The background of the slide is a high-angle aerial photograph of a coastline. The ocean is a deep blue, with a prominent red and orange plume of water extending from a point near the shore towards the horizon. In the distance, a range of mountains is visible under a clear sky.

Development of a Raman Spectroscopic Sensor and Library for Classification of HAB Species and Toxins

Scott Gallager

Woods Hole Oceanographic Institution and CoastalOceanVision, Inc
and Donald Anderson, WHOI

and

Richard York, Department of Natural Resources, Town of Mashpee

NESSA Conference April 10, 2019

Harmful Algal Blooms (HAB)

Non-toxic but can cause anoxic conditions



Noctiluca scintillans

Non-toxic to humans but harmful to fish

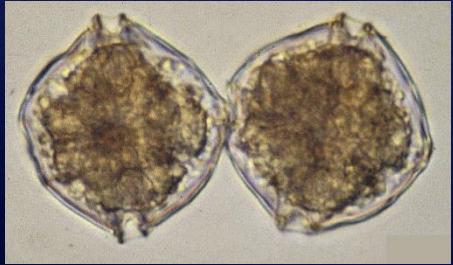
Cochlodinium polykrikoides



What is the efficacy of
Raman spectroscopy
for cell and toxin
identification ?

Paralytic Shellfish Poisoning (PSP)

Alexandrium catenella



Produce very potent toxins

Neurotoxic Shellfish Poisoning (NSP)

Karenia brevis



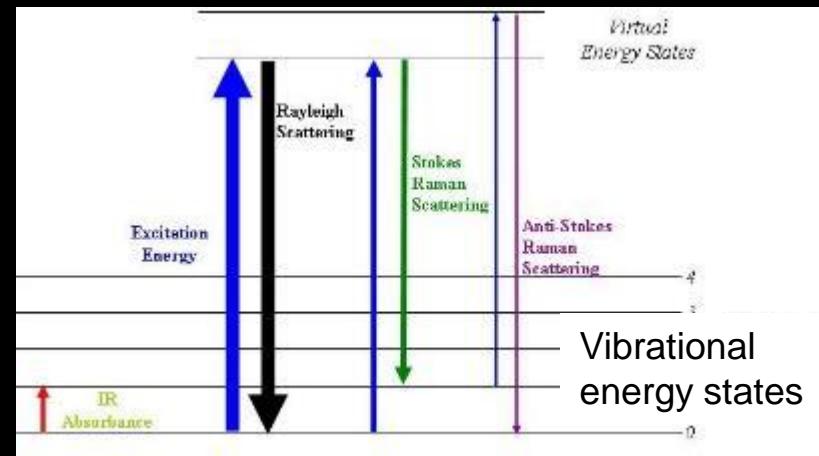
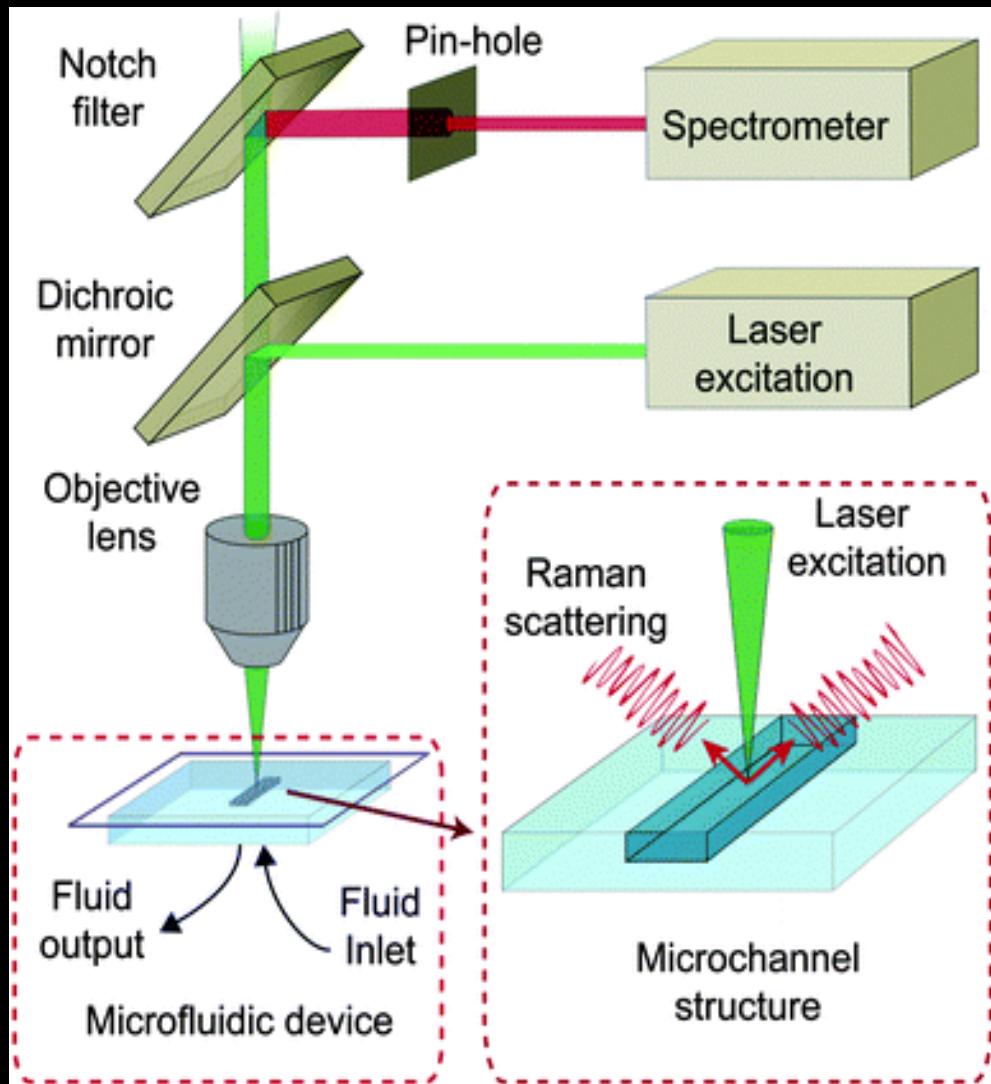
Domoic Acid
Pseudo-nitzschia spp.



Objectives

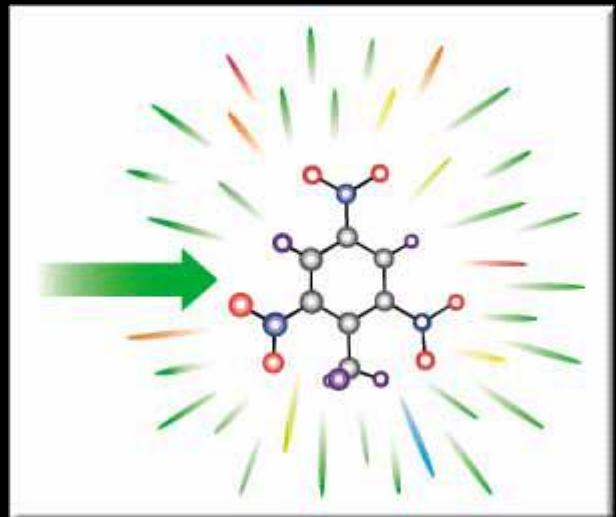
1. To combine Raman spectroscopy with image analysis to discriminate between HAB species using Deep Learning
2. To miniaturize and package the sensor to allow hand held, in situ, and distributed arrays of real-time instruments

Raman Spectroscopy

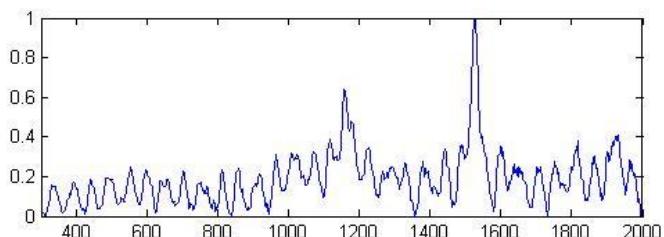
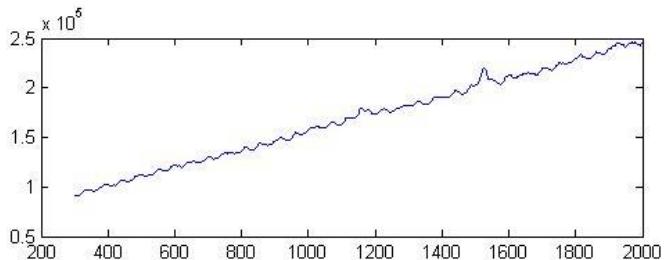


Raman Shift ν (cm⁻¹)

$$\text{U} = \frac{1}{\lambda_{\text{incident}}} - \frac{1}{\lambda_{\text{scattered}}}$$



Processing a Raman Spectra



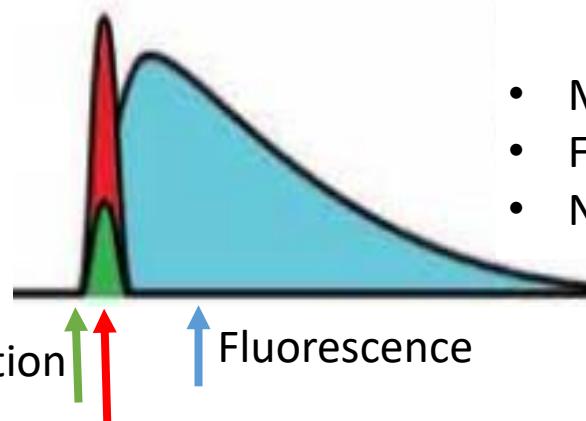
Standard Continuous Excitation Raman

Remove:

- cosmic ray events
- background fluorescence
- S/N depends on many variables

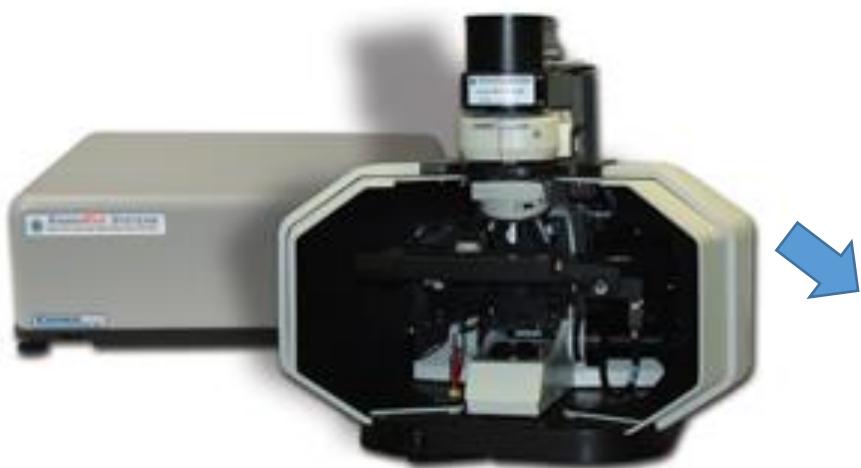
Time-Domain Excitation and Emission Raman

- Measure fluorescence separately from Raman
- Flattens baseline
- No need to use longer wavelength low energy laser (e.g., 785, 1025)

