Towards Ultrasensitive, High-speed Diagnostics: Nanoscience Meets Health Care

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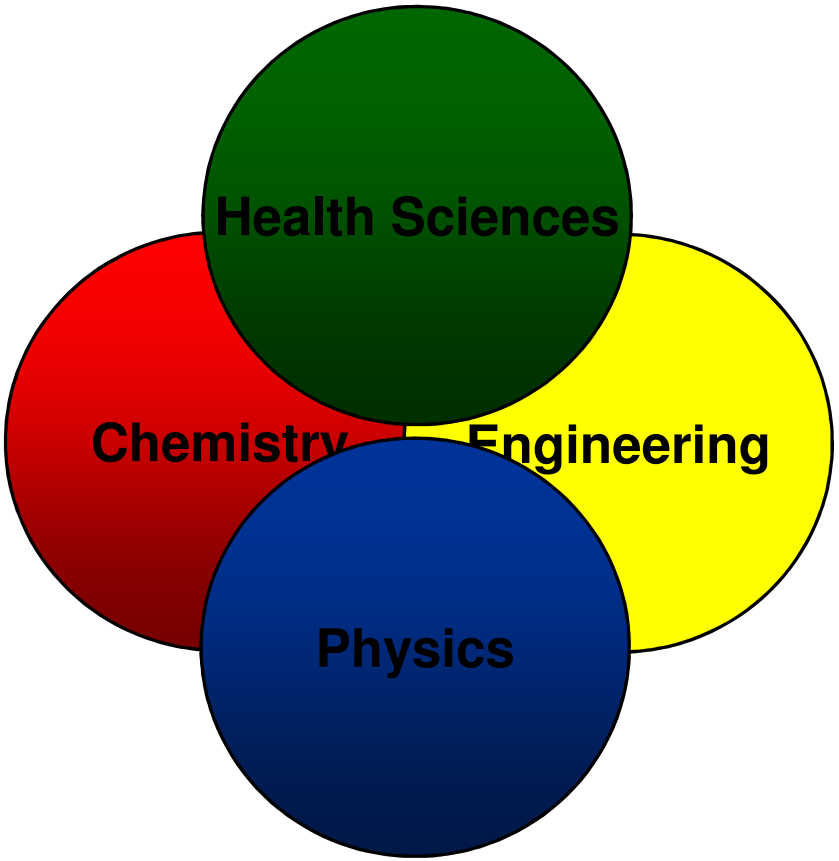
R21-EB016925
Kerith Foundation
Center on Aging (UofU)

Disease Diagnostics: Team Sport



- Goals and Challenges in Diagnostic Tests
- Nanodiagnostics - Surface-enhanced Raman Scattering (SERS)
  - Sample Pretreatment
  - Solid Phase MicroExtractions (SPME)
- What Are We Measuring Anyway?

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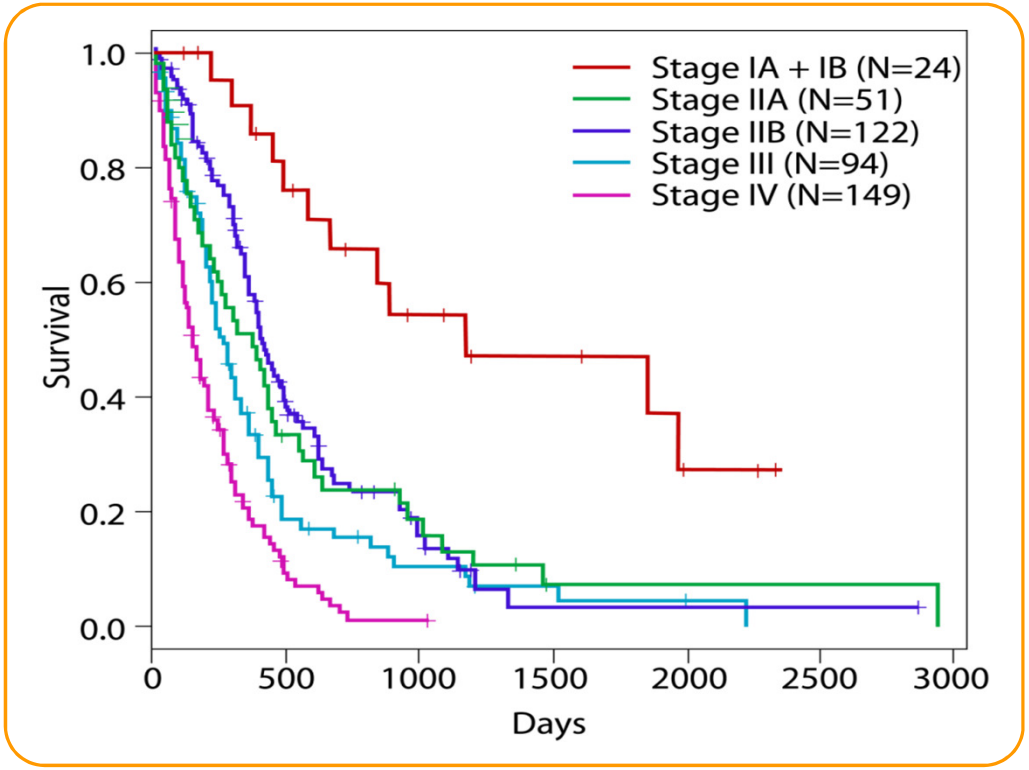
Personalized healthcare – diagnostics for everyone

- Dave is a blue-collar worker in early stages of pancreatic cancer (PC), but is not aware of his affliction.
- PC is the fourth most common cause of cancer deaths in men and women.
- PC has a 4% five-year survival rate, and its onset is asymptomatic.
- Dave has yet to exhibit clinically suspect signs and will not be diagnosed until there is obvious need to seek medical care.
- Dave's cancer is unfortunately at an advanced stage, and he is no longer eligible for tumor resection. He then receives palliative care, succumbing to his cancer within six months.

Early diagnosis is paramount

Stage-specific survival: 440 pancreatic ductal adenocarcinoma (PDA or simply PC) patients



Detection when tumor is small and confined to pancreas (IA+IB) improves survival rates.

Current staging does not optimally stratify patients.

Novel paradigms (multiplexing) needed for early diagnosis and for better treatment options.

Kaplan-Meier Survival Curve: University of Utah SOM and HCI

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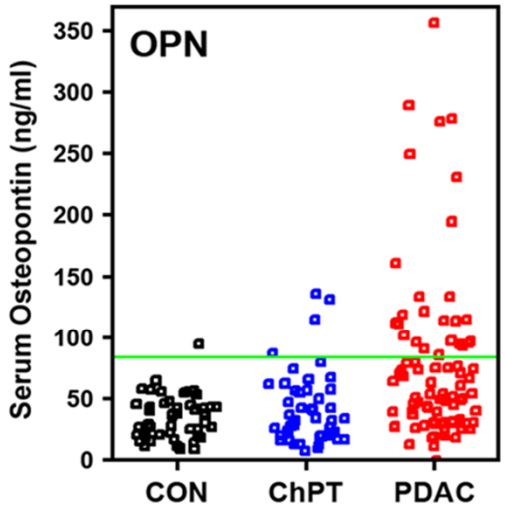
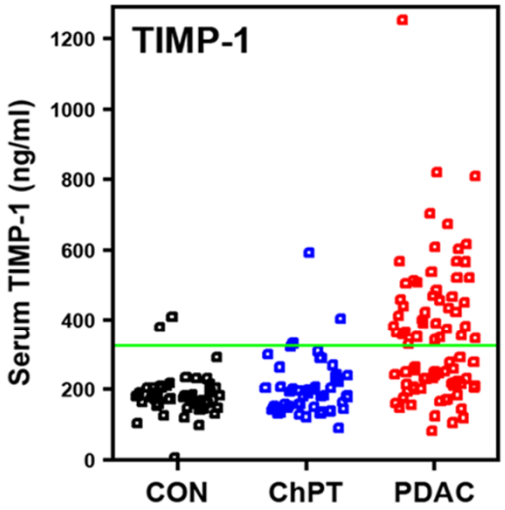
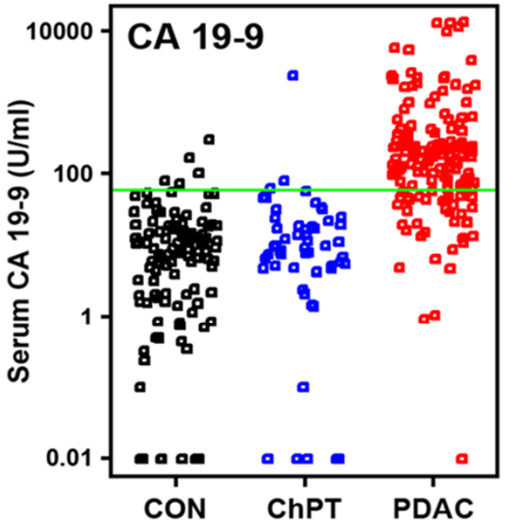
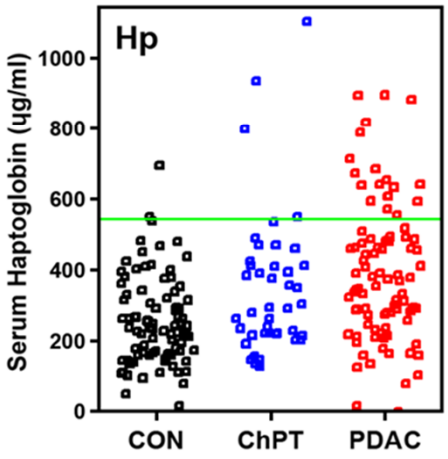
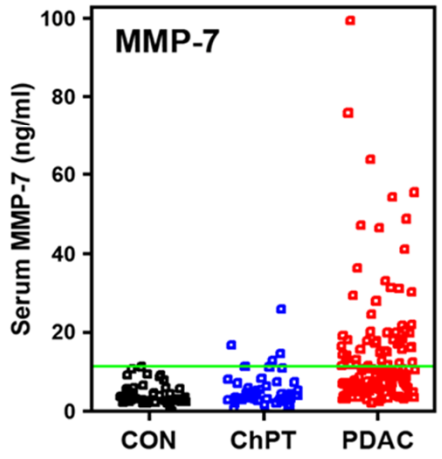
Relative distributions for PC diagnostic biomarkers











Large overlap of control (CON), chronic pancreatitis (ChPT) and pancreatic ductal adenocarcinoma (PDAC - PC) in patients.

Green lines indicate 95% specificity threshold.

M. A. Firpo, et al. *Proc. Pancreatic Cancer: Progress & Challenges,* Am. Assoc. Can. Res., Reno, NV, 2012.

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Definitions and Binary Classifiers

Analytical sensitivity: slope of calibration plot

Limit of detection (LOD): analyte concentration with response equal to the blank signal plus 3× the standard deviation of the blank signal

Clinical sensitivity: fraction of infected people identified being infected
It equals the number of TPs vs. number of TPs and false negatives (FNs).

$$\text{Sensitivity} = TP/(TP + FN)$$

Clinical specificity: fraction of uninfected people correctly identified uninfected
It equals the number of True Negatives vs. number of TNs and false positives (FPs).

$$\text{Specificity} = TN/(TN + FP)$$

PC's clinical sensitivity/specificity problem

Asymptomic - The Need to Test Everyone

- **Population of 100M individuals -50 years or older tested for PDA**
- **Prevalence: ~4 in 10,000 or 40,000 in a population of 100M**
- **Assume 90% test sensitivity and specificity**

	Binary Classifier: Condition	
	Positive Patients	Negative Patients
Positive test outcomes	True positive (TP = 36,000)	False positive (FP = 9,996,000)
Negative test outcomes	False negative (FN = 4,000)	True negative (TN = 89,964,000)
Total	40,000	99,960,000
	Sensitivity = TP/(TP+FN) = 90%	Specificity = TN/(FP+TN) = 90%

The false positive problem

100,000,000 Subjects Tested

4 in 10,000 Prevalence

40,000 with PDA | 99,960,000 without Disease

Test Sensitivity & Specificity

36,000 TP	*4,000 FN	90%	89,964,000 TN	†9,996,000 FP
39,600 TP	400 FN	99%	98,960,400 TN	999,600 FP
39,960 TP	40 FN	99.9%	99,860,040 TN	99,960 FP

* The false negative problem: patients fail to receive treatment

† The false positive problem: (1) severe patient stress
(2) $5.5 billion annual cost for a single CT follow-up screen based on 2011 Medicare reimbursement rate of $554/CT

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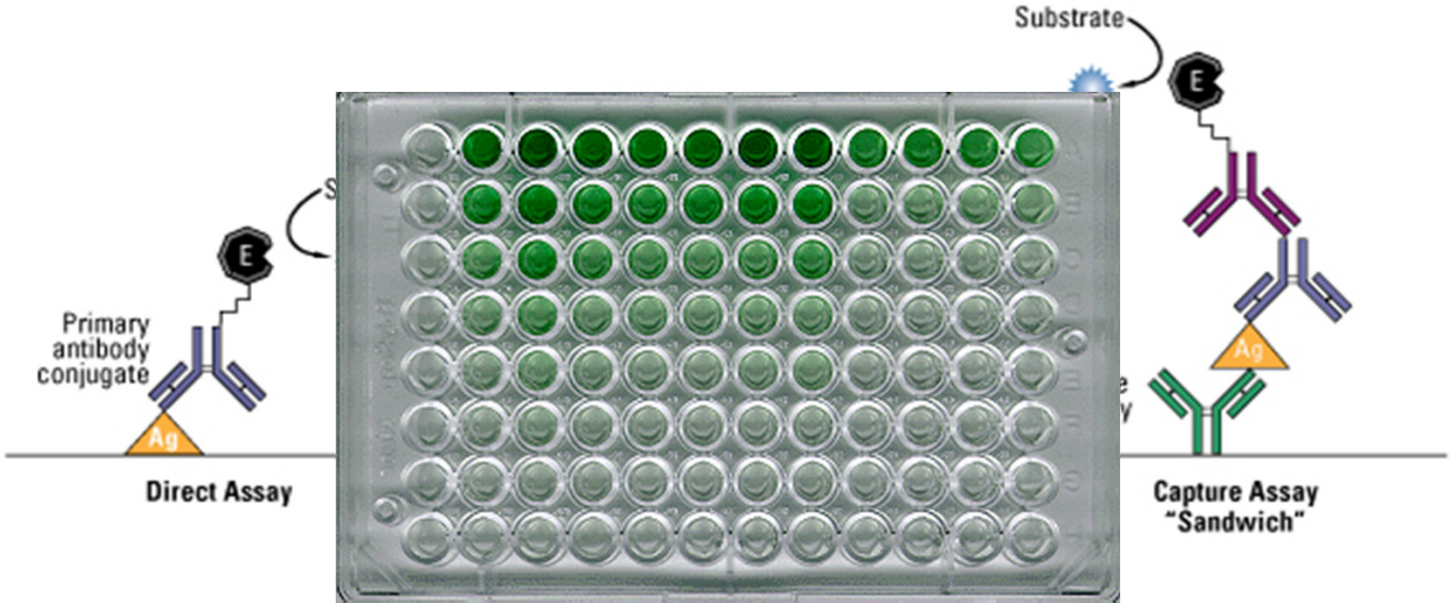
Target Attributes for Advanced Diagnostics

- Multiplexing
  - Everyone is different
  - Signal overlap at multiple addresses: cross reactivity and/or non-specific binding
  - One marker may be sufficient for some diseases (tuberculosis)
  - Multiple marker signature may be needed for other diseases (pancreas cancer)
  - Low limits of detection (LOD)
  - Sample volume: sample scarcity
- Instrumentation
  - Simple, cost-effective
  - Short time to results
- Complex sample matrices (more later)

C. Haglund et al. *Br J Cancer*. **1986**, *53(2)*, 197-202.

The Gold Standard: ELISA

- Enzyme-linked Immunosorbent Assay (ELISA)
  - Antibody: specific capture of target antigen
  - Solid phase (sorbent): wash off of material not specifically captured
  - Enzymatic amplification: transforms capture into quantifiable visible color



Adapted from C. Roth, Rutgers University

SERS: Robust Hardware Platform

Sensitivity

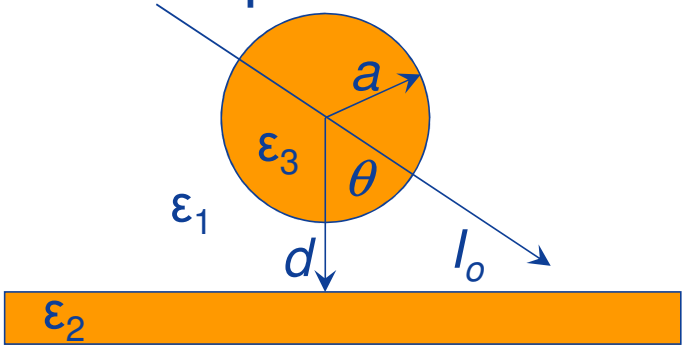
Pico to femtomolar detection

Multiplexing

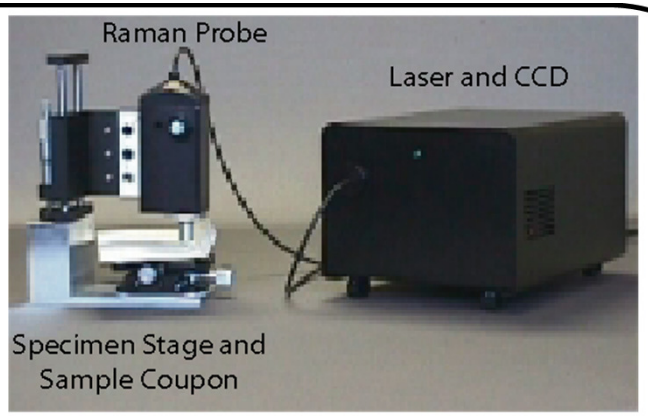
Raman bands 10-100 times narrower than fluorescence

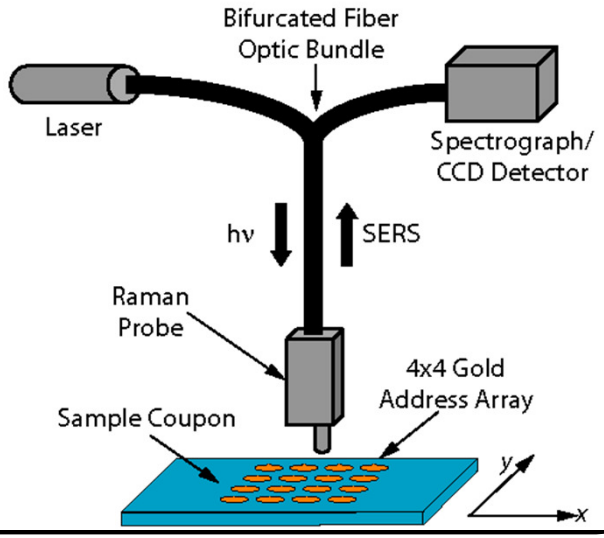
Hardware Simplicity

Excitation wavelength is substrate-dependent



Schatz & Van Duyne, Hndbk Vibrational Spec. 2002, 1.

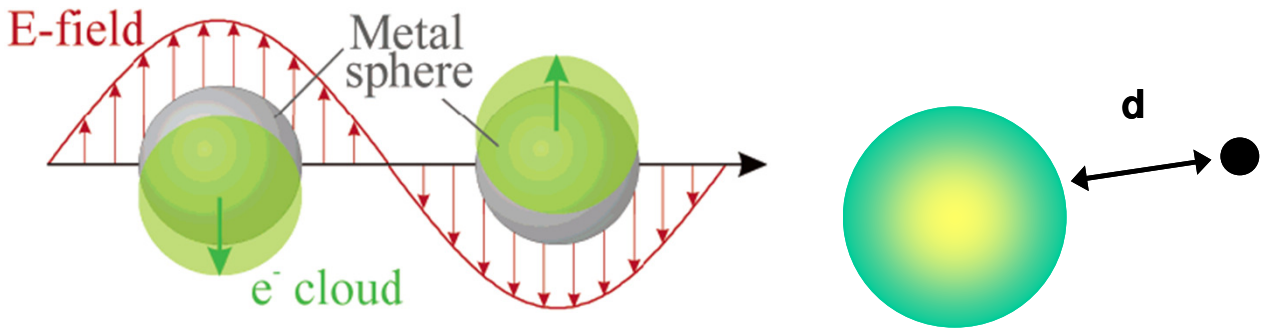




Origin of Surface Enhanced Raman Scattering (SERS)

Electromagnetic Enhancement (dominant)

- Incident radiation is resonant with surface plasmon of conductors
  - plasma resonance: oscillation of conduction electrons
  - nanoparticles smaller than excitation wavelength

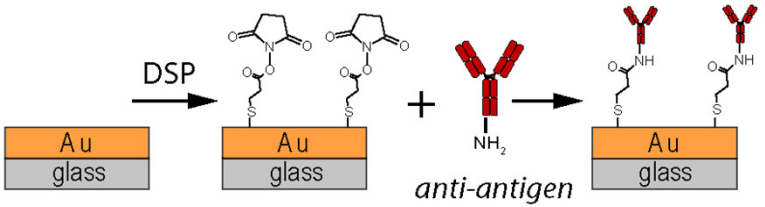


- Raman signal increased by 10^6-10^7
- Enhancement decays by d^{-10}

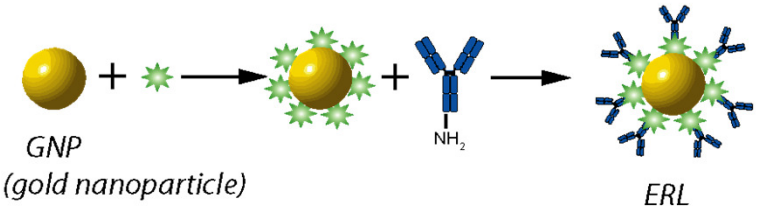
Kneipp, Chem. Rev. 1999, 99, 2957; Moskovits, Rev. Mod. Phys. 1985, 57, 783; Kelly, J. Phys. Chem. 2003, 107, 68.

SERS: Extensible Assay Platform

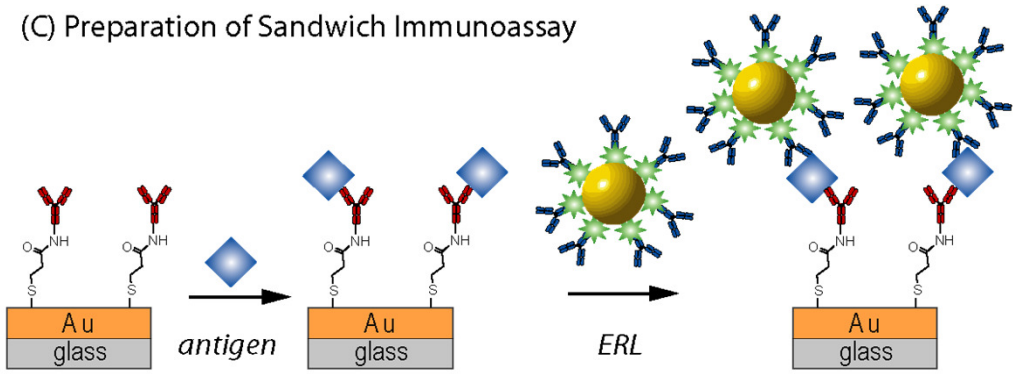
(A) Preparation of the Capture Substrate



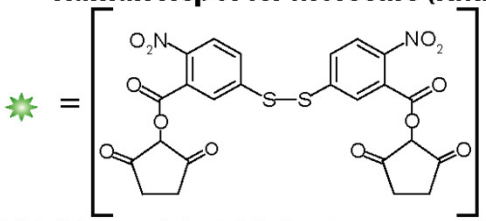
(B) Preparation of the Extrinsic Raman Labels (ERLs)



(C) Preparation of Sandwich Immunoassay

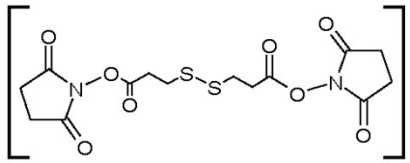


Raman Reporter Molecule (RMM)



dithiobis(succinimidyl nitrobenzoate (DSNB)

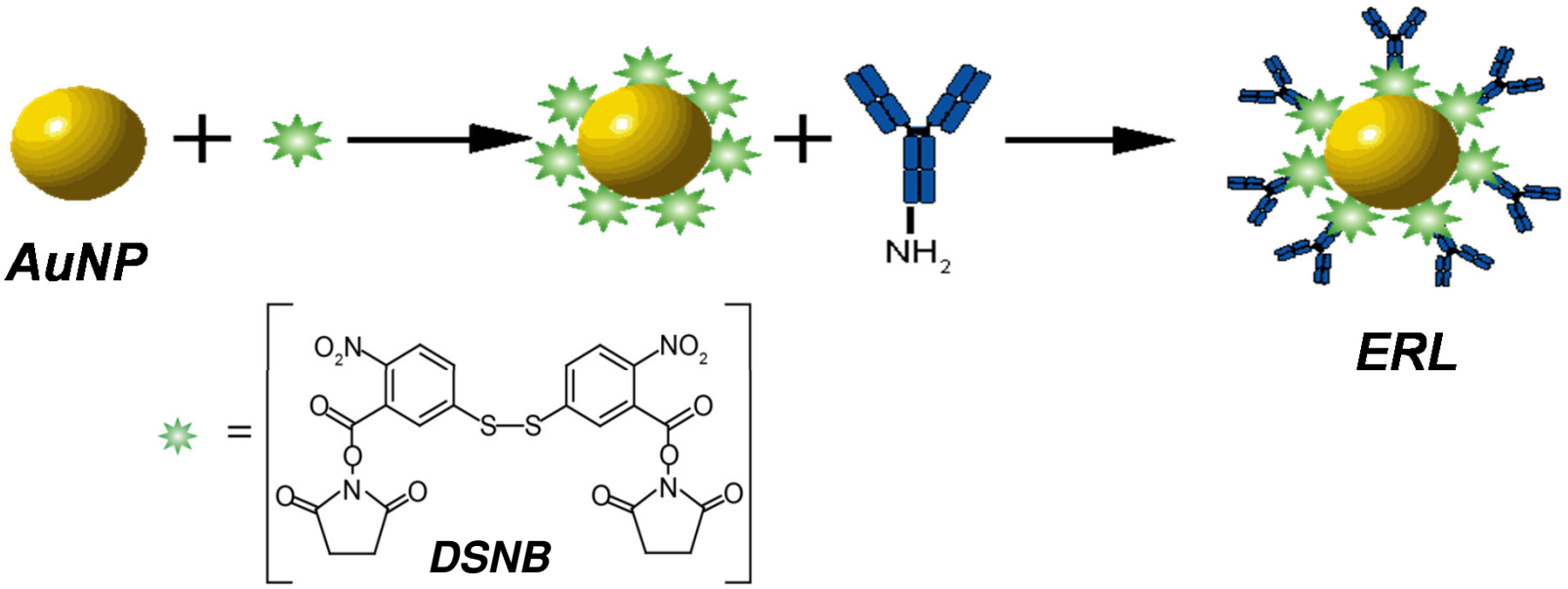
DSP =



dithiobis(succinimidyl propionate)

1. **20-µL sample**
2. **Rinse**
3. **20-µL suspension of ERLs**
4. **Rinse**
5. **Measure Raman spectrum**

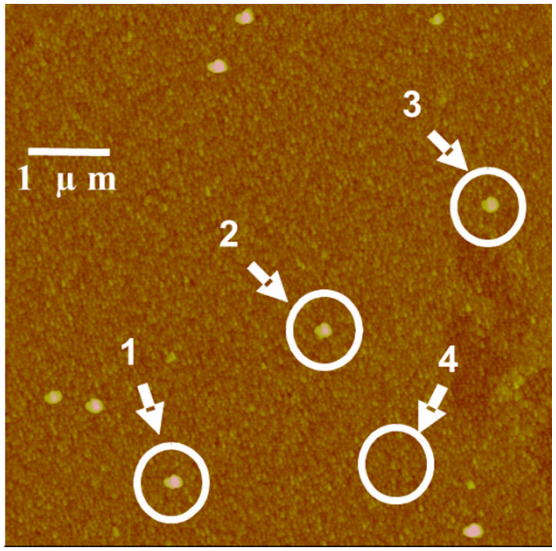
Extrinsic Raman Label (ERL) Preparation



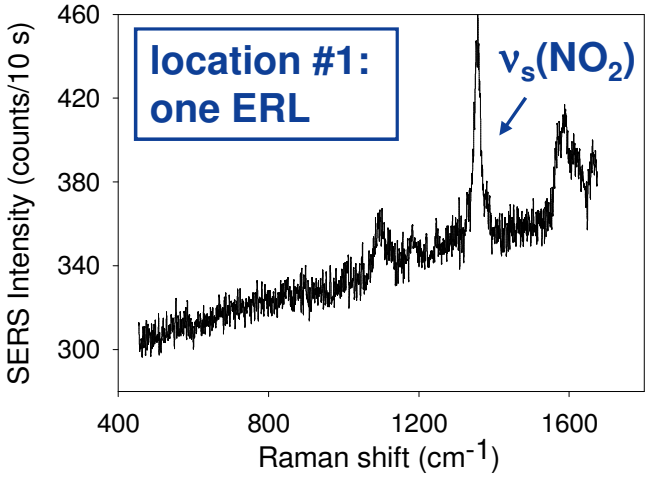
Self-assembled monolayer on Au
Scatterer close to Au surface
$4.0 \pm 0.2 \times 10^4$ RRMs per particle
Red excitation for Au NPs

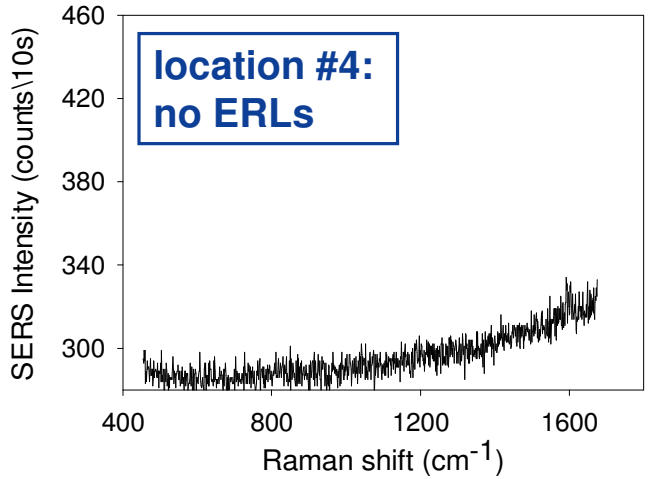
Intrinsically strong Raman scatterer: $\upsilon_s(NO_2)$
Small particle size dispersity
190 ± 20 mAbs per particle

SERS: Single Binding Event Detection



AFM: DSNB-coated ERLs tethered to aminoethanethiol adlayer on smooth Au





H.-Y. Park, R. J. Lipert, and M. D. Porter, "Single Particle Raman Measurements of Gold Nanoparticles Used in Surface-Enhanced Raman Scattering (SERS)-Based Sandwich Immunoassays," in Nanosensing: Materials and Devices; M. Islam and A. Dutta (Eds.), *Proc. SPIE*, **2004**, *5593*, 464-77.

