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RC Anderson and S. A. Connors

MICROBIAL HABITABILITY OF KILIMANJARO'S GLACIERS AND SOILS



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Near Sterile Environments

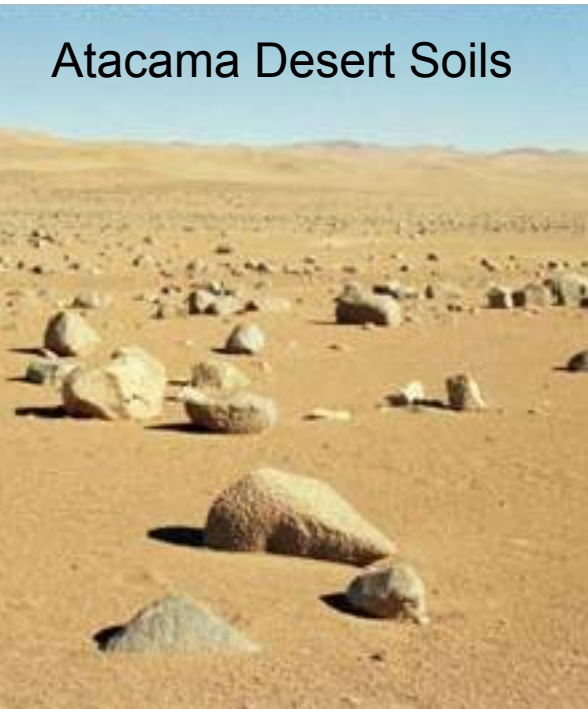
Mars Analogs for Life Detection



- Near sterile soils and ices (<10 CFU/g).
- Found in extreme deserts, glaciers, and volcanoes.
- Above 10^3 CFU/g, strong CFU to TOC correlation.
- Contain CFU hot spots → patchy microhabitats.
- Culturing is standard for viability assessment, but VBNC population is >90% of environmental microbes.



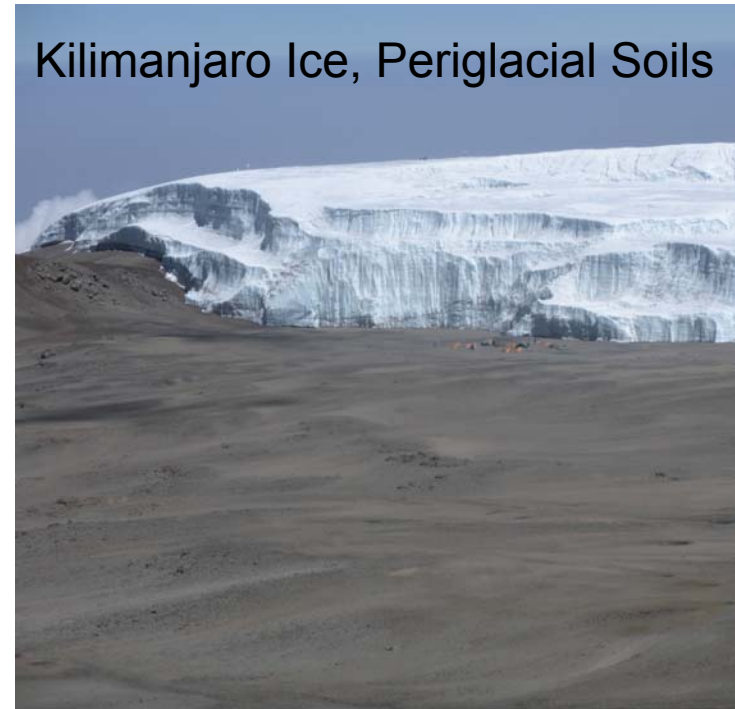
Atacama Desert Soils



Polar Ices



Kilimanjaro Ice, Periglacial Soils

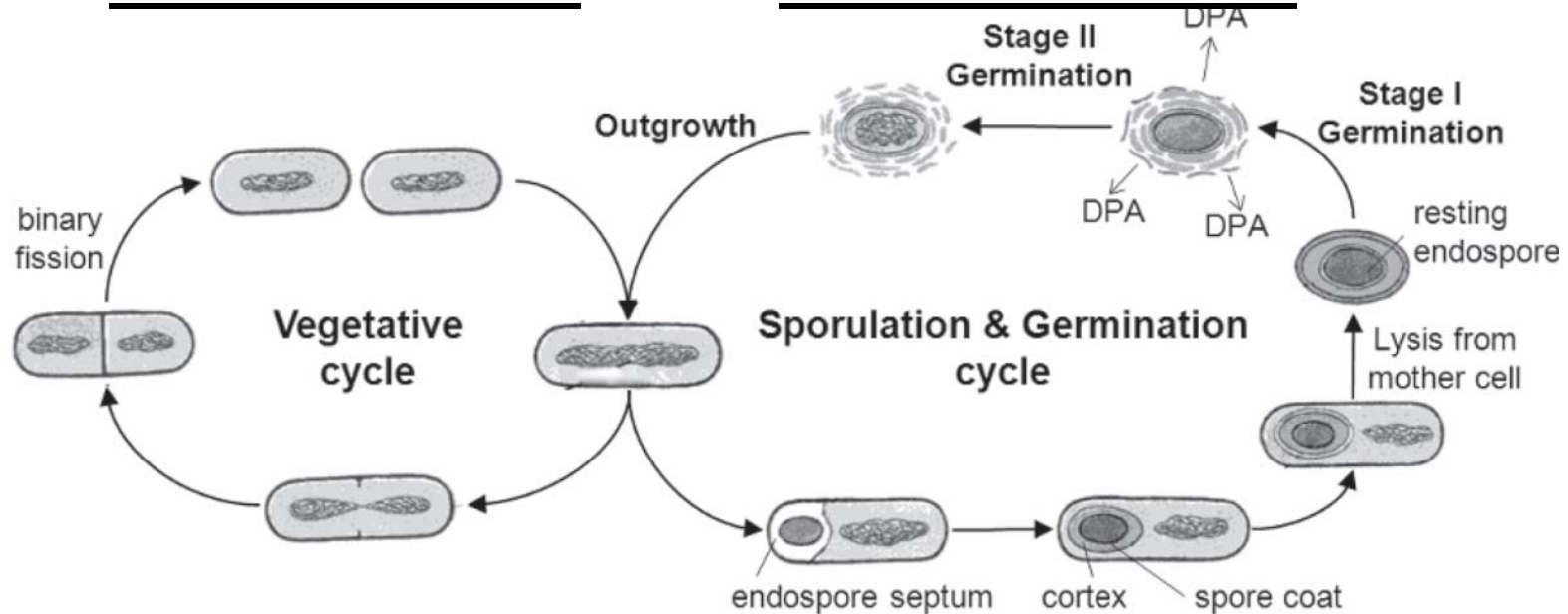




Viability Assessment

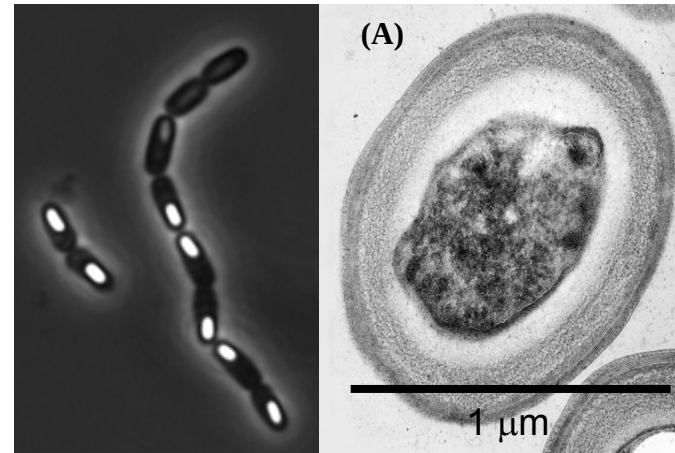
Growth Phase

Dormant Phase



- Measure germinable spores, including germinable-but-not-culturable population.

- Aerobic species: *Bacillus*
- Anaerobic species: *Clostridium*





Bacterial Spores

The Toughest Microbial Form of Life

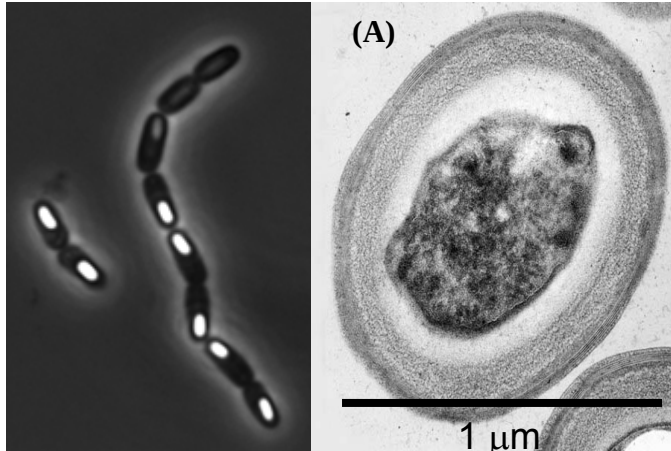
Planetary Protection



Biodefense and Restoration



Survival and Growth in Extreme Environments



Bacillus and Clostridium species

Increasing Resistance

**Bacterial
Spores**

Cryptosporidiidae

Mycobacteria

Small Viruses

Fungi

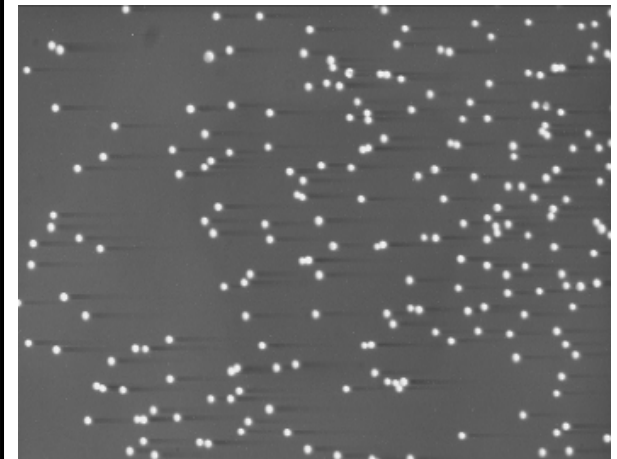
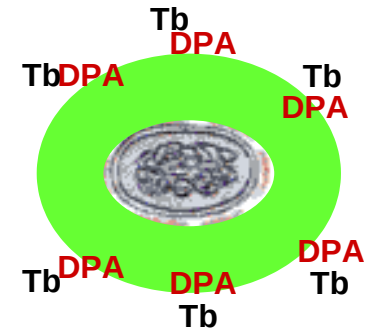
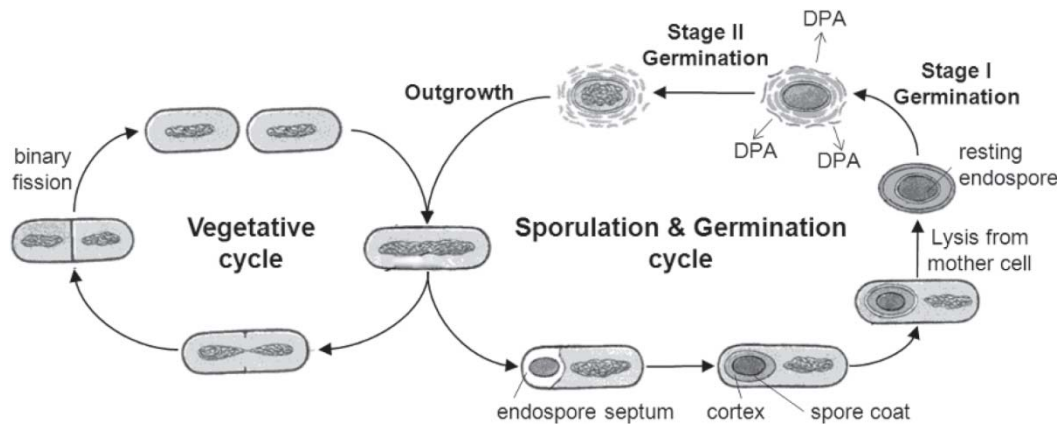
Vegetative bacteria

Lipid Viruses

Germinable Spore Assay



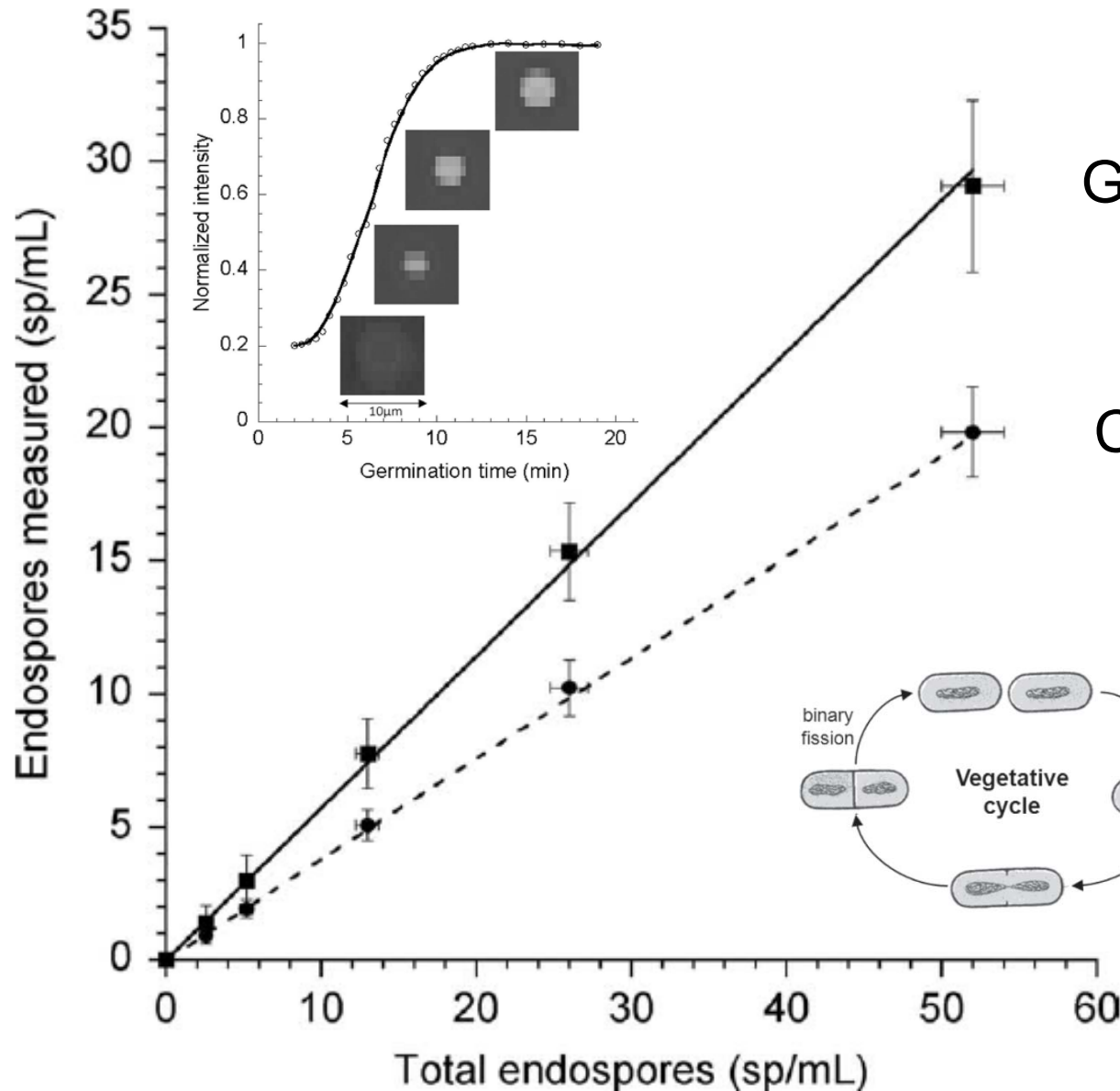
Fast Validation of Pathogen Inactivation:



Germinable Spores

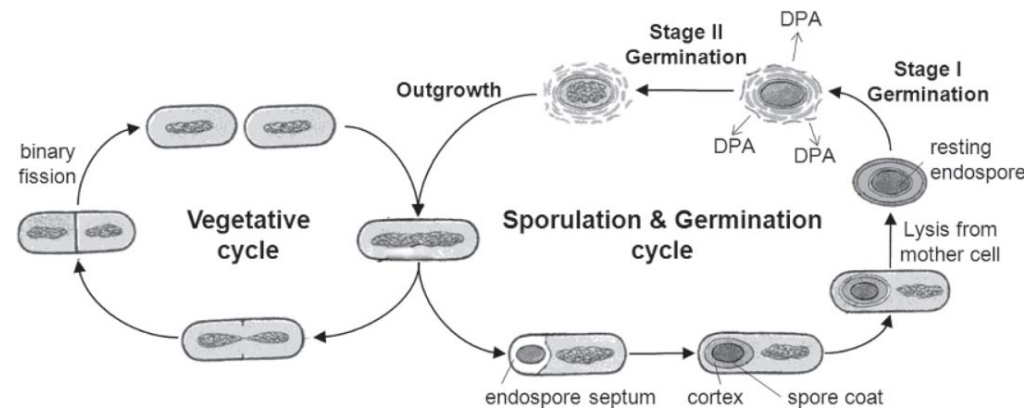
Germinable Spore Assay

Validation Experiments



Germination

Colony Formation

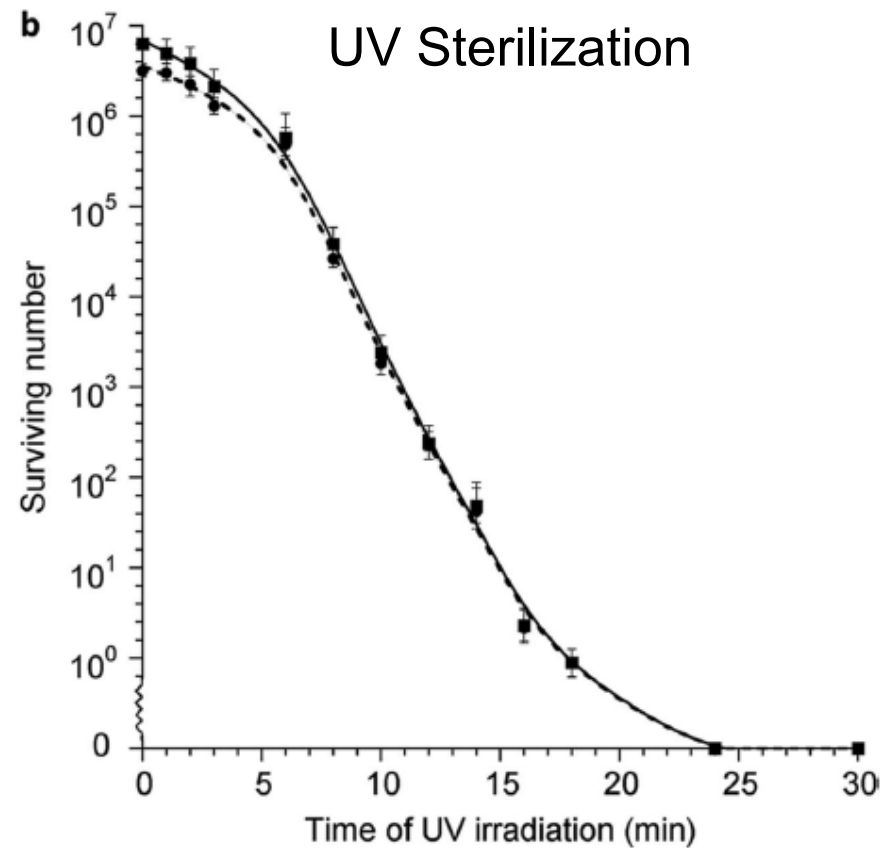
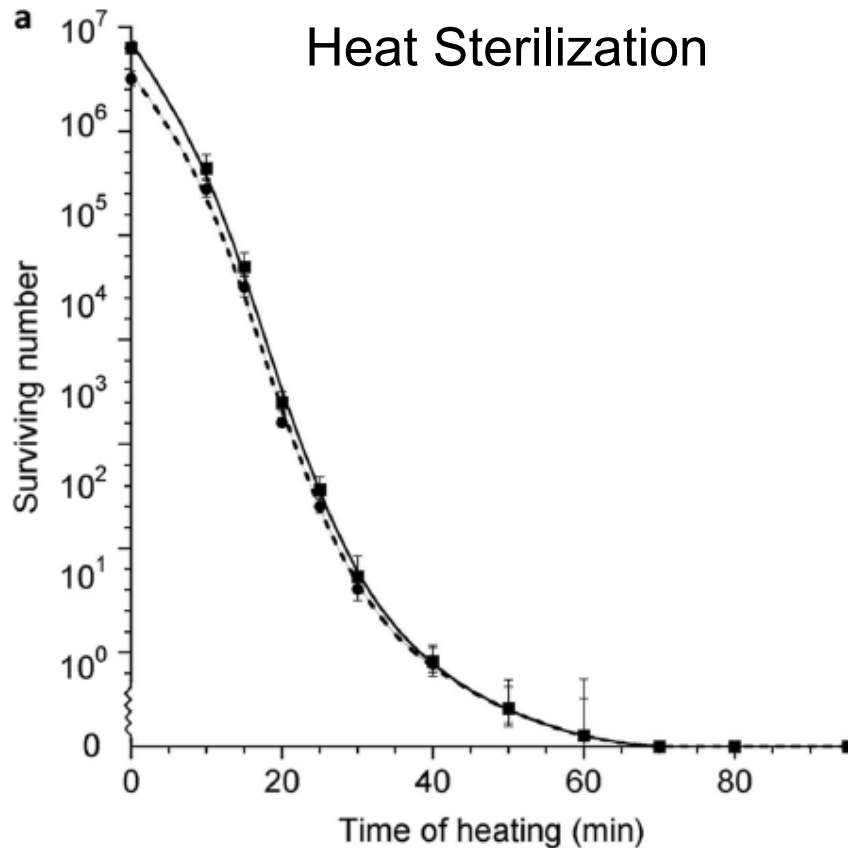




