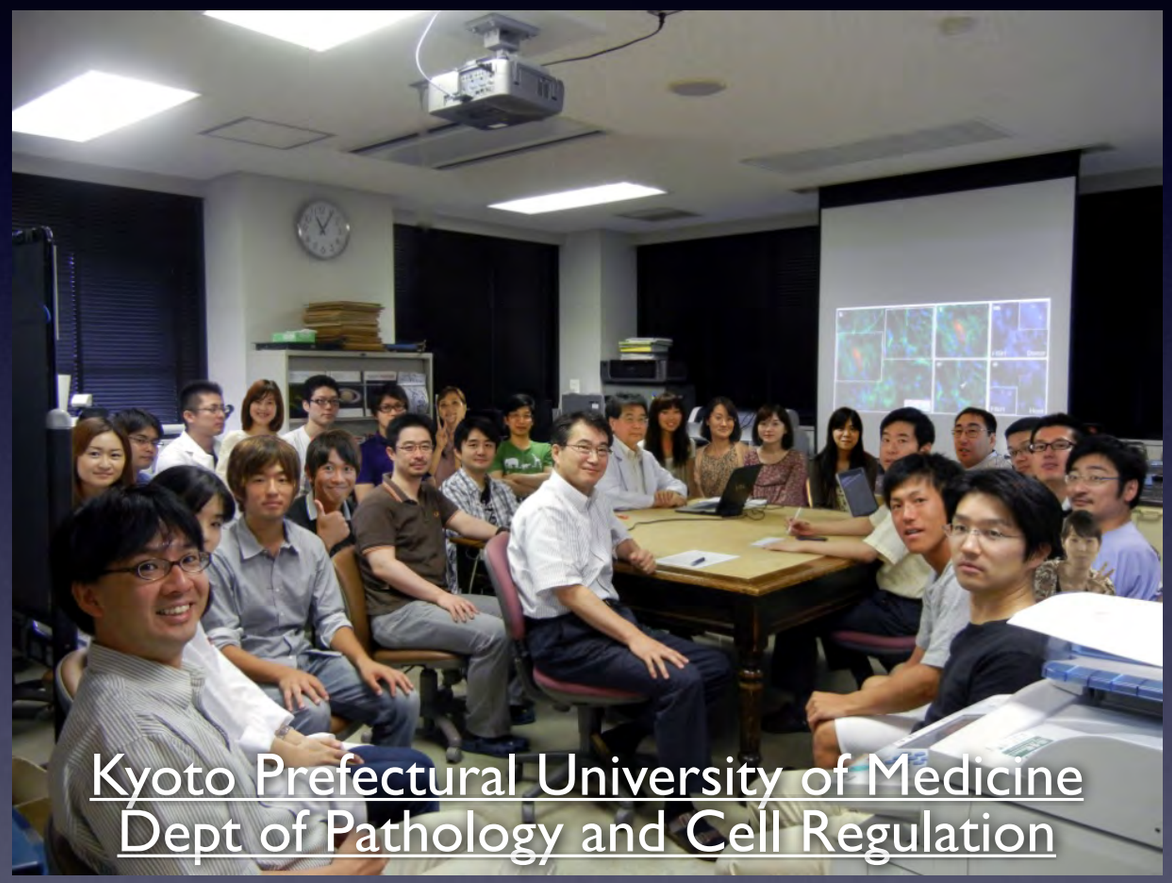
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