Cancer Diagnostics: Pancreatic Cancer (PC) Markers[1,2]

LODS [ng/mL (pM)] for PC cancer markers in human serum

MARKER[3]	SERS	ELISA	SERS/ELISA
CA19-9	0.040 (0.20)	1.10 (5.5)	~30
MMP-7	0.003 (0.16)	0.041 (2.3)	~15

Data for MMP-7 and CA 19-9 in patient samples.[1]

Specimen	Antigen	Concentration (ng/mL)	
		SERS	ELISA
DIDM199	MMP-7	3.53 ± 0.12 (3.42%)[4]	3.91 ± 0.16 (4.24%)
DIDM30		4.77 ± 0.12 (2.60%)	4.36 ± 0.26 (5.91%)
DIDM17		1.92 ± 0.08 (3.97%)	2.79 ± 0.16 (5.90%)
DIDM199	CA 19-9	50.4 ± 2.9 (5.7%)	30.7 ± 2.0 (6.40%)
DIDM30		ND[5]	ND
DIDM17		ND	ND

MUC4 not detected with ELISA and Radioassays in sera, but is with SERS.[2]

(1) Granger, Porter et al., Analyst, 2013, 138, 410-16. (2) Wang, Porter et al., Anal. Chem., 2011, 83, 2554-61.
(3) MW: 210 kD for CA19-9; 29 kD for MMP-7. (4) %SD. (5) ND: not detected.

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Diagnosis - Public Health for Everyone

Sanura lives in a rural Kenya village, with her husband and 3 young children.

She has been complaining of general malaise and cough, finally traveling to the closet clinic 4 h away by bus to visit a care giver.

At the clinic, she provided a sputum sample for tuberculosis (TB) testing by acid fast bacteria (AFB) smear and culture.

Sputum microscopy was negative for AFB, and she was treated empirically with antibiotics for suspected pneumonia and returned home.

At two weeks, the culture grew Mycobacterium tuberculosis, but Sanura could not be located to begin proper treatment.

What can be done to give every Sanura a fighting chance?

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Tuberculosis (TB)

- TB has killed more people than any other pathogen in history
- TB is the world's deadliest infectious disease
  - 1.6 million deaths/year[1]
  - 30% of people globally are infected with latent TB
  - Economic burden estimated at $1-3T in next 10 years[2]

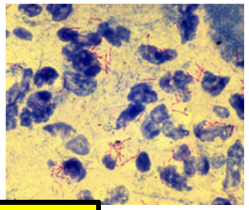
- WHO urges ban on TB blood tests (July 20, 2011)
- Diagnosis in epidemic regions relies on symptoms and sputum (phlegm) microscopy
- Today's methods cannot detect components of the TB organism for reliable early diagnosis of active nad/or latent infection
- 15-20% of TB infections are extrapulmonary only

[1]Global Tuberculosis Report, 2018, WHO, 2018. [2]WHO TB Diagnostics Economic Working Group (http://www.who.int/tdr/publications/documents/tbdi.pdf)

Today's TB Diagnostics Landscape

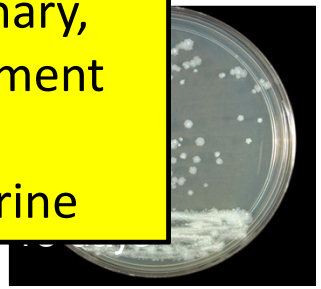
Sputum (Phlegm) Smear Microscopy

- Fast and low cost, but poor sensitivity (20-80%)



Culture

- Gold



Bottom Line: No all encompassing test for all forms of TB (pulmonary, extrapulmonary and latent) or for response to treatment

Our Approach: Markers of TB microorganism in blood serum and urine

Nucleic Acid (DNA) Amplification Tests (GeneXpert Mtb/RIF)

- High sensitivity in sputum; ~120 min
- Ineffective for specimens from children and HIV patients due to extrapulmonary infection
- Cost: Instrument: $17,000 USD (actual: ~$65k USD)
  Cartridge: $9.98 USD per test (actual: ~$100-200 USD)

Cochrane Database Syst Rev. 2013; www.finddiagnostics.org

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Ideal Attributes: Point-of-Need (PON) Diagnostics

Assay

- Simple/no sample preparation (matrix agnostic)
- Short sample-to-answer (<5 min)
- Accessible Cost (1-2 days of economic income – goal of $2-3 USD per test)
- No refrigeration needed to store reagents - break cold chain
- Multiplexed detection (disease signature)

Readout

- Lightweight: < 2 lbs (0.9 Kg)
- Battery or solar powered
- Ethernet or WiFi connectivity (telemedicine)
- Embedded software and database for Yes/No answer (easy-to-use)

High Clinical Accuracy

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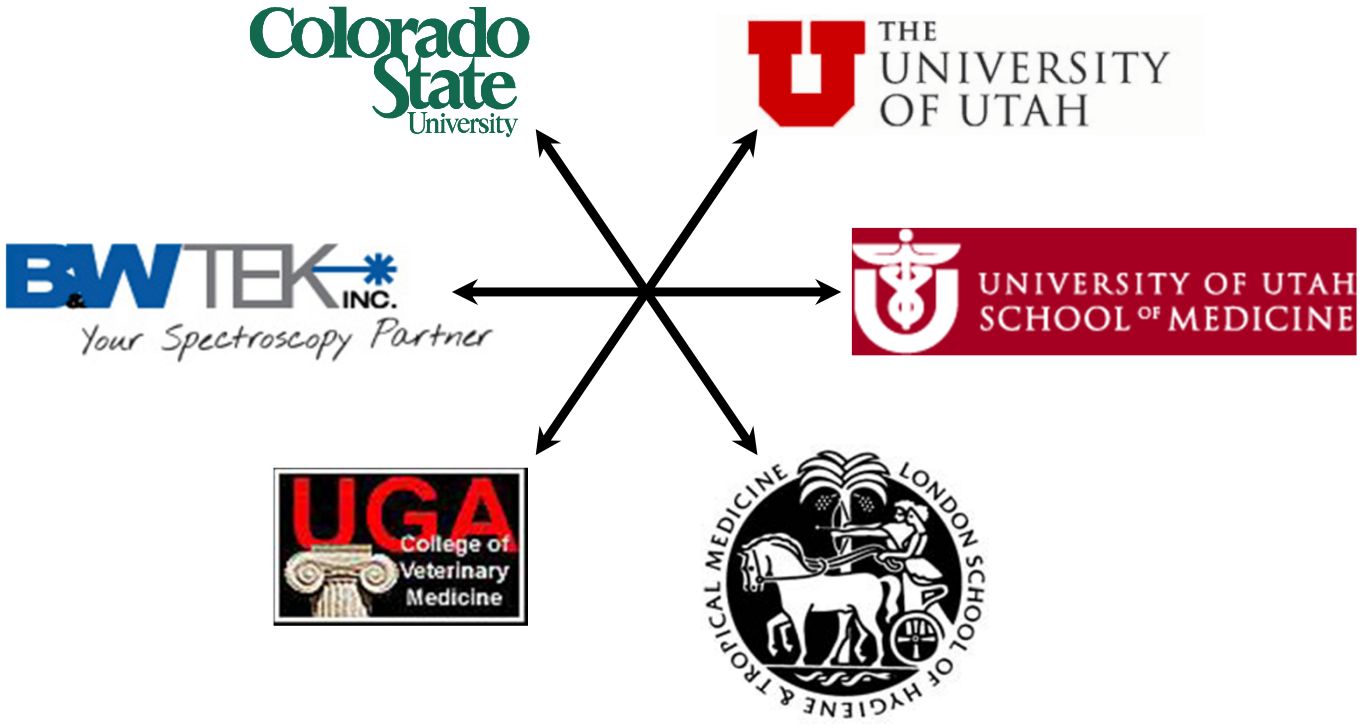
TB Diagnostics Consortium



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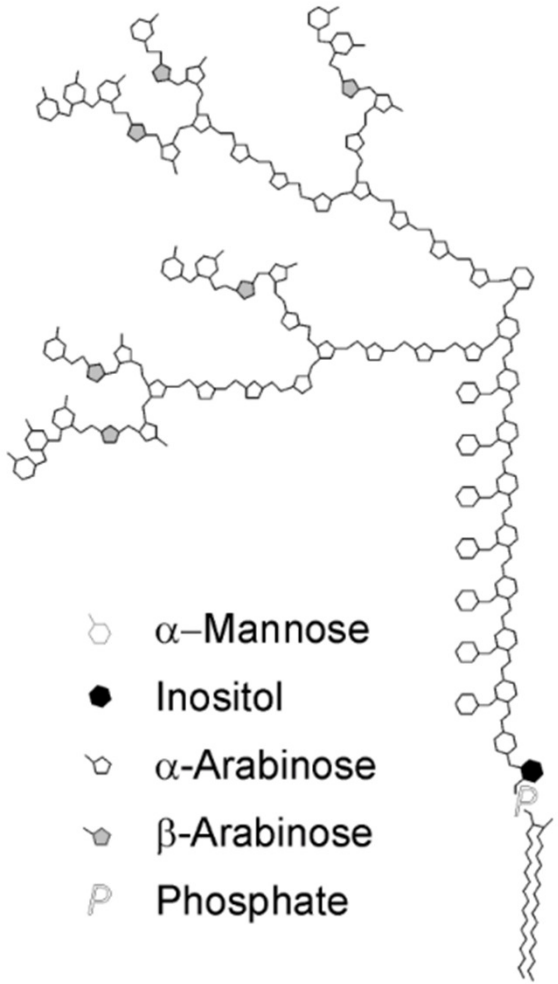
University of Medicine & Dentistry of New Jersey

DENVER HEALTH
Level One Care for ALL

Biomarker: Mannose-capped Lipoarabinomannan (LAM)