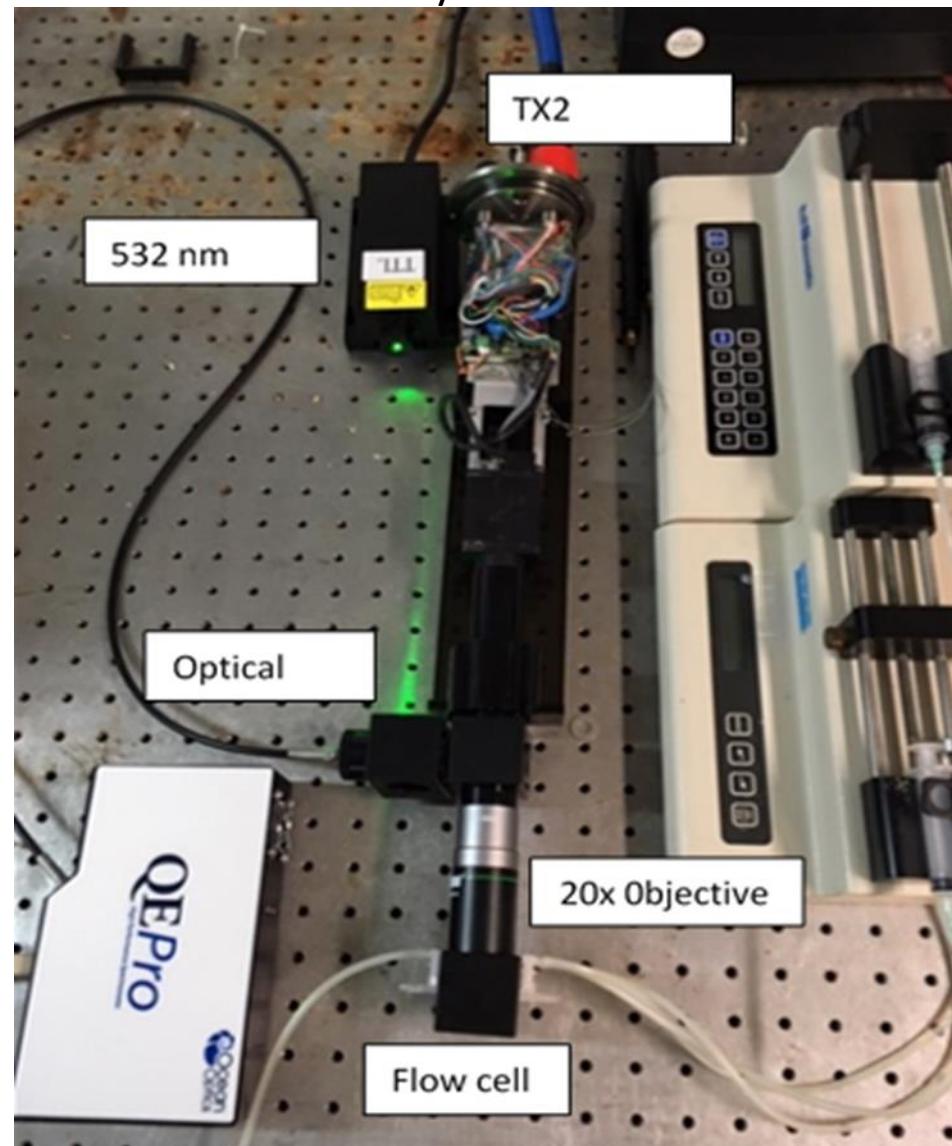


Time (ns) ->

Single Cell Analysis on commercial
micro-Raman Spectrometer



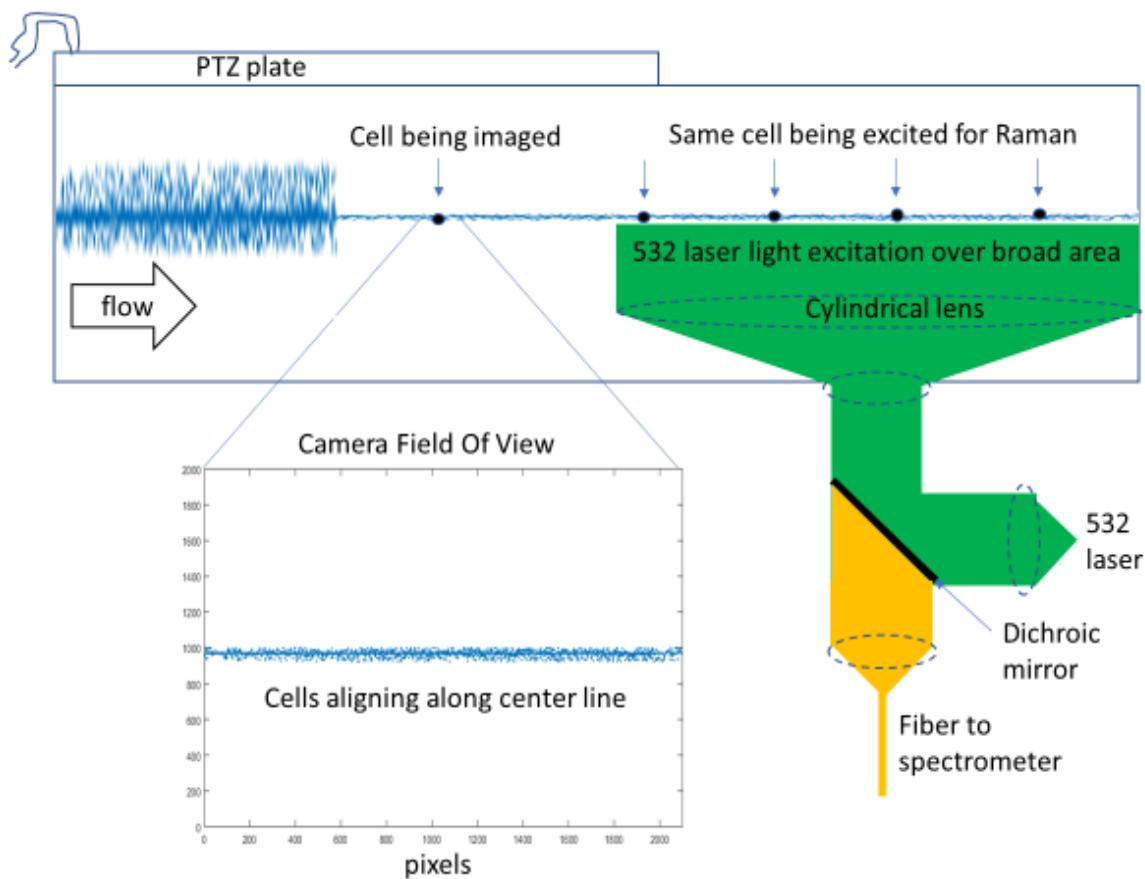
Phase I SBIR:
Prototype time-domain Raman Flow Cell
Cytometer



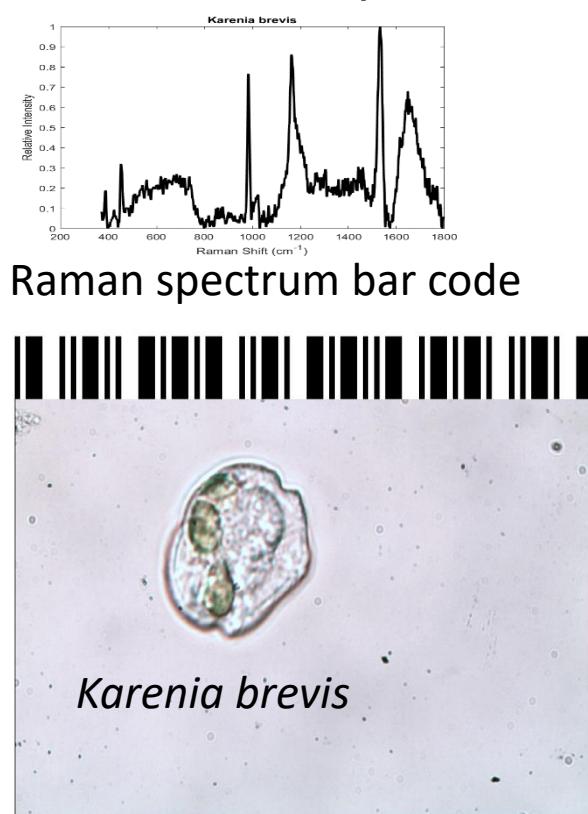
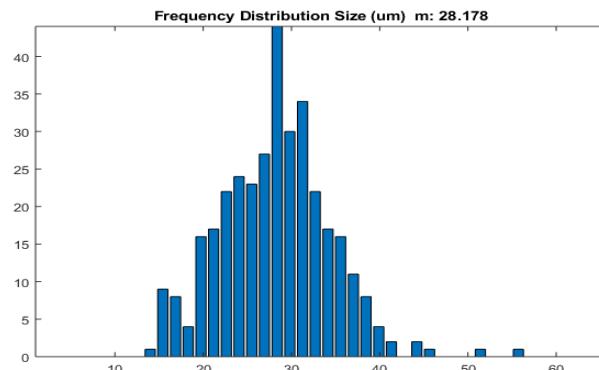
Phase II SBIR Goal:
Population level
Analysis @ 532 nm
in a hand held instrument
or distributed network of sensors



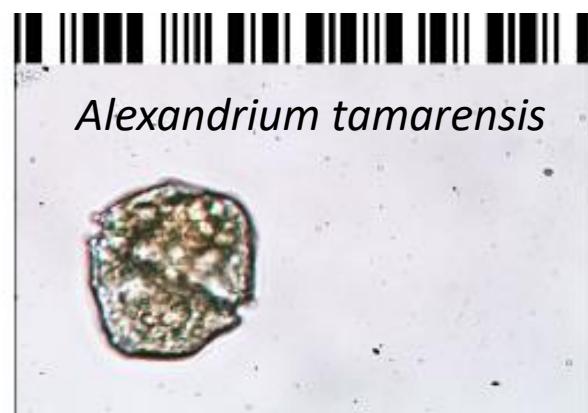
Raman flow cytometer is very different from standard flow cytometer