Germinable Spore Assay

Monitoring Inactivation Processes

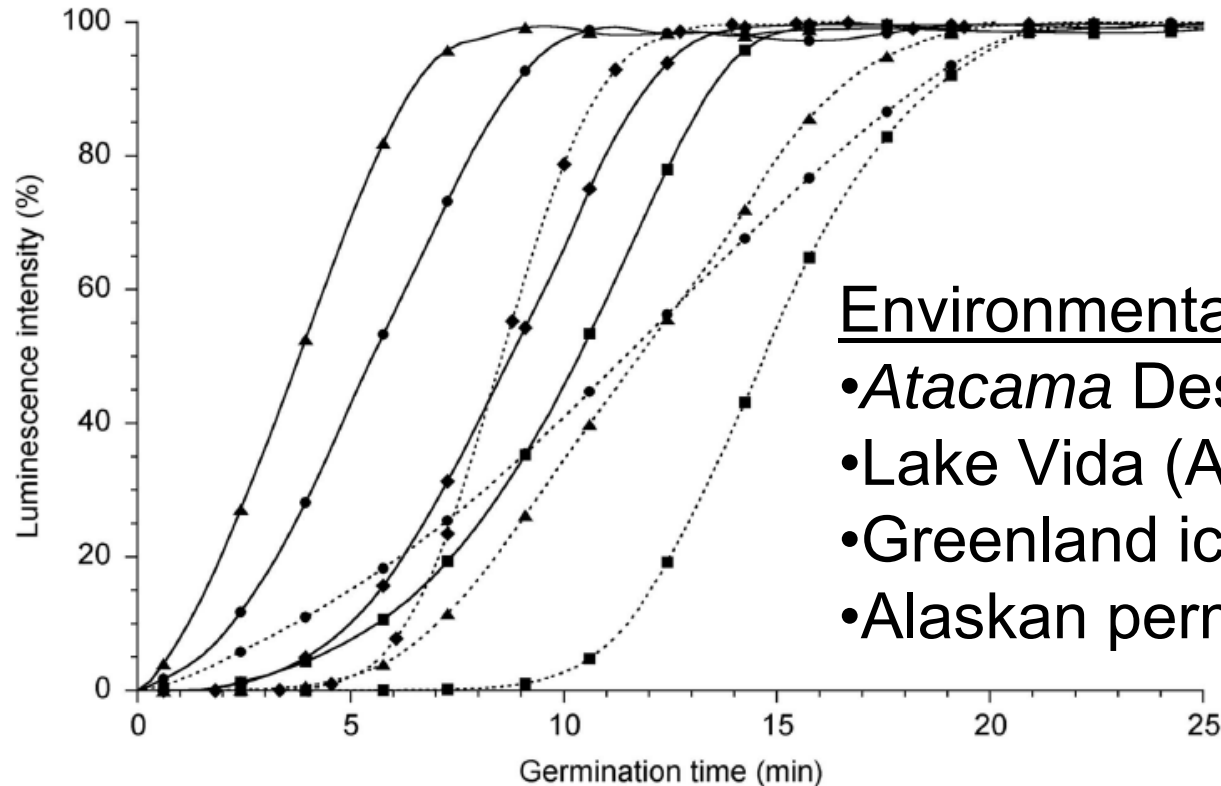
Bacillus spores in aqueous suspension





Germinable Spore Assay

Samples from Extreme Environments



Environmental Samples

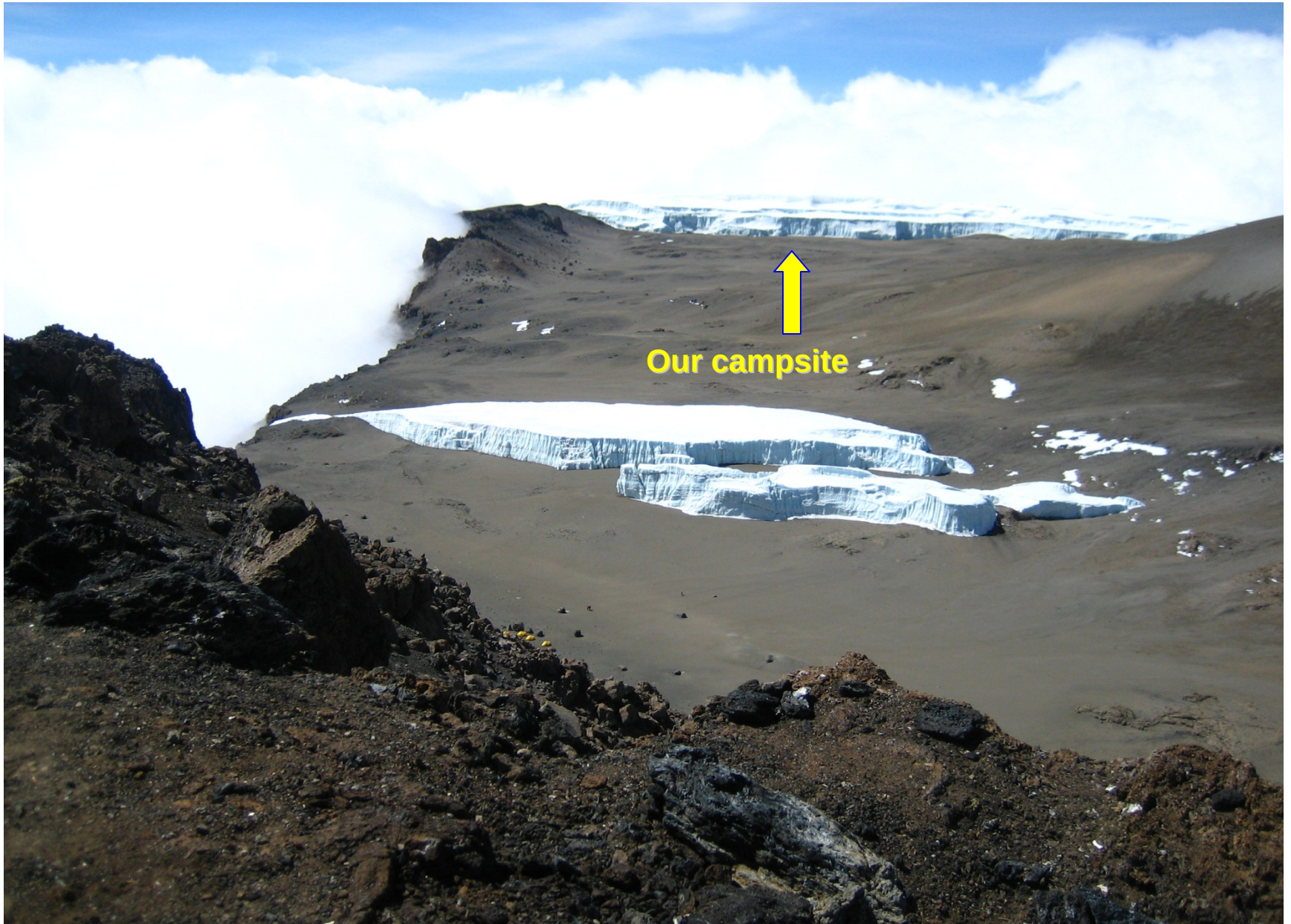
- Atacama Desert (Chile)
- Lake Vida (Antarctica)
- Greenland ice core (GISP2)
- Alaskan permafrost (Barrow)