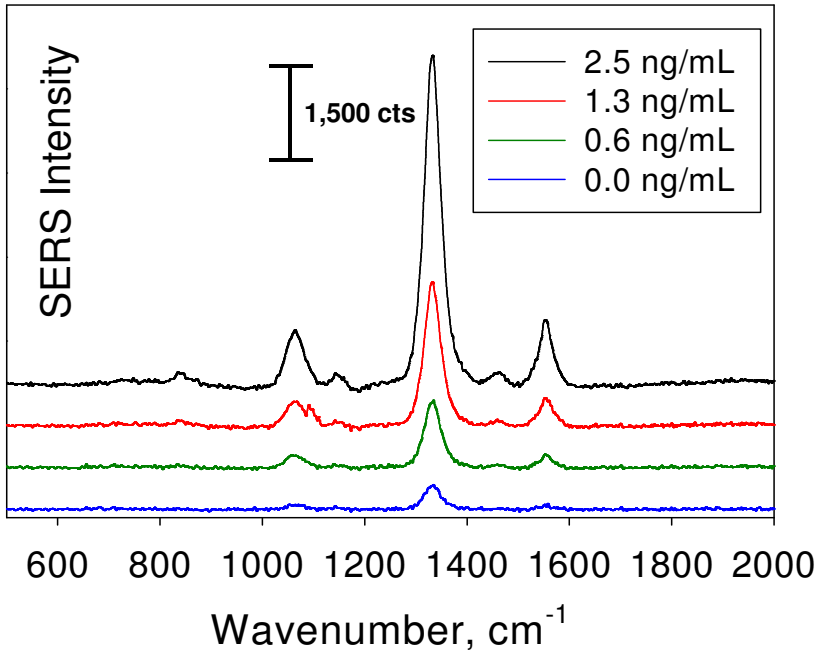


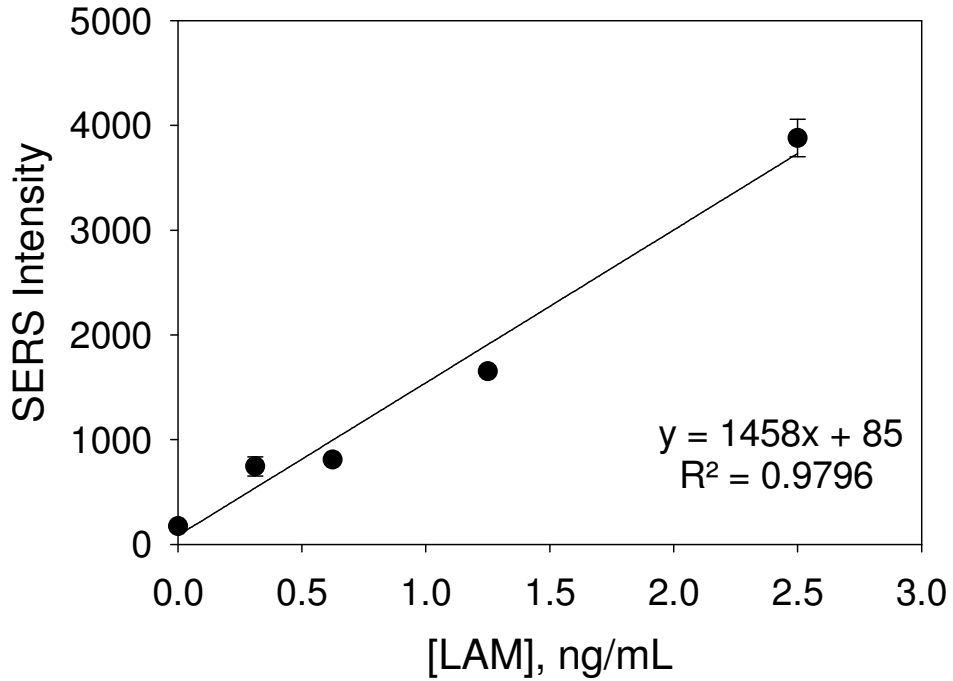
- 17.5 kDa structure[1]
- Phosphatidylinositol anchor with mannosyl backbone and arabinosyl side chains
- Unique to mycobacteria
- ~40% of cell wall composition (1.5% total mass)
- Diagnostic potential
  - ELISAs (96-well microplate) & LFAs (DipStick)
    - Limit of Detection (LOD): ~1 ng/mL
    - LAM detection in urine[2]
      - » SN 13-93%, SP 87-99%

1. Chatterjee D. et al. JBC 1992, 267, 6228
2. Minion et al. Eur Respir J 2011, 38, 1398

LAM Image: Achim Treumann and Steve Homans

LAM spiked into Saline (PBS) Solution





Saline calibration run: 0.0 to 10.0 ng/mL LAM LOD: 0.2 ng/mL

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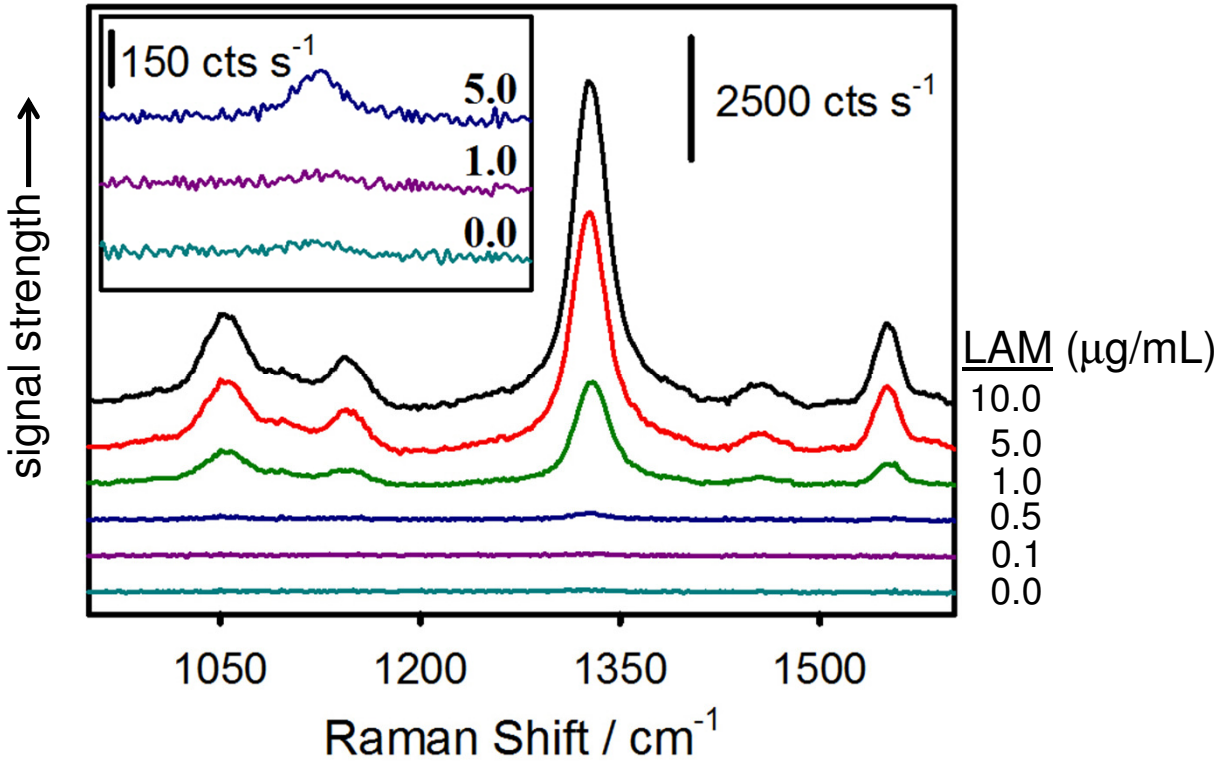
LAM Spiked into Blood Serum of Healthy Individuals



Serum calibration run: 0.0 to 10.0 µg/mL LAM LOD: 800 ng/mL

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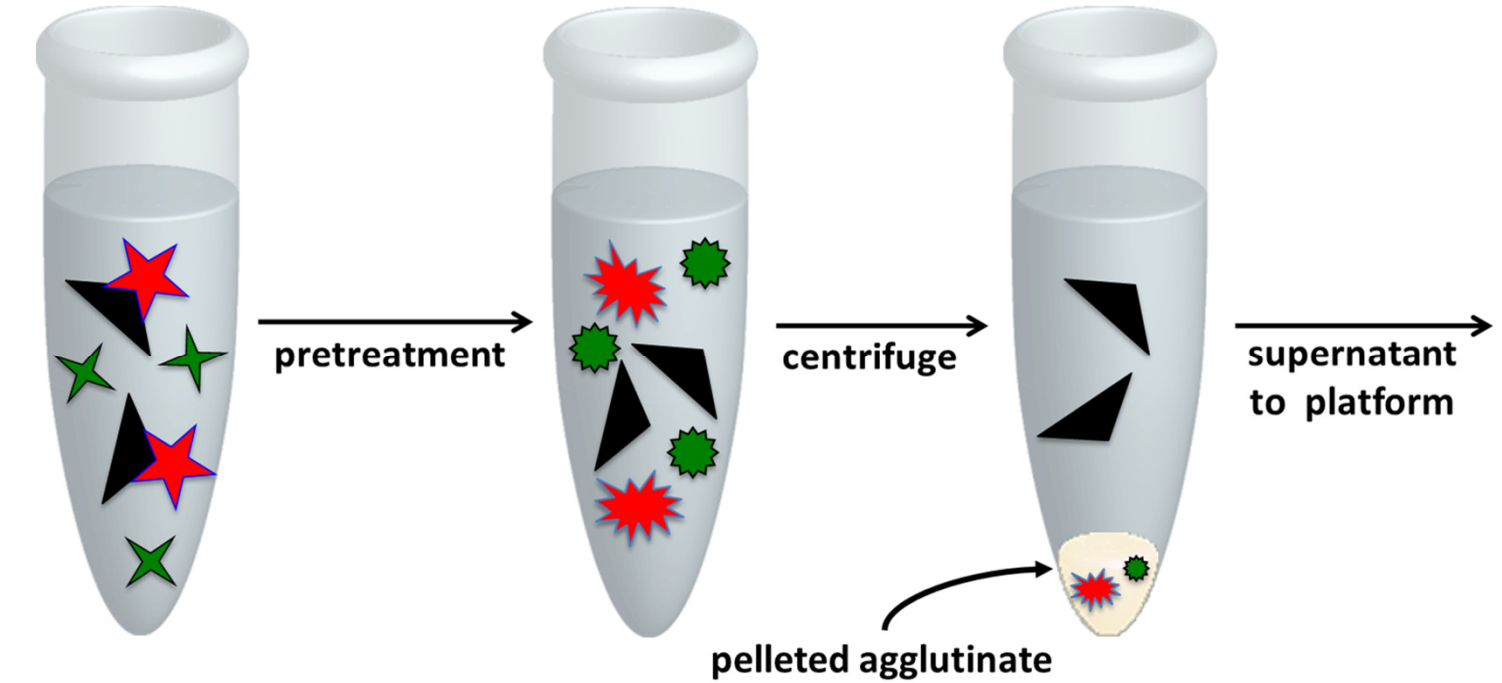
Pretreatment Method