Size frequency distribution



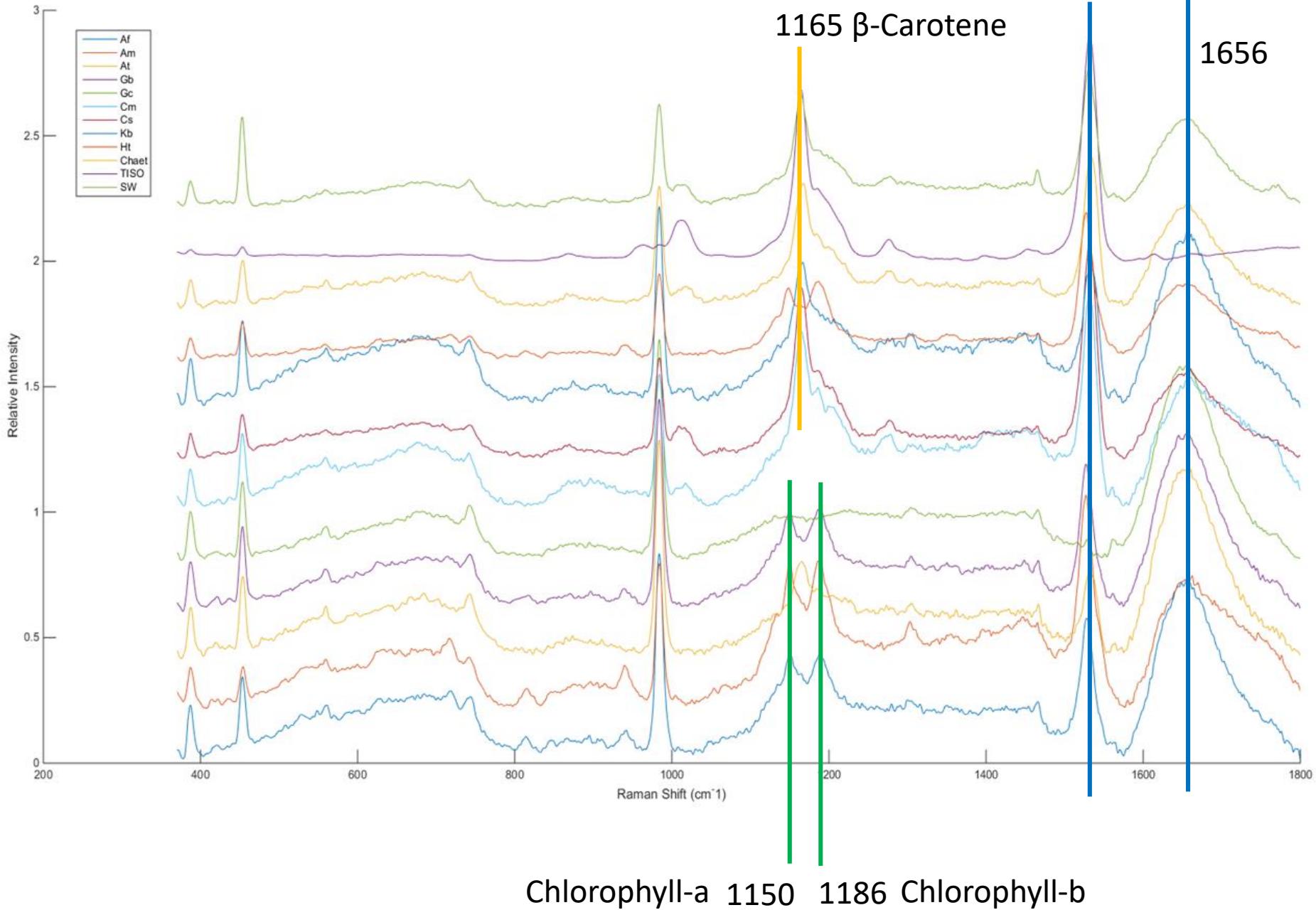
Karenia brevis



Alexandrium tamarensis

Characteristics of 12 Species at 532 nm

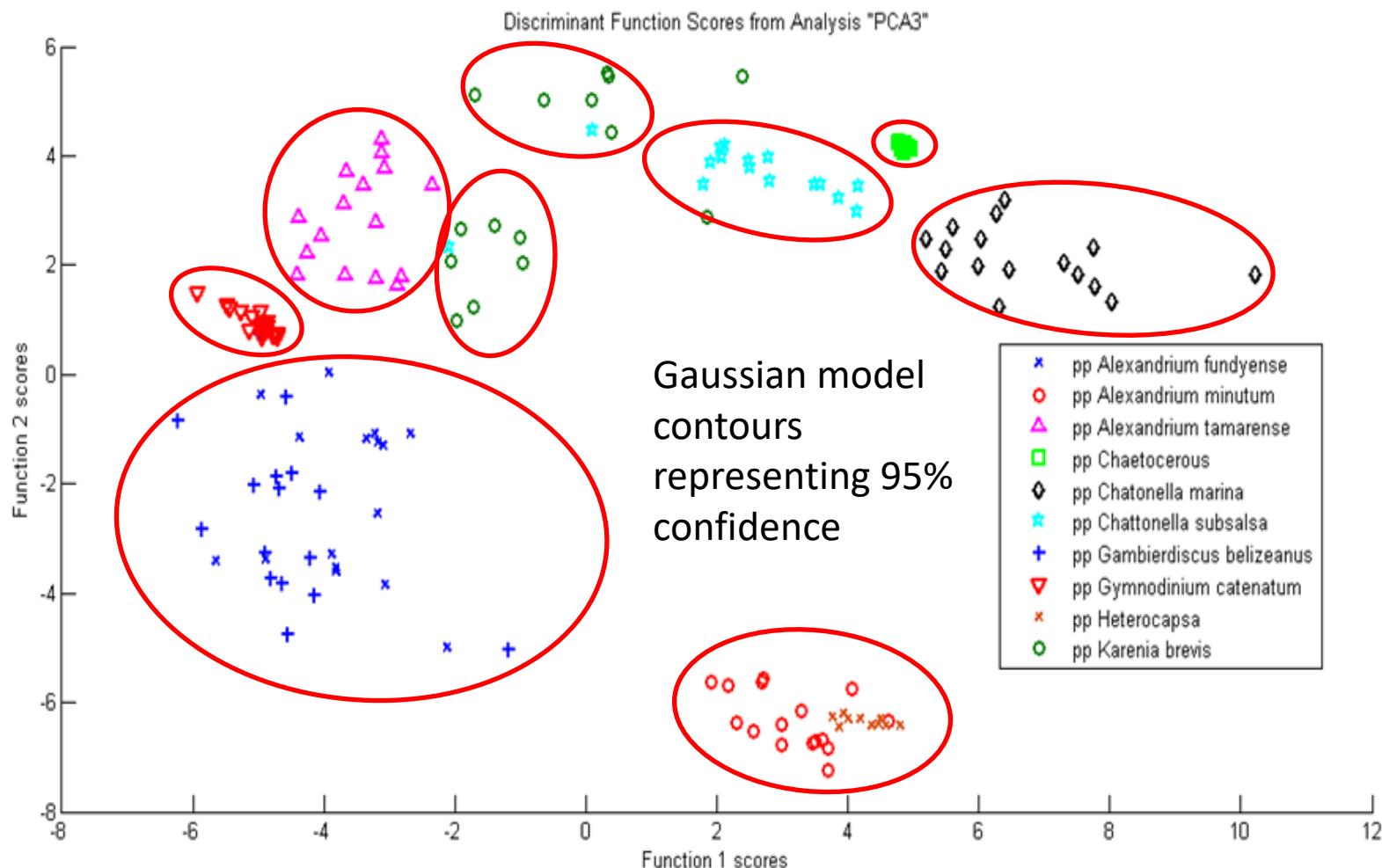
1467 triglycerides



Gambierdiscus belizianus
Alexandrium catenella ATMP7
Alexandrium catenella 1911
Alexandrium minutum
Heterocapsa sp.
Chaetoceros sp.
Alexandrium tamarensse
Chattonella subsalsa
Chattonella marina
Isochrysis galbana
Karenia brevis
Pseudo-nitzschia subcurvata 92
Pseudo-nitzschia subcurvata 13
Gymnodinium catenatum

Raman Shift														Compound	Molecular assignments		
388	0.23	0.26	0.26	0.20		0.04	0.25	0.13	0.19	0.05	0.24			0.22		v2 bending reduction in symmetry of the	
454	0.38	0.39	0.39	0.21		0.08	0.39	0.21	0.35	0.06	0.41			0.36			
744	0.29	0.47	0.47	0.33			0.32	0.19	0.37		0.22			0.26	chlorophyll a-b		
940				0.21	0.09											v(C-C), alpha-helix	
987	0.95	0.94	0.94	1.00		0.12	0.98	0.46	0.62		0.92			1.00	chlorophyll a-b		
1000	0.03	0.04	0.05	0.12	0.02	0.08	0.04	0.07	0.03	0.12	0.13	0.13	0.15	0.07	chlorophyll a-b	v3	
1017											0.86				Gyroxanthin		
1150	0.45	0.47	0.48	0.66	0.41										β -Carotene	v2 C14-C15 δC15-H	peridinin?
1165						0.70	0.45	0.78	0.81	0.77	0.67	0.54	0.59				
1186	0.45	0.47	0.48	0.68	0.45				0.56						chlorophyll a-b	20δ(CH), vN21-C4	
1190						0.32	0.22	0.35	0.52	0.31	0.44	0.32	0.27		β -Carotene	δ(C10-H), δ(C11-H)	
1200						0.28	0.26	0.33	0.51	0.26	0.38	0.31	0.25				
1278						0.12					0.17	0.11			fucoxanthin		
1289															chlorophyll a-b	CH3 bend,(10+5)δ(CH), vN23-C14	
1303				0.41		0.26			0.30		0.35			0.24		Carbonate antisymmetric stretching	
1348					0.07										chlorophyll a-b	CH3 bend (20+5+10)δ(CH), vN21-C4	
1467	0.25	0.33	0.33	0.54	0.05	0.06	0.24	0.15	0.36	0.04	0.24	0.06	0.04	0.25	triglyceride	C-C saturated	
1495		0.12	0.12	0.12		0.12			0.12						chlorophyll a-b	vC12-C13, vC1-C20, vC5-C6(81+82+121)CH3 b	
1520									0.12		0.12				β -Carotene	v(C13=C14), v(C11=C12)	
1529	0.66	0.64	0.65	0.98	1.00	1.00	0.40	0.97	0.97	0.99	0.64	0.97	1.00	0.10	chlorophyll a-b		
1656	0.86			0.82				0.44	0.73						triglyceride	cis C=C stretching vibration unsaturated	

Can Raman distinguish between species?