1. Yang, WW.; Ponce, A. *Appl. Environ. Microbio.*, 2011 (in press).
2. Yang, WW.; Ponce, A. *Int. J. Food Microbio.*, 2009, 133, 213-216.
3. Yang, WW.; Crow-Willard, E.; Ponce, A. *Appl. Microbio.*, 2009, 106(1), 27-33.
4. Detection and viability assessment of endospore-forming pathogens. Ponce, A.; Connon, S.A.; Yung, P.T. In *Principles of Bacterial Detection: Biosensors, Recognition Receptors and Microsystems*, Eds. Zourob, M.; Elwary, S.; Turner, A., Springer Science Business Media, 2008.
5. Yung, P.T.; Ponce, A. *Appl. Environ. Microbio.*, 2008, 74(24), 7669-7674.
6. Connon, S.A.; Lester, E.D.; Shafaat, H.S.; Obenhuber, D.C.; Ponce, A. *J. Geophys. Res.*, 2007, 112(G4): Article No. G04S17.
7. Yung, P.T.; Shafaat, H.S.; Connon, S.A.; Ponce, A. *FEMS Microbiology Ecology*, 2007, 59, 300-306.
8. Lester, E.D.; Satomi, M.; Ponce, A. *Soil Biology & Biochemistry*, 2007, 39, 704-708.
9. Shafaat, H.S.; Ponce, A. *Appl. Environ. Microbio.*, 2006, 72(10), 6808-6814.