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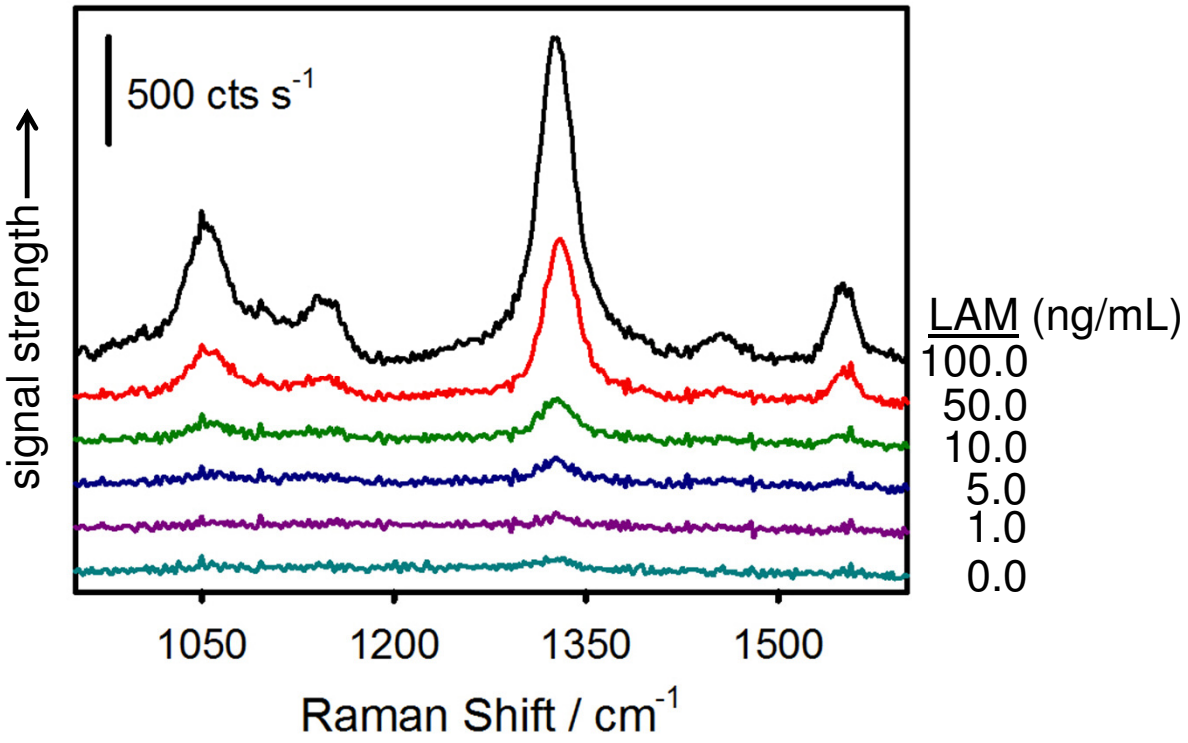
LAM Spiked into Blood Serum and Pretreated



Serum calibration run: 0.0 to 100.0 ng/mL LAM	LOD: 2 ng/mL

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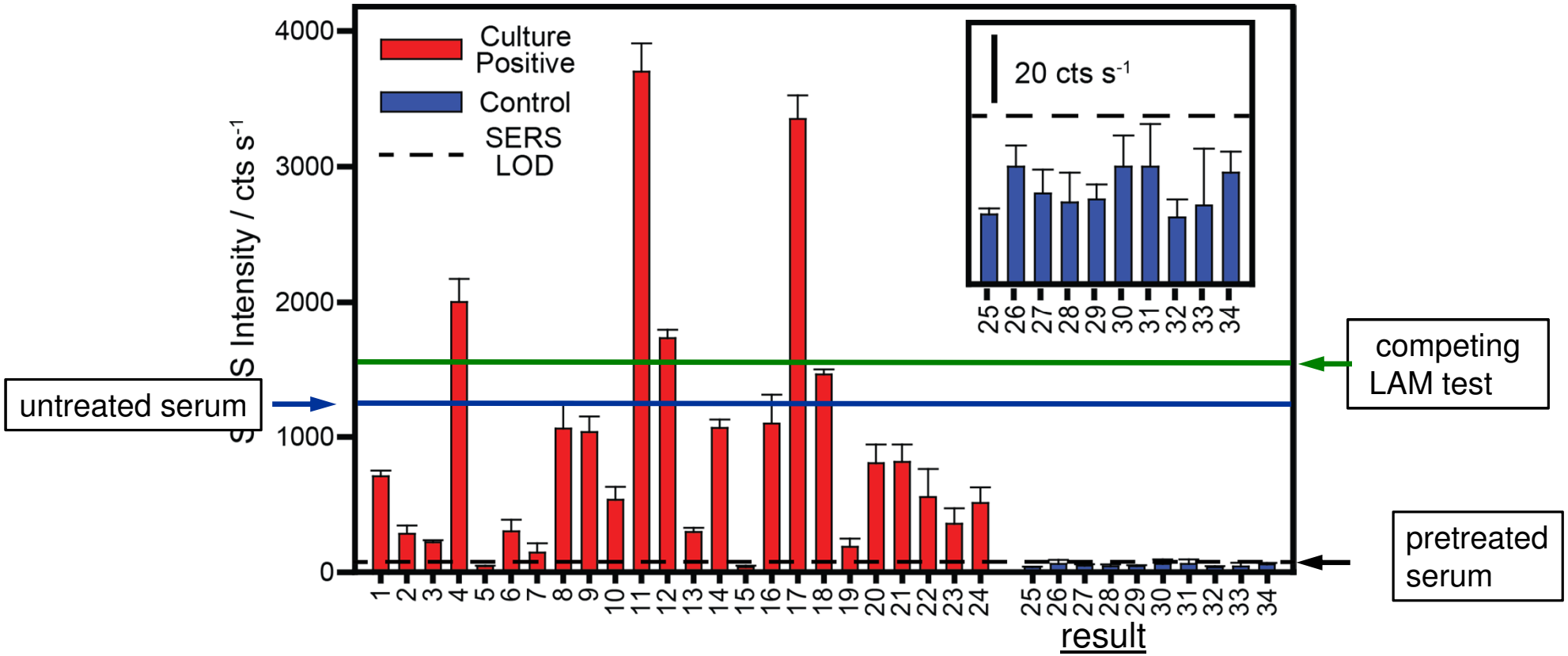
LAM Detection in TB Patient Serum



	result
24 TB-positive subjects: adult, HIV-negative:	21/24
10 TB-negative subjects: adult, healthy:	10/10

Reducing the global burden of tuberculosis: the contribution of improved diagnostics

Authors: **Emmett Keeler,[1] Mark D. Perkins,[2] Peter Small,[3] Christy Hanson,[4] Steven Reed,[5] Jane Cunningham,[6] Julia E. Aledort,[1] Lee Hillborne,[1] Maria E. Rafael,[1] Federico Girosi[1] & Christopher Dye[6]**

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[4]United States Agency for International Development, 1300 Pennsylvania Avenue North West, Washington DC 20523, USA
[5]Infectious Disease Research Institute, 1124 Columbia Street, Seattle, Washington 98104, USA
[6]World Health Organization, Avenue Appia, 1211 Geneva 27, Switzerland

Nature Medicine: 2006, 49-57

Box 1 | Key messages

- A rapid and widely available diagnostic for tuberculosis (TB) with ≥85% sensitivity for smear-positive and smear-negative cases, and 97% specificity, could save ~400,000 lives annually.
- Ideally, new diagnostics for TB should require no electricity, refrigeration or access to clean water, and should be easy to use with minimal or no training. Test results should be available within 1 h.
- All the parameters examined in this study (test performance, speed and access) are important for achieving gains, and improvements can be realized in any of these areas. The best health outcomes result from implementing multiple solutions.

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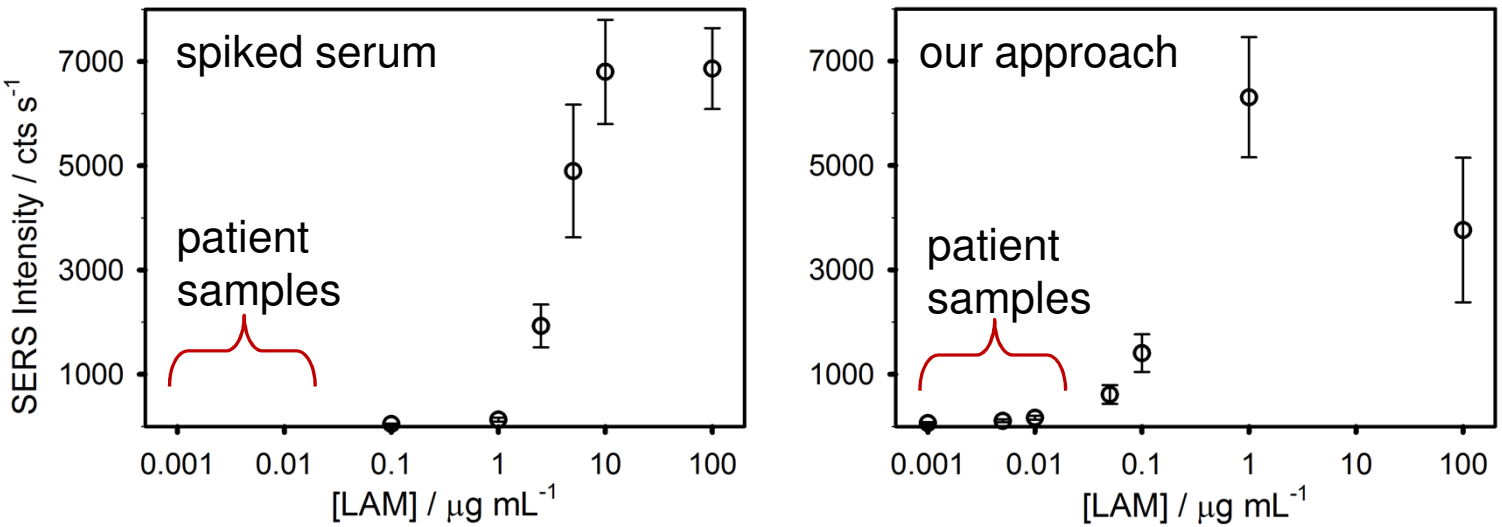
LAM detection in serum before/after serum pretreatment



- Signal plateaus at similar SERS intensities
- Spiked serum has a ~10 x dynamic range
- Spiked serum (our approach) has >100x dynamic range
- LOD after pretreatment is 250x lower than without pretreatment

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What Next?