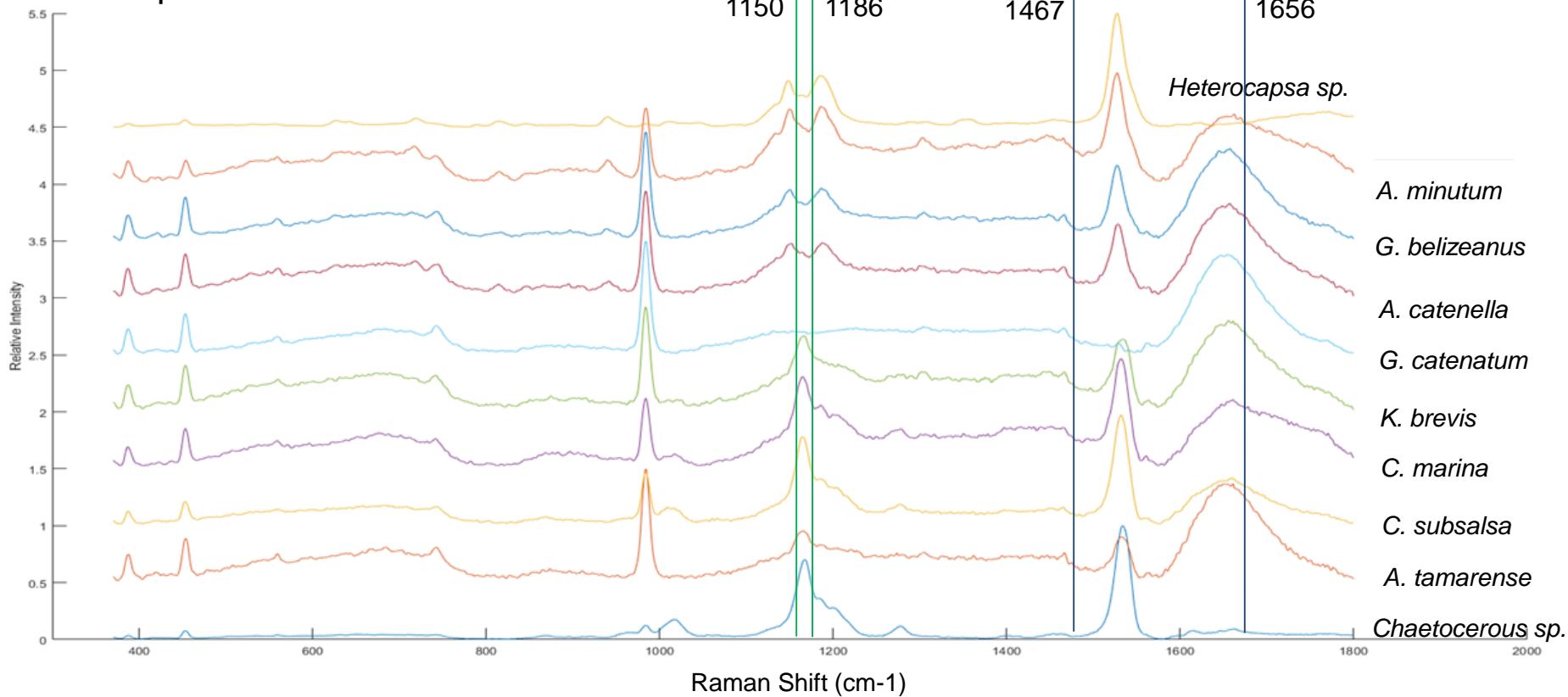


LOO-Cross Validation of Linear Discriminant Function

- 8 toxic, 3 non-toxic species

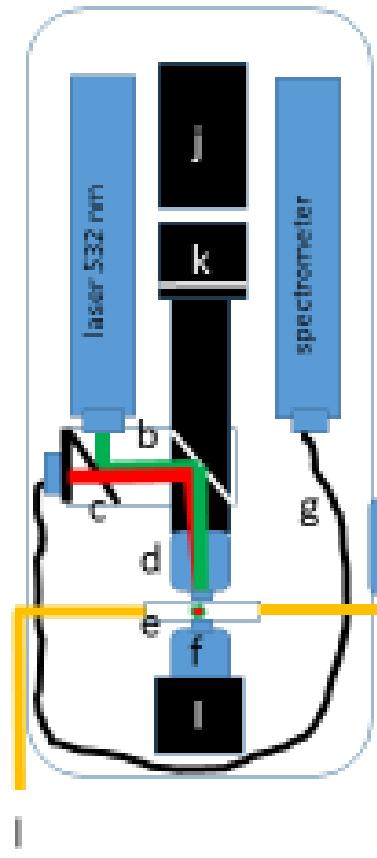
Result: 100% discrimination between toxic and non-toxic Alex. complex discriminated

Species											% Accuracy
<i>Alexandrium catenella</i>	16	0	0	0	0	0	0	0	0	0	100
<i>Alexandrium minutum</i>	0	16	0	0	0	0	0	0	0	0	100
<i>Alexandrium tamarensense</i>	0	0	15	0	0	0	0	0	0	1	94
<i>Chaetoceros sp.</i>	0	0	0	16	0	0	0	0	0	0	100
<i>Chatonella marina</i>	0	0	0	0	16	0	0	0	0	0	100
<i>Chattonella subsalsa</i>	0	0	0	0	1	13	0	0	0	2	81
<i>Gambierdiscus belizeanus</i>	0	0	0	0	0	0	14	2	0	0	88
<i>Gymnodinium catenatum</i>	0	0	0	0	0	0	1	15	0	0	94
<i>Heterocapsa sp.</i>	0	0	0	0	0	0	0	0	16	0	100
<i>Karenia brevis</i>	0	0	0	0	1	1	0	0	0	14	0
<i>Isochrysis galbana TISO</i>	0	0	0	0	0	0	0	0	0	16	100

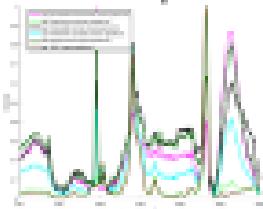


Feature Sets for Training and Classifying Cells using Deep Learning

Feature Type	Feature	Source	Number Features
Morphology	Geometry	3-D image	128
	Shape (FFT)		>1000
	Texture		256
	Invariant Moments		128
	Color pattern		156
	Fractal index		>128
	Cell volume		1
	Cell length		1
	Cell area		1
Chemical Content	Pigmentation (β -Carotene, Chlorophylla,b,c, Fucoxanthin, Xanthophylls, Phycobiliproteins etc.)	Raman & Florescence	~128
	Triglycerides	Raman	21
	Toxins (saxitoxin, ciguatera, Domoic Acid, etc.)	Raman	>100



Raman spectra

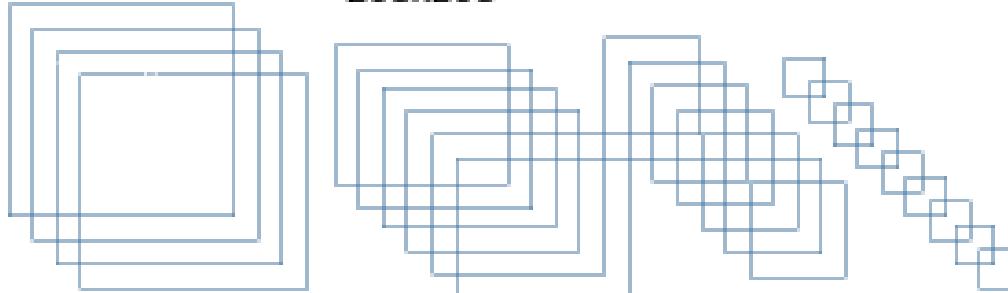


Raman spectra encoded in barcode

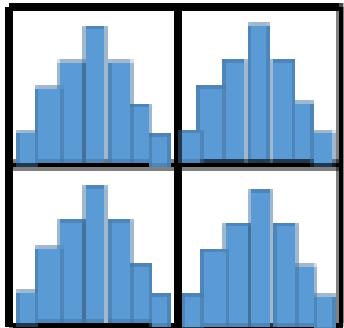


Barcode included
with cell image

Darknet19 Deep Learning network
416x416 208x208 104x104 ... 13x13



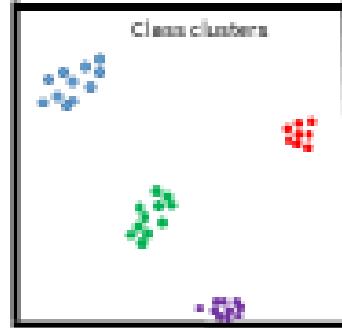
Size distribution per class



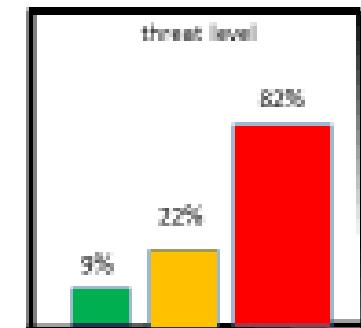
Abundance per class
cells/mL

<i>A. tamarense</i>	20
<i>A. fundyense</i>	1
<i>A. rodemansi</i>	39
<i>K. brevis</i>	0
Pseudo-nitzschia sp.	0