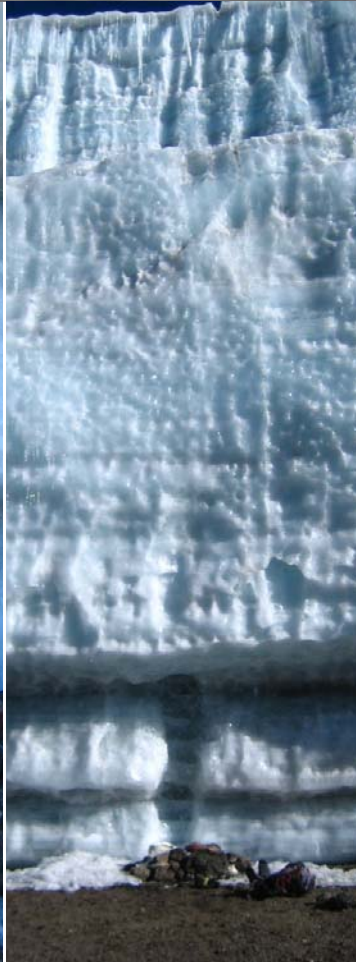
Kilimanjaro







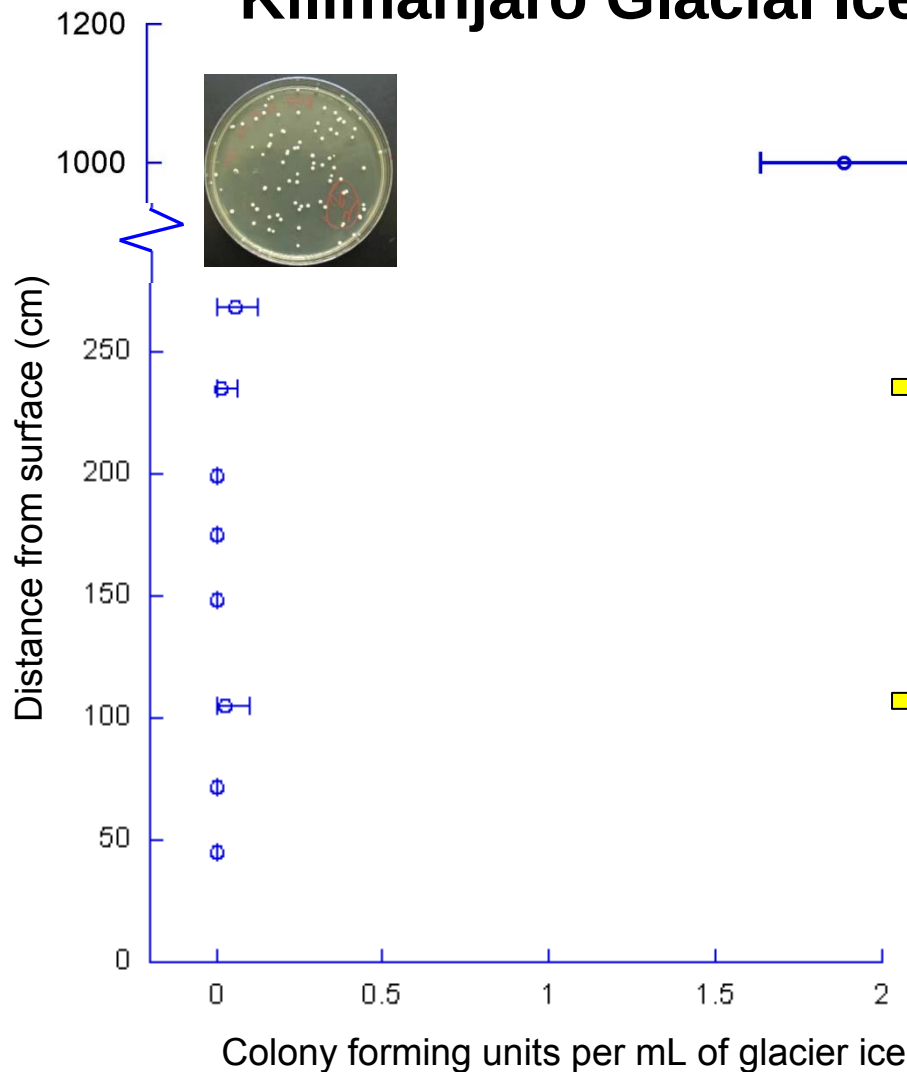






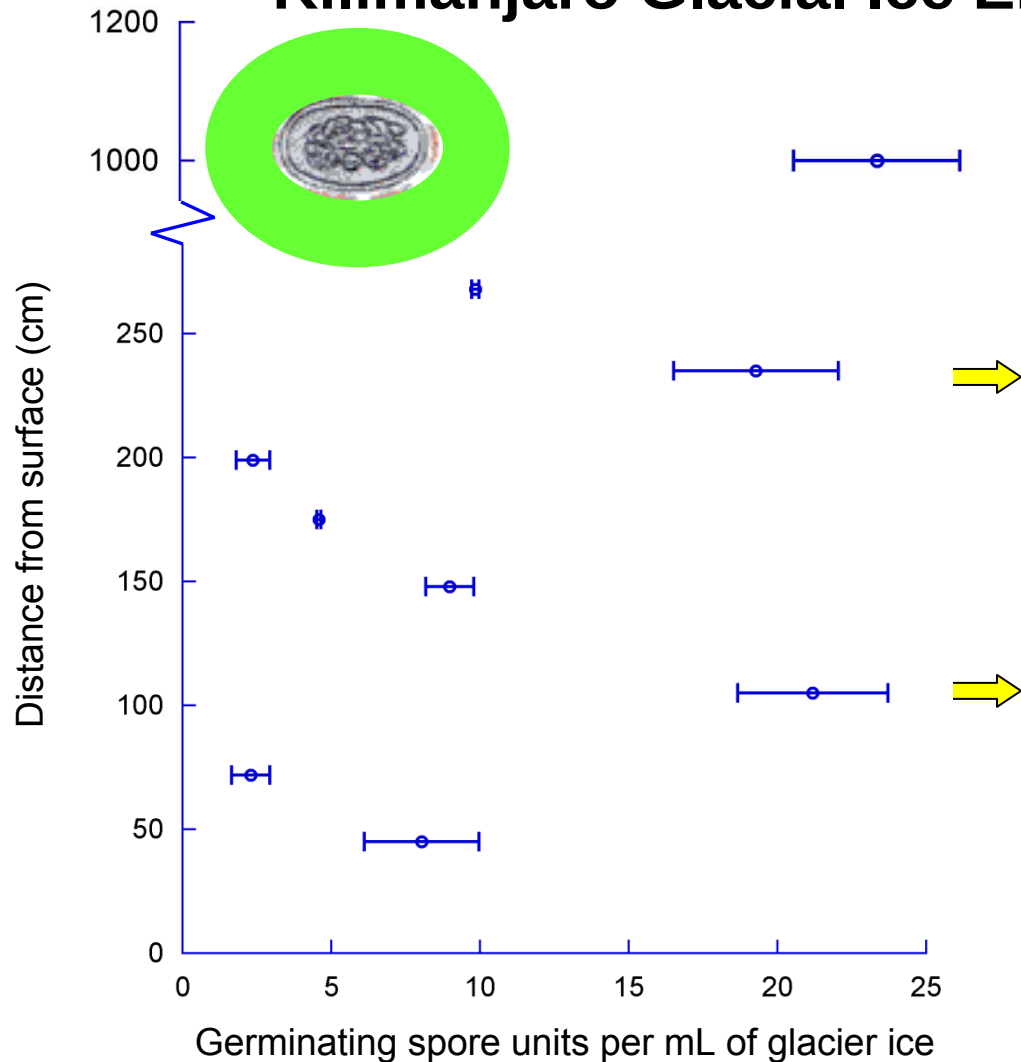


Kilimanjaro Glacial Ice Endospore Concentrations



Culturable population in ice is ~0 spores/L.

Kilimanjaro Glacial Ice Endospore Concentrations



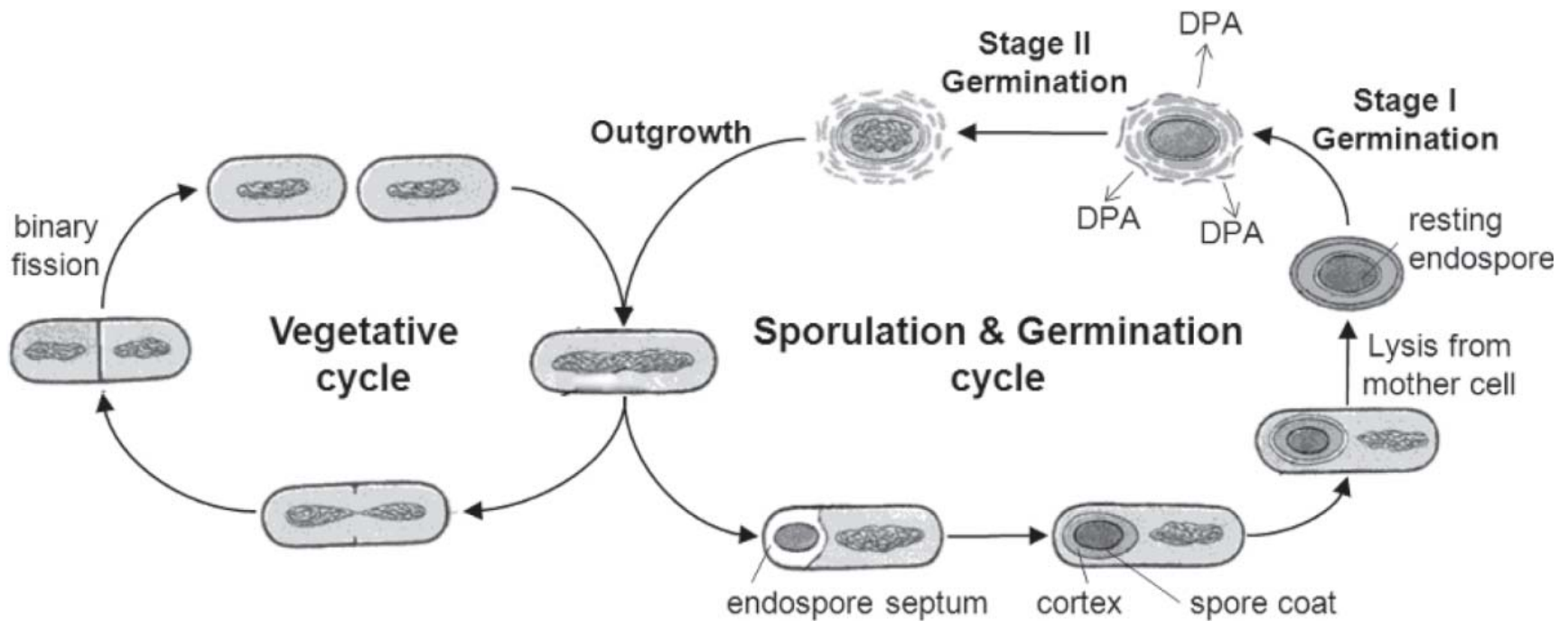
- Germinable-but-not-culturable population in ice is $\sim 10^3$ - 10^4 spores/L.



Viability Assessment

Growth Phase

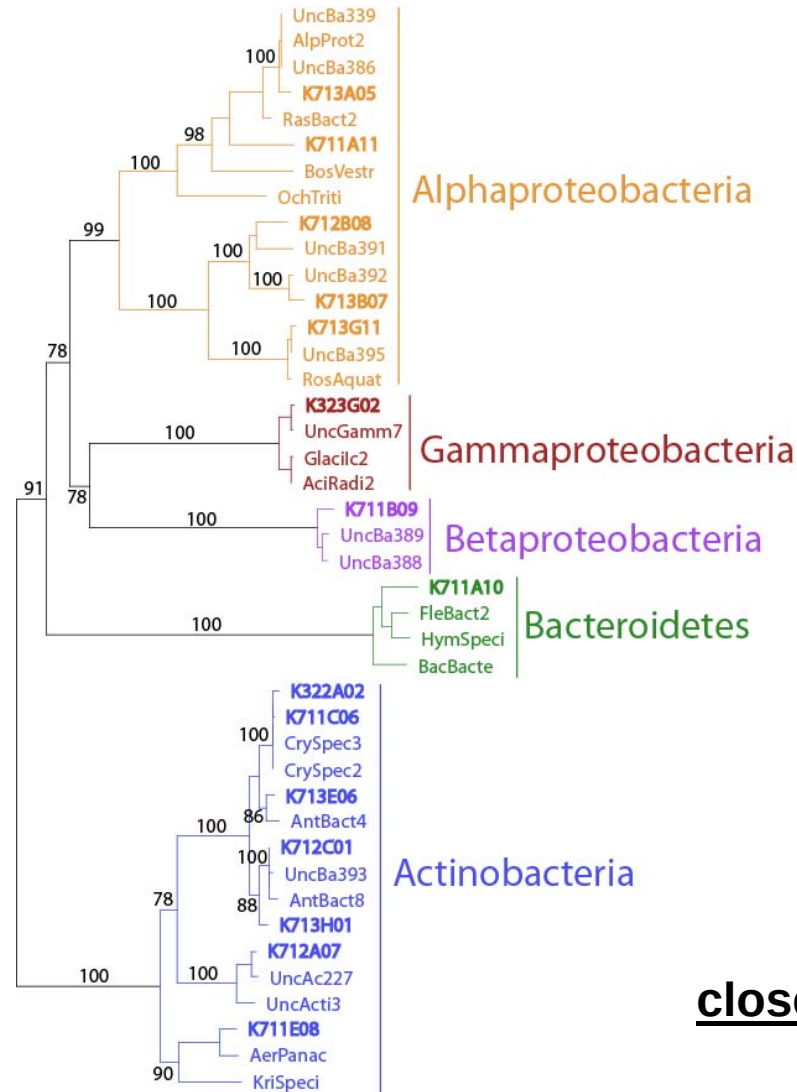
Dormant Phase



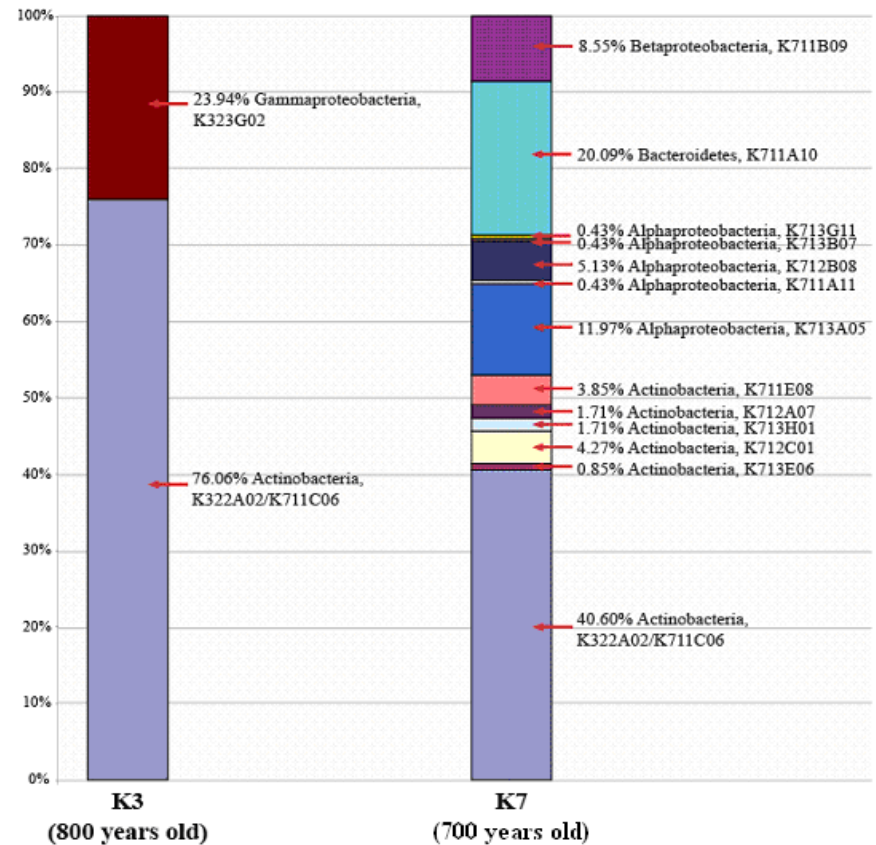


Supraglacial Habitat

Microbial Growth in Meltwater Ponds



— 0.01 substitutions/site



closest relative from a cold water environment

100%

55%

Supraglacial Habitat

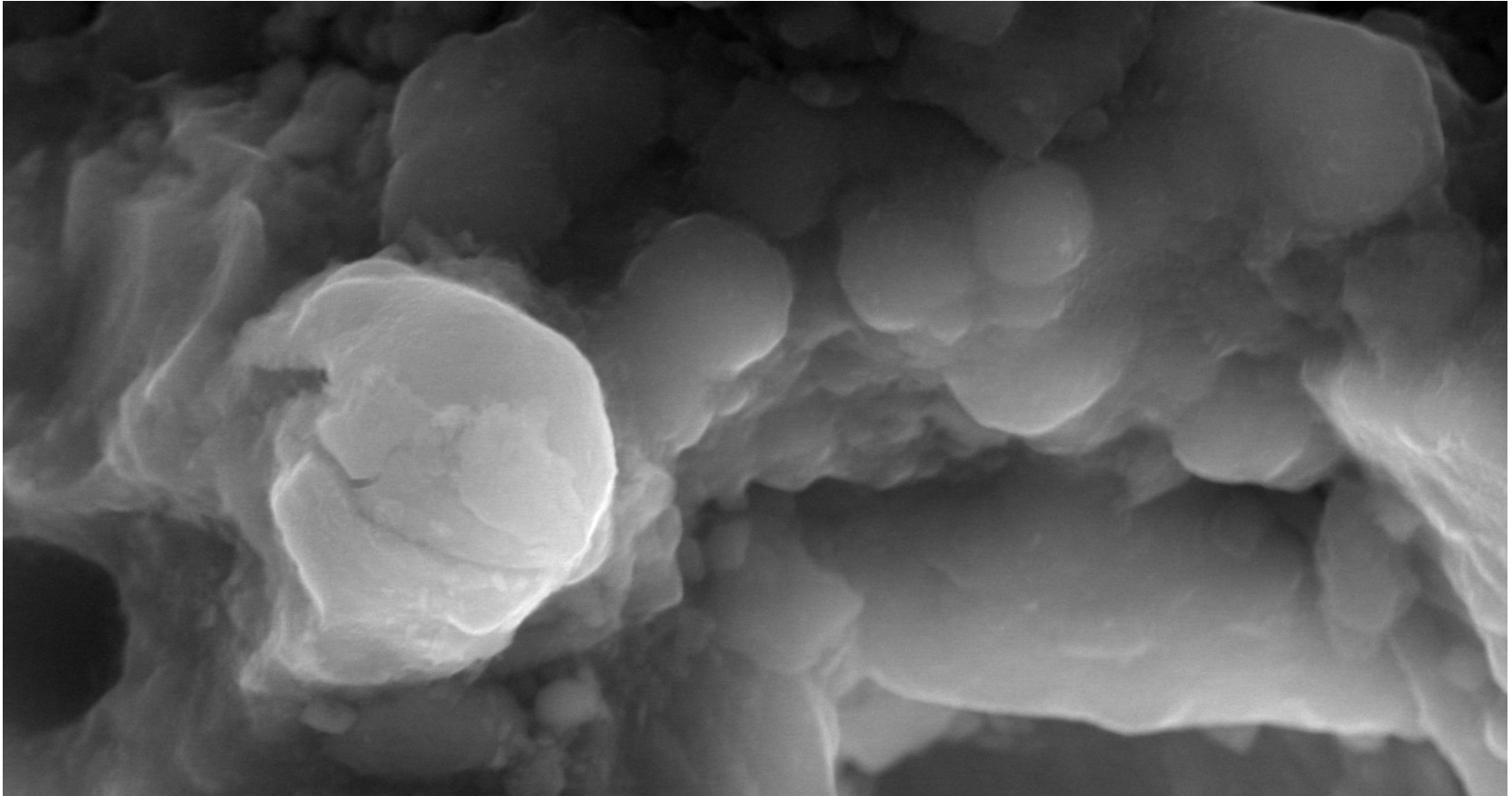


- Large fraction of cold-water species suggests microbial activity in a cold-water ecosystem at the time of deposition.
- Discovered a 1-foot deep, mud-rich meltwater pond on glacier.





Growth in Periglacial Soils



- Periglacial soils and ice dust are volcanic ash.
- Biofilm on some particles (Morphology, EDX).
- No pollen.



Conclusions

- Dust layers contain volcanic ash, not soil particles from regions below summit.
- Germinable-but-not-culturable population in ice is $\sim 10^4$ spores/L.
- At the time of dust layer formation, the glacier surface hosted an active microbial, cold-water ecosystem.
- Radiocarbon dating of near basal dust layers suggest that NIF glacier formed around 12th century AD. Kilimanjaro ice core record should be reevaluated.
- Periglacial volcanic soils contain biofilms, indicating microbial activity.



Kilimanjaro as a Mars Analog

Future Directions



- Further investigate microbial diversity/activity of glacial dust layers, supraglacial pond and periglacial soils.
 - Which species are there? How are they making a living?
 - How are they interacting? Novel organisms special adaptations?
- High-elevation periglacial soils are among the most extreme but least studied soil systems on Earth.
 - Volcanic ash Fe, Al-silicate soils
 - Low organic carbon soils (<1mg/g)
 - Low water activity ($a_w < 0.6$)
 - Daily extreme freeze thaw cycle (-26 °C to 5 °C)
 - Vegetation coverage: None
 - High UV flux
 - Dynamic system, glaciers disappearing in 20-50 years.
 - Gradients in temperature (fumaroles) and water (melting glacier) enables investigations of a continuum of microhabitats.
- Better understanding habitability in Mars special regions, where oligotrophic, phyllosilicate rich regolith abuts polar ice caps.



\$ FUNDING \$
 NASA Astrobio, PP Programs
 DHS Biodefense Programs



CONGRATULATIONS!
YOU ARE NOW AT

UHURU PEAK, TANZANIA, 5895M AMSL.

AFRICA'S HIGHEST POINT
WORLD'S HIGHEST FREE-STANDING MOUNTAIN



Kilimanjaro as a Mars Analog

Characteristics



Site Name	Kibo
Latitude, longitude	Latitude: -3.06667, Longitude: 37.35
Elevation, Areal Extent	5.9 km, 2 km by 2 km
Prime Science Questions	What is the organic carbon threshold in Fe-, Al- silicate soils for microbial viability?
Distance of Science Targets from nearest road or airstrip	From nearest road, it is a 6 days hike to reach Kilimanjaro summit.
Environmental characteristics	Max temp: 5 °C, Min temp: -26 °C Precipitation: 100 mm, Vegetation coverage: None
Previous studies	2 manuscript in preparation.
Primary Landing Site Target	Phylosilicate rich landing sites.

Near Sterile Environments

Mars Analogs for Life Detection

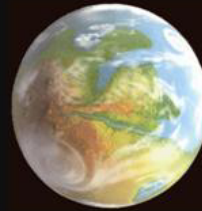


- Near sterile soils (<10 CFU/g) are only found in some extreme arid desert and volcanic soils.
- Culturing is standard method for viability assessment, but VBNC population is >90% of environmental microbes.
- Spores are toughest form of life and most likely interplanetary travelers to survive.
- Germinability assay detects much greater fraction of viable sporeforming microbes.
- In near sterile soils, hot spots due to favorable microhabitats dominate viable microbe distribution.
- Growth vs. dormancy.
- Growth limited by a_w , T, Corg and Delta(Eredox).
- Kilimanjaro has gradients in a_w , T, Corg across large range to enable systematic studies of microhabitats.
- Diversity on, in and around glacier and near fumaroles.²⁹

Mars Astrobiology Questions



← Viable Microorganisms →



Early Mars

- **Panspermia**
- **Natural transfer**
- **Strongly coupled biospheres**
- **Interplanetary lithoincocolation**

Earth Astrobiology Questions

Mojave Desert



Atacama Desert



Survival/Growth in Extreme Deserts.

Ice Cores

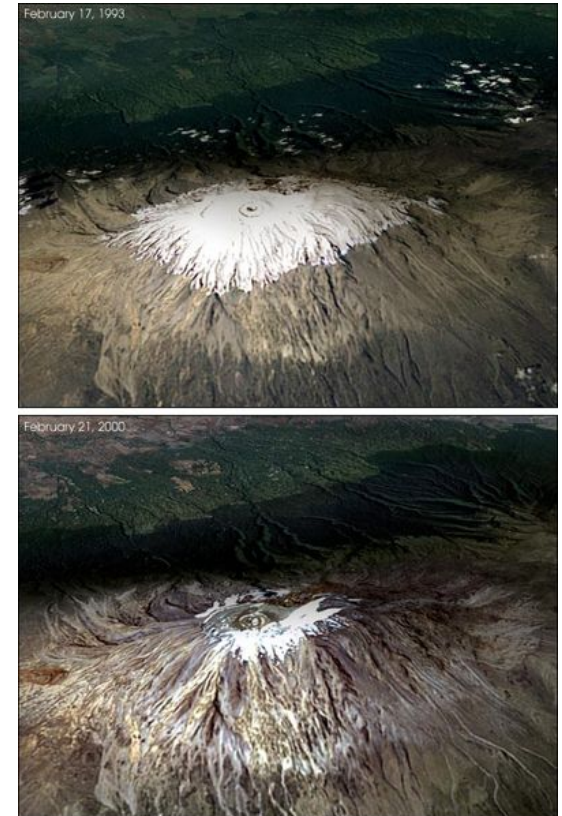