Ongoing/planned work:

A. Unravel complexation mechanism - LOD

B. Test Development and Automation

C. Test Validation (3200 specimens)

- HIV positive/TB positive
- HIV negative/TB positive
- Serum/urine longitudinal studies
- Patients treated with antibiotics
- Pulmonary vs. Extrapulmonary
- BCG vaccinated

C. Innovations for field deployment

TB ID

B&W TEK INC.
Your Spectroscopy Partner

Goal: Improved TB diagnostics could help save ~400,000 lives/yr

Distribution Challenge: Estimated PON distribution at ~80M tests/yr

Overall Goal: Add malaria and dengue fever [$36B (USD) in 2010]

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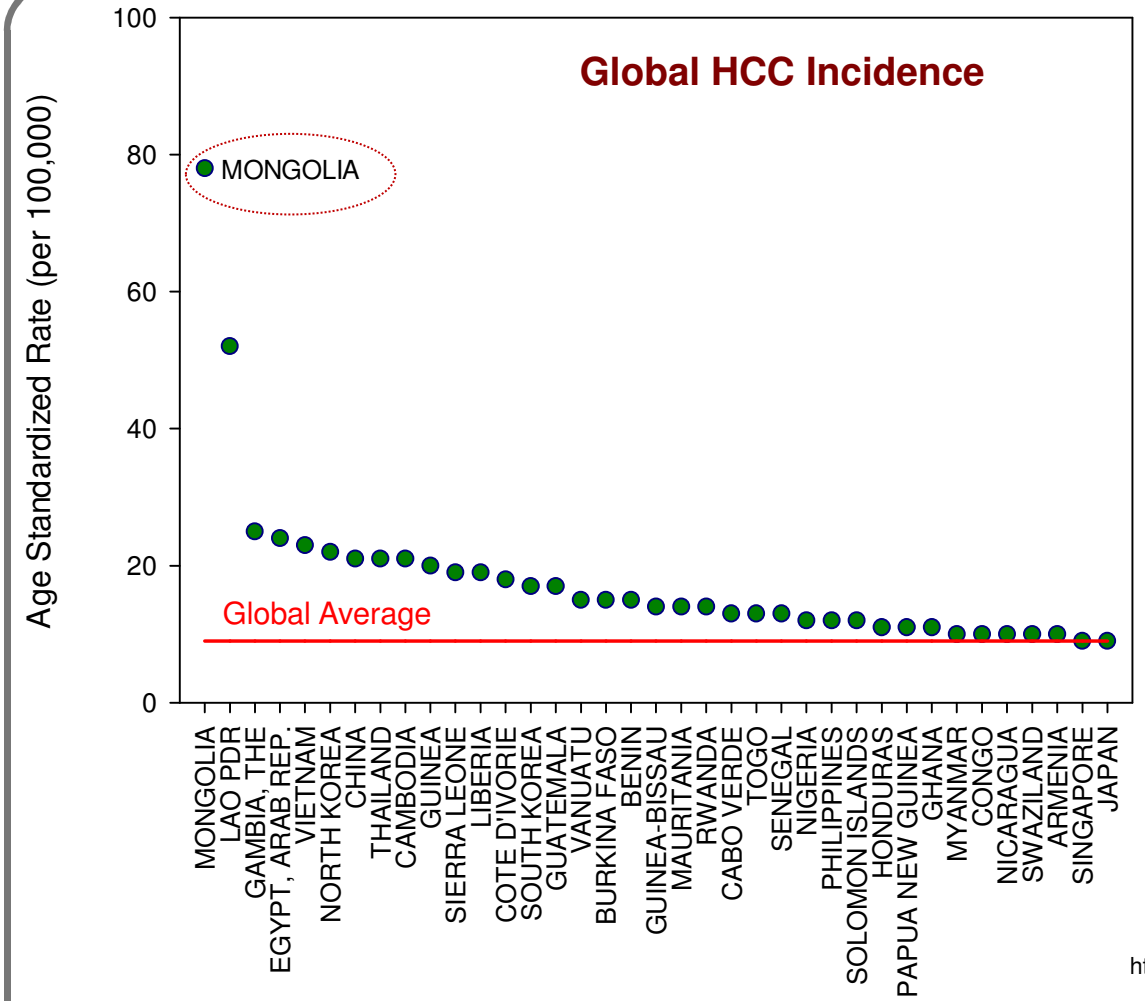
Motivation for Point-of-Need Diagnostic Tests



- Liver cancer is world's 3rd most prevalent cancer.
- Patient survival compounded in Mongolia by limited access to preventative care.
- Nearly all HCC cases in Mongolia are referred to the National Cancer Center of Mongolia (NCCM) in Ulaanbaatar.
- ~90% of patients seeking HCC treatment at NCCM are late stage (*i.e.*, tumors >2 cm) and not eligible for resection or other curative treatments.
- ~40,000 new cases in USA in 2017

http://www.wcrf.org/int/cancer-facts-figures/data-specific-cancers/liver-cancer-statistics

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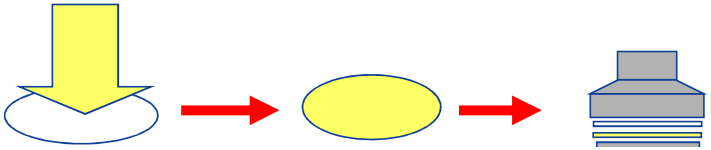
Proposed Panel Screen for PON Screening of Liver Cancer

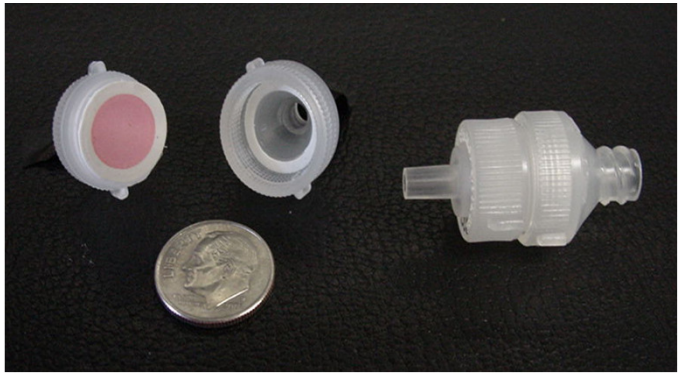
Biomarker	Abbreviation	Function	Target Levels for Risk Assessment
Alphafetoprotein	AFP	fetal glycoprotein that is increased in HCC	≥10 ng/mL
Lens-culinaris agglutinin binding Alphafetoprotein	AFP-L3	fucosylated variant of AFP that has a high affinity to Lens culinaris produced by malignant hepatocytes	>10% of total AFP (1 ng/mL)
Des-Gamma-Carboxy Prothrombin	DCP	arises from an acquired defect in post-translational carboxylation of the prothrombin precursor in malignant hepatocytes	≥10 ng/mL
Core Antibodies to Hepatitis B Virus	HBV	small enveloped DNA virus that can lead to HCC	5 ng/mL
Core Antibodies to Hepatitis C Virus	HCV	small enveloped DNA virus that can lead to HCC	5 ng/mL

C-SPE: Technology to Monitor ISS Crew Health

Prior to analysis: membrane impregnation with colorimetric reagent and load in filter holder

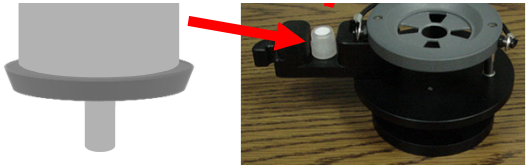


Low LoDs for biocides in drinking water on ISS (2 ppb of iodine) in ~2 min from concentration strengths of SPE and high rates for reactions in confined domains.



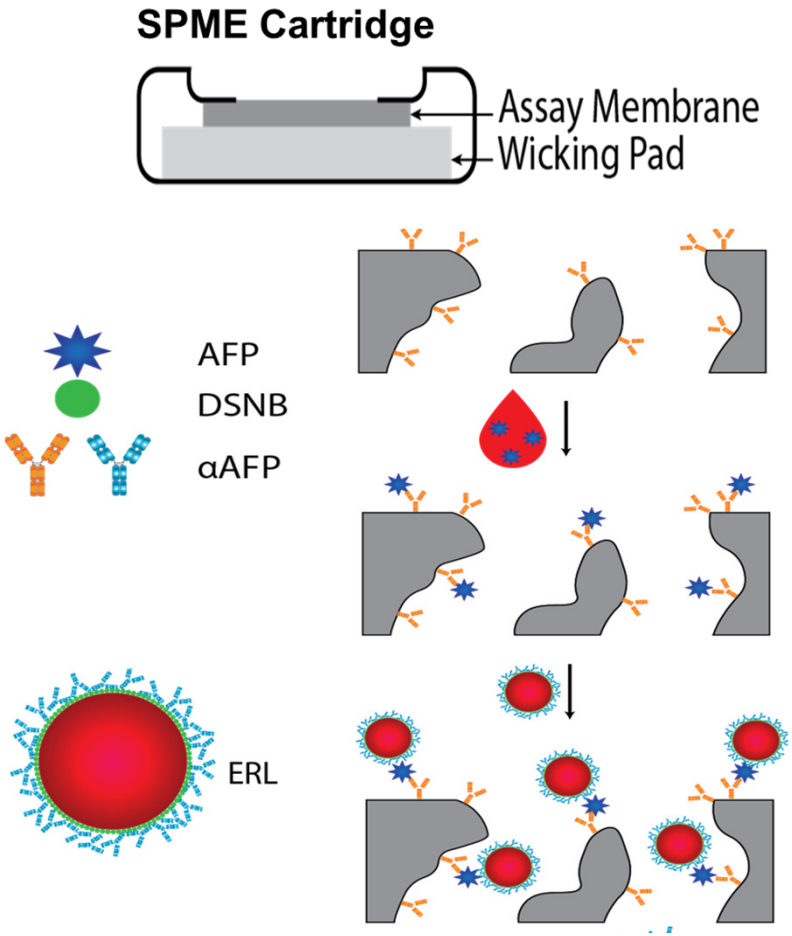
Step 1: draw sample into syringe

Step 2: pass sample through disk



Step 3: acquire reflectance spectrum with portable spectrometer

SERS and SPME



STEP 1: Sample drawn through membrane address by wicking action of absorbent pad, <10 s).