Class clusters



threat level



Classify Manager - v1.1-20181020

CPICS DataSet: HABStats

Refresh CPIC

- Manage Sets
- Extract Features
- Auto Classify
- Manually Classify
- Build/Train Model
- View Results

1) Select TrainingSet TS Subset 2
 2) Select Image Class on Right
 3) Tag the Images Below and press either
 Delete_Images OR
 Move_Images to Class R_Amatarenis OR
 Add_Images to Dest TrainingSet TS Subset 2

View Images: ByTime BySize (len pixels) min: max:

Thumbnail Size: Normal ▾

Admin Options: Hide Show

Classification: R_Amatarenis

Select all ROIs | Clear All ROIs

Classify Manager - v1.1-20181020

CPICS DataSet: HABStats

Refresh CPIC

- Manage Sets
- Extract Features
- Auto Classify
- Manually Classify
- Build/Train Model
- View Results

1) Select TrainingSet TS Subset 2
 2) Select Image Class on Right
 3) Tag the Images Below and press either
 Delete_Images OR
 Move_Images to Class R_Amatinum OR
 Add_Images to Dest TrainingSet TS Subset 2

View Images: ByTime BySize (len pixels) min: max:

Thumbnail Size: Normal ▾

Admin Options: Hide Show

Classification: R_Amatinum

Select all ROIs | Clear All ROIs

Instrument website

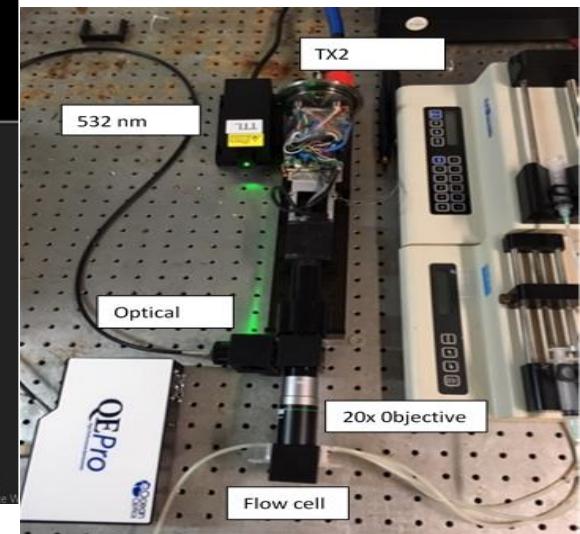
*sort images with Raman bar codes

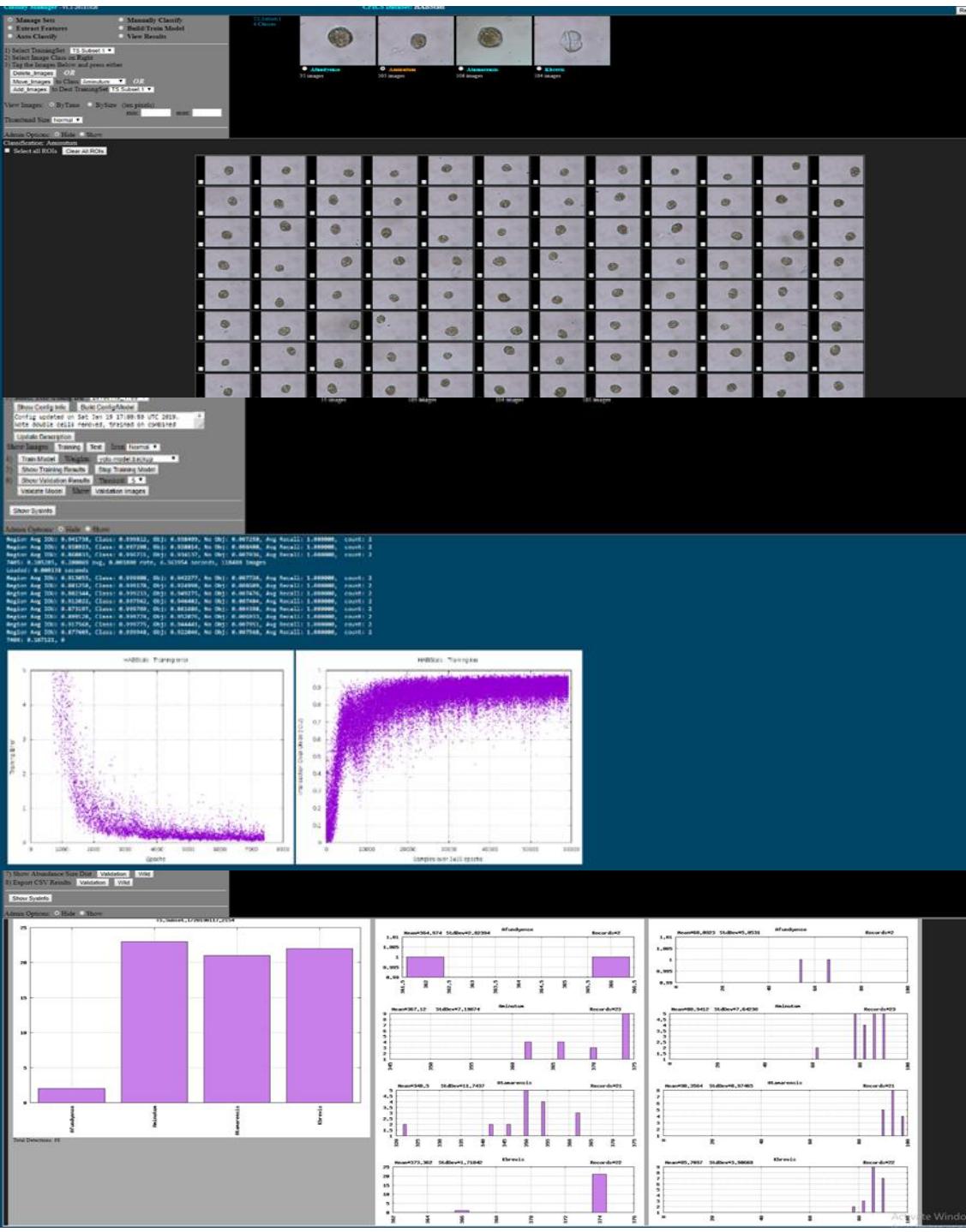
*build training sets

*build Deep Learning models

*run cross-validations

*classify unknown cells





Instrument website

- *build Deep Learning models

- *run cross-validations

- *classify unknown cells by matching against spectral library in real-time at 10 to 50 cells /sec

Marine Raman and Image Library

1	Alexandrium tamarens		saxitoxin
2	Alexandrium tamarens		saxitoxin
3	Alexandrium tamarens	1771	saxitoxin
4	Alexandrium tamarens	1493	saxitoxin
5	Alexandrium minutum	1598	saxitoxin
6	Gambierdiscus belizeanus	ATSW01-2	ciguatera
7	Gambierdiscus belizeanus	113	ciguatera
8	Alexandrium fundyense	401	saxitoxin
9	Alexandrium fundyense	401-K	saxitoxin
10	Pyrocystis noctiluca	ATMP7-E8	
11	Karenia brevis	1911-K	brevitoxins
12	Karenia brevis	Florida, 08/02/18	brevetoxins
13	Karenia mikimotoi	2229-K	brevitoxins
	Karenia mikimotoi	718	brevitoxins
14	Gymnodinium catenatum	429	gonyautoxin
15	Chattonella subsalsa	430	brevetoxin-like
16	Chattonella subsalsa	1937	brevetoxin-like
17	Chattonella marina	217	brevetoxin-like
18	Chattonella marina	217-K	brevetoxin-like
19	Pseudonitschia subcurvata	2050-K	domoic Acid
20	Aureococcus amophageffreus	2050	non-toxic but produces fish kills through anoxia

Non-Toxic

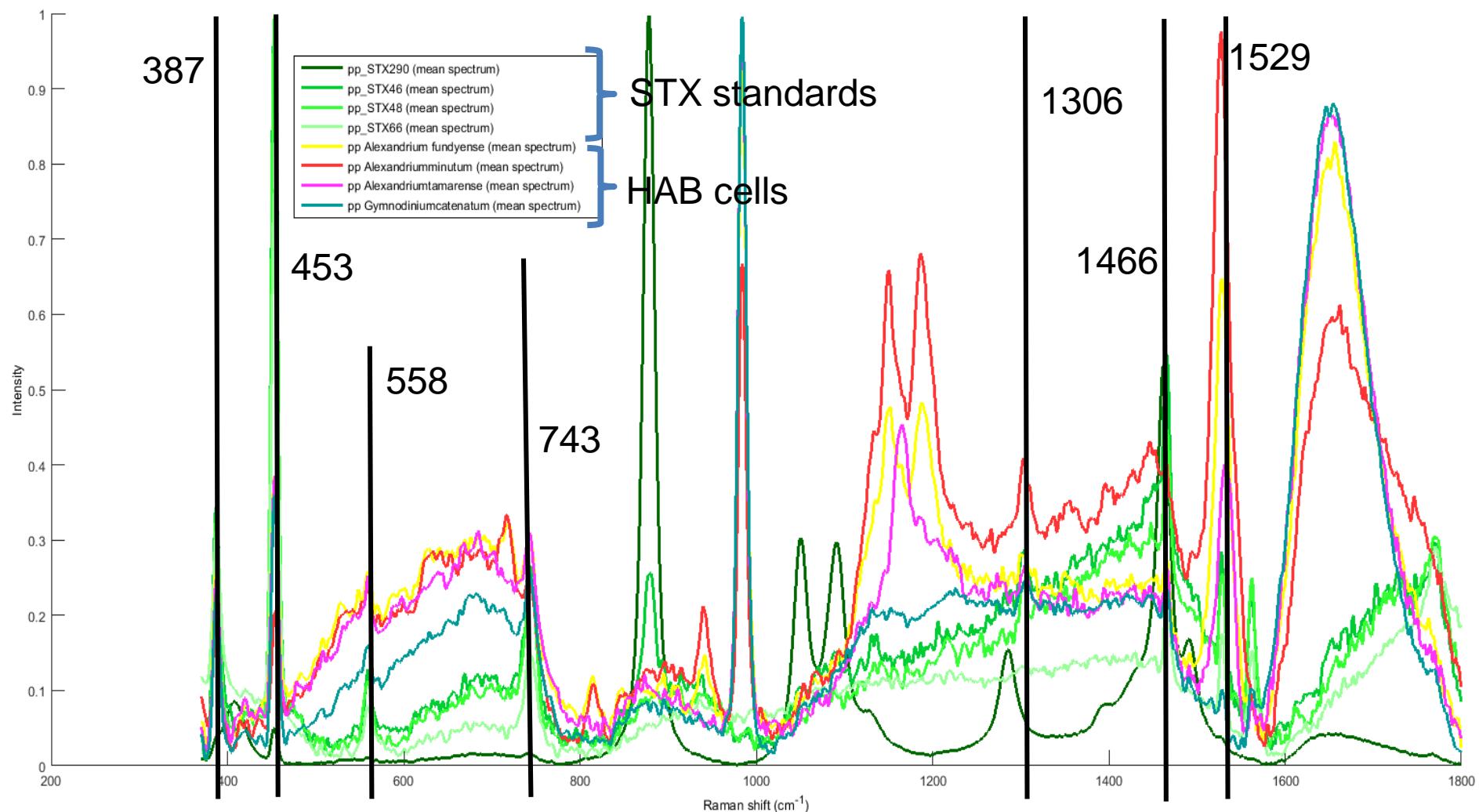
1	Isochrysis galbana	Tiso
2	Chaetoceros	chaet
3	Tetraselmis suecica	tet
4	Rhodomonas salina	1319-rhodo
5	Heterocapsa sp.	Het

Freshwater Raman and Image Library

1	Anabaena cylindrica	B 1611 UTEX	various cyanotoxins- microcystins
2	Anabaena variabilis	B 377 UTEX	nodularin
3	Anabaena sp.	1448 UTEX	anatoxins
4	A. spiroides	B 1552 UTEX	saxitoxins
5	Microcystis aeruginosa		cylindrospermopsins
6	M. aeruginosa	LB 2061 UTEX	lyngbytoxin a
7	M. aeruginosa	LE3 S. 3462	aplysiatoxins
8	M. flos-aquae	LB 2673 UTEX	
9	Nostoc		
10	Nostoc sp.	LB 2210 UTEX	
11	Nostoc sp.	B 2211 UTEX	
12	Oscillatoria lutea	B 1814 UTEX Texas	
13	Volvox	Carolina biological	not toxic
14	Cylindrospermopsis raciborskii		cylindrospermopsin, anatoxin-a
15	C. raciborskii	LB 2897 UTEX	
16	C. raciborskii	Thai W. Carmichael Toxic?	
17	Nodularia harveyana	B 2093 UTEX Washington	
18	Calothrix parietina	LB 1952 UTEX swimming pool	
19	Microcoleus	LB 2220 UTEX	
20	Aphanizomenon flos-aquae	AFA S.Wilhelm	
21	A. flos-aquae	LB 2557 UTEX Toxic Hebgen Lake, MT	
22	A. flos-aquae	LB 2558 UTEX NON-TOXIC Hebgen Lake, MT	
23	A. flos-aquae	B 1444 UTEX Not Listed Mississippi	
24	Microcystis flos-aquae	LB 2673 UTEX Wisconsin	
25	Cyanophora paradoxa	LB 555 UTEX not listed Not listed	
26	Cylindrospermum sp.	LB 942 UTEX not listed California	
27	A. catenula	B 375 UTEX Netherlands	
28	Synechocystis sp.	2470 UTEX not listed California	
29	Synechococcus sp.	LB 2537 UTEX not listed Vancouver Island	
30	Planktothrix	Tim Davis Lab	

Can toxins be detected in single HAB cells?

At least 7 peaks for Saxitoxin (STX) standards align with cells known to be toxic