STEP 2: Biolyte captured by immobilized antibodies as sample flows through membrane.

STEP 3: Buffer rinse, followed by antibody-labeled ERLs to tag captured biolyte at an enhancement factor of ~10^6.

STEP 4: SERS signal measured with handheld Raman spectrometer at high accuracy and low limit of detection.

Total assay time with a little practice: ~2 min

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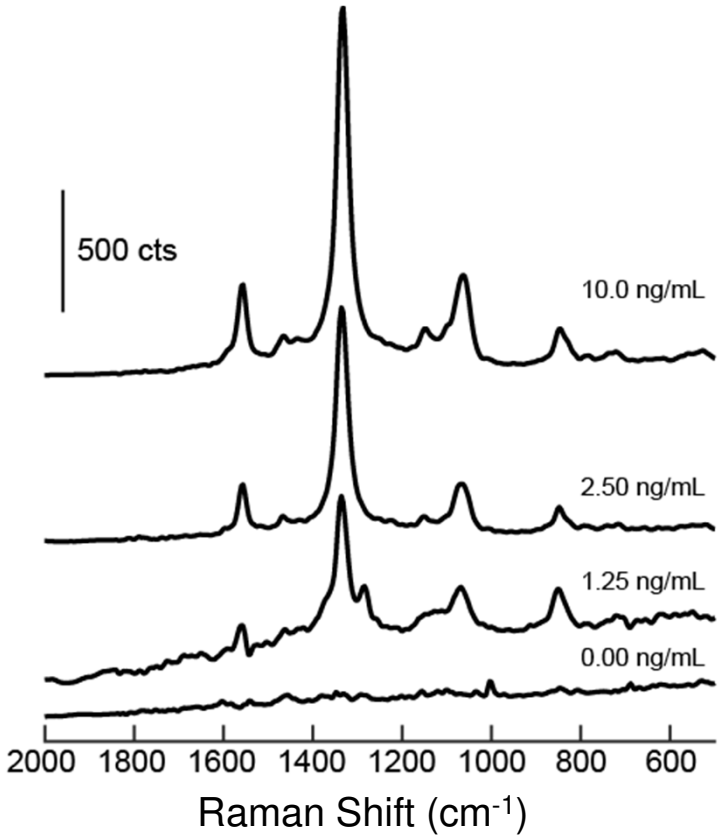
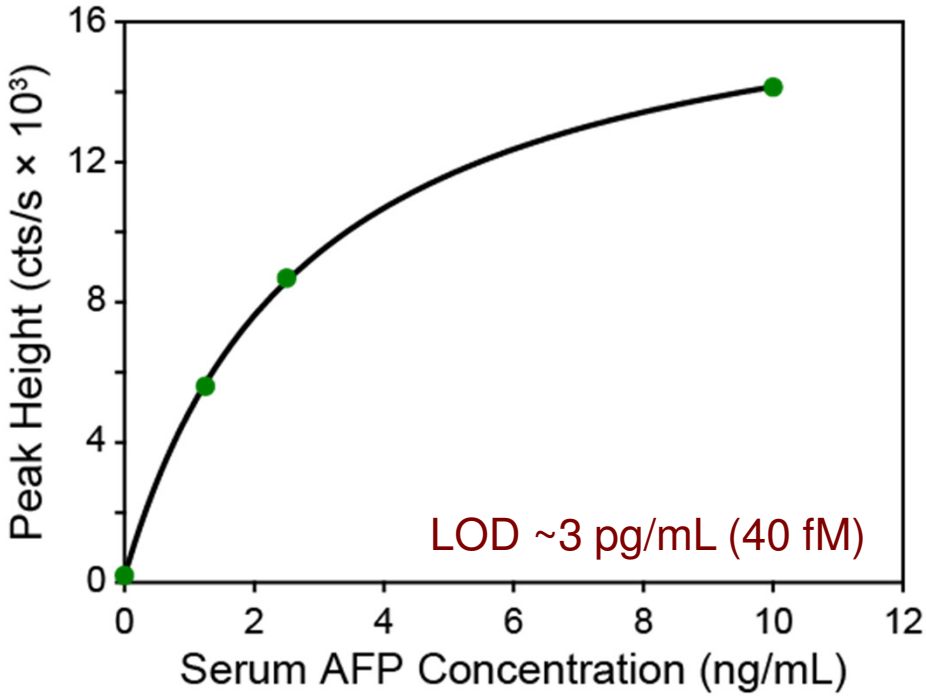
R-SPE: AFP Directly from Human Serum (10 µL)



Bovine Serum Albumin as a blocking agent



Why?

The Nuts and Bolts of R-SPE (SERS Version)

1. Binding affinity (K_d = 1.67 x 10^{-10} M)

2. SERS enhancement (~10^6)

3. Concentration factor (>100×)

4. “Blank” blank (immeasurable level of nonspecific adsorption)

5. Fast on (capture) rate coupled with reactions in confined submicron channels

6. Very slow off rate (k off: 2 x 10^{-4} s^{-1} or a $t_{½}$ of ~60 min)

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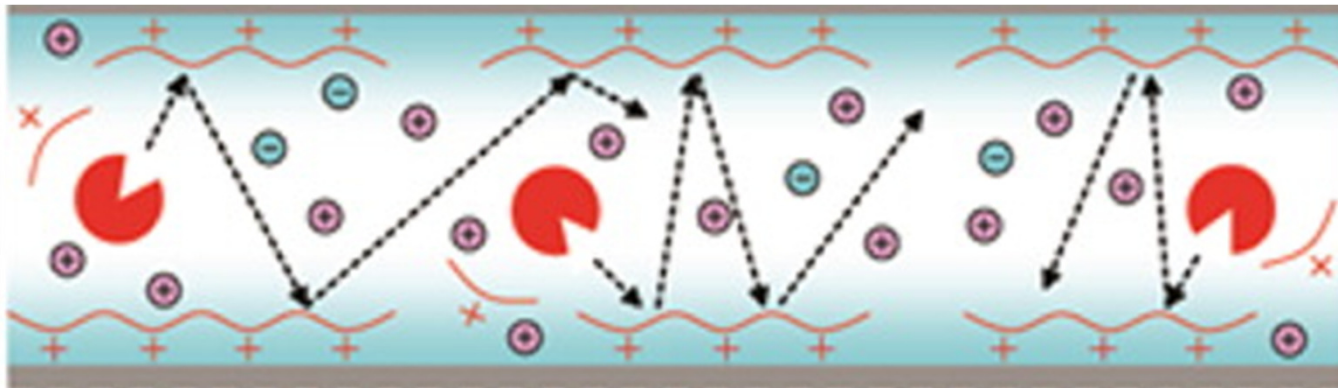
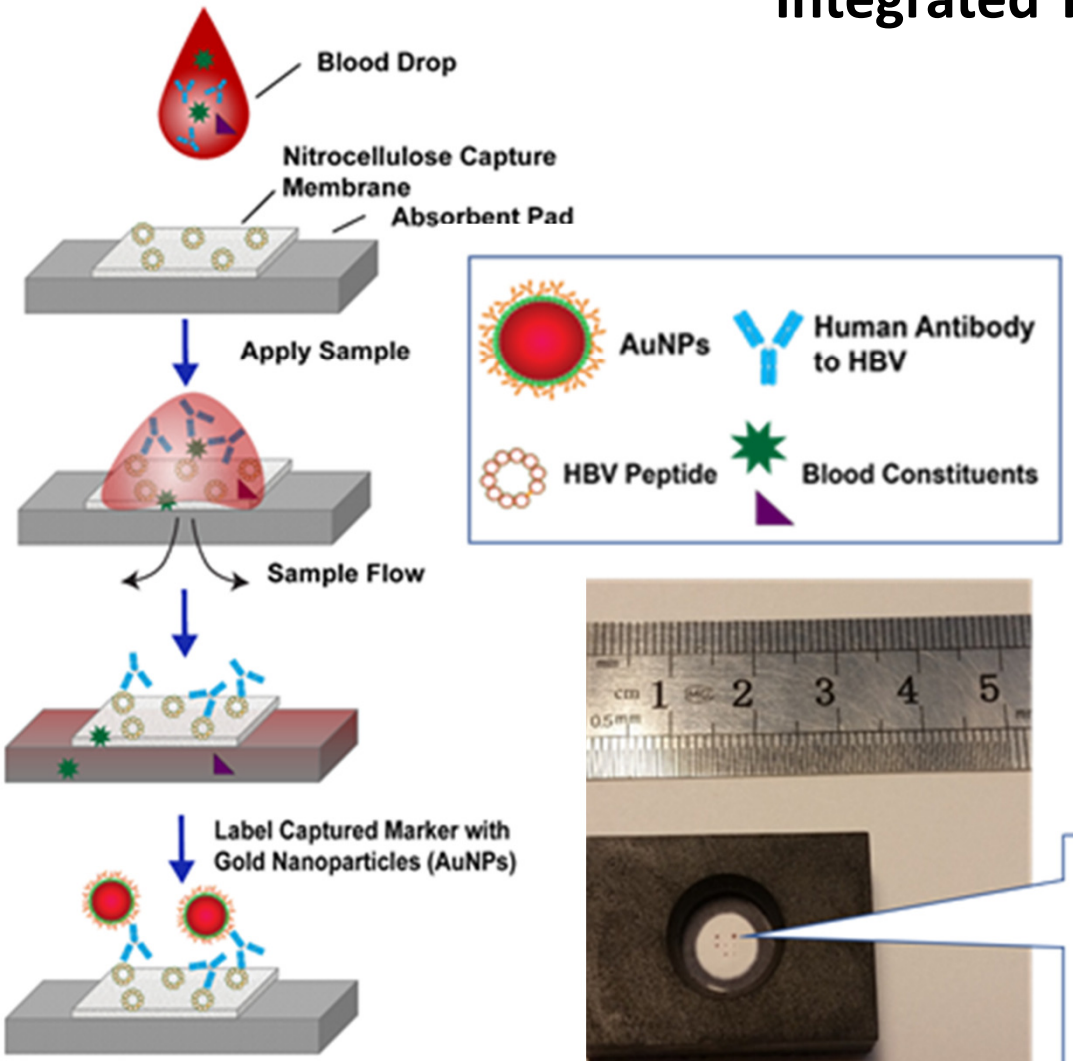
Reactions in "Nanodomains" - Large Surface Area to Volume Ratio

Reaction rates enhanced up to 1000 times and more



D. Chuanhua, et al, *ACS Nano* **2016,** 10, 7476-84.

Integrated Test Kit



B&W Tek's TacPico

Porter Medical Technologies