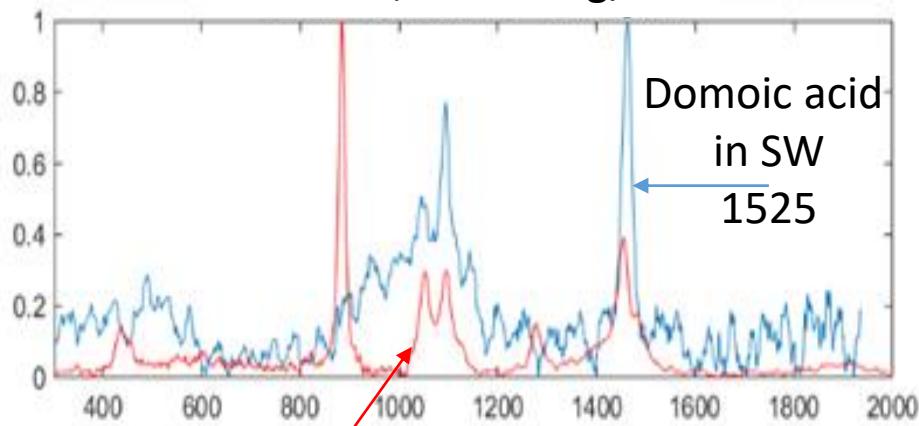


Single cell analysis of *Pseudo-nitzschia* from Woods Hole harbor

February 2017

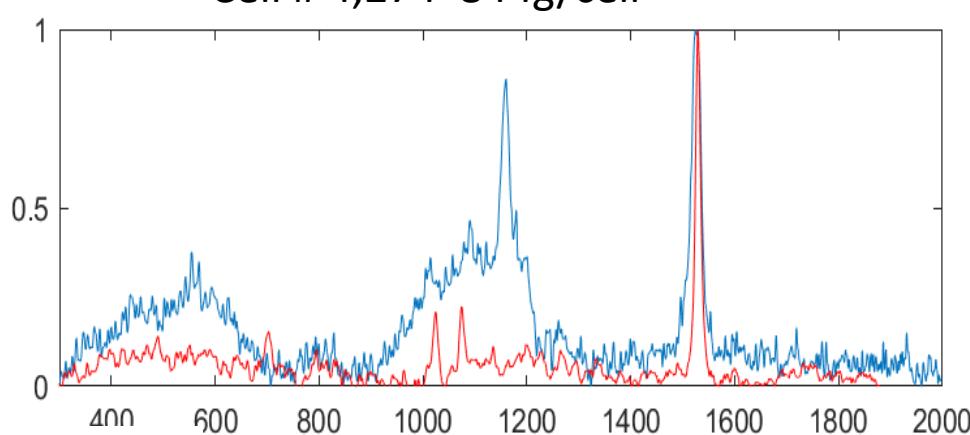


Cell # 28,473 9.5 fg/cell

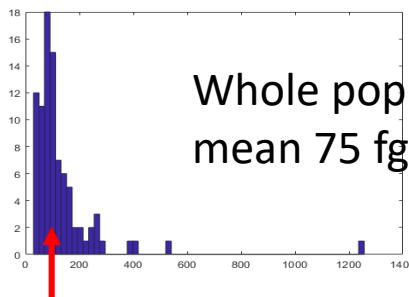


PN cell

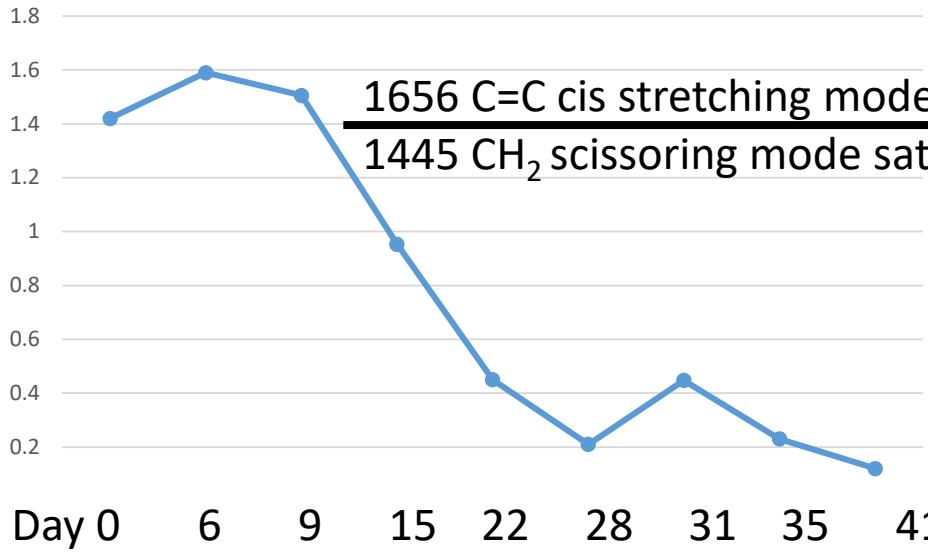
Cell # 4,274 84 fg/cell



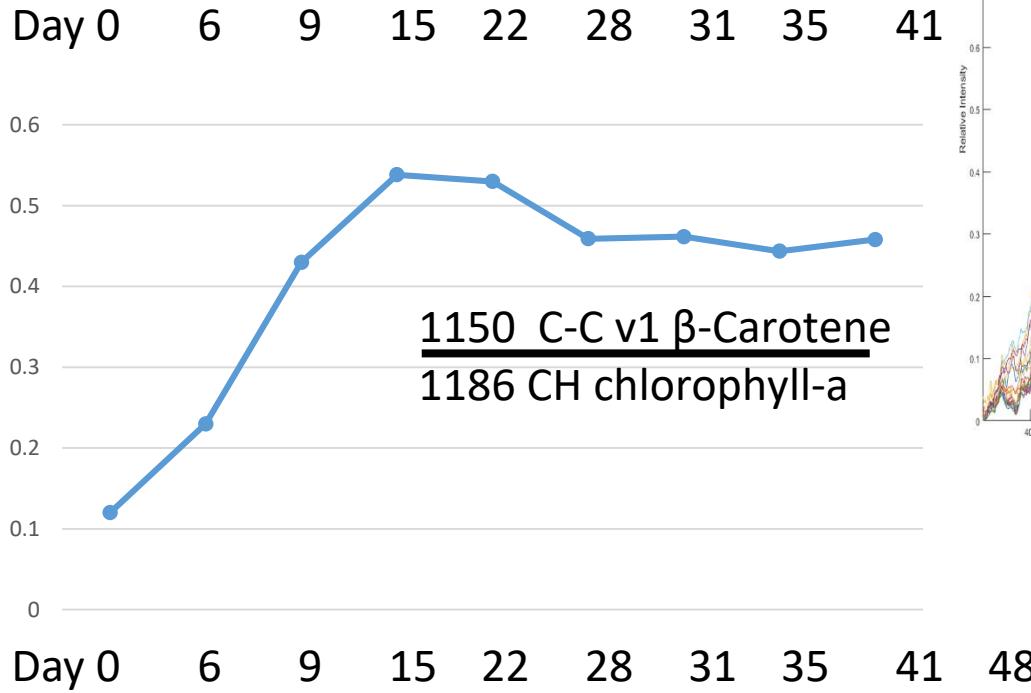
Whole population distribution
mean 75 fg/cell 166 cells



Can We Track Cell Dynamics in a Bloom?

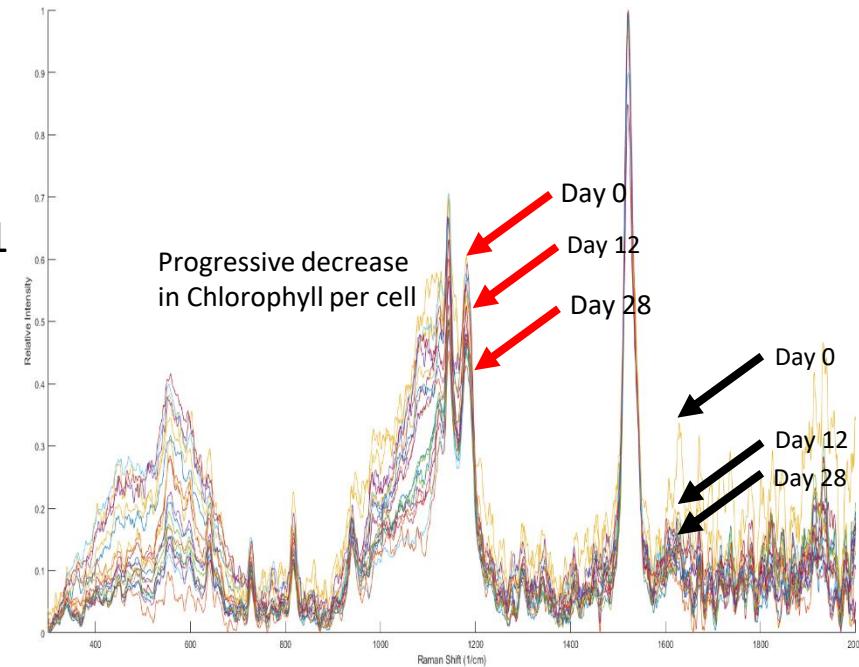


1656 C=C cis stretching mode unsaturated FA
1445 CH₂ scissoring mode saturated FA



1150 C-C v1 β-Carotene
1186 CH chlorophyll-a

Alexandrium catenella ATMP7-E8



Progressive decrease
in degree of unsaturation

Conclusions and Implications for Further Research

- Raman spectroscopy can be used directly in natural seawater to discriminate between HAB species and non-toxic cells, and some toxins.
 - Real-time, automated distributed array of sensors to detect HABS on large scales when HAB species are present before they form blooms.
 - Future work: continue peak database, miniaturize flow cytometer
- Need beta testers to use instrument under various conditions- this summer

Acknowledgements



SBIR program

STTR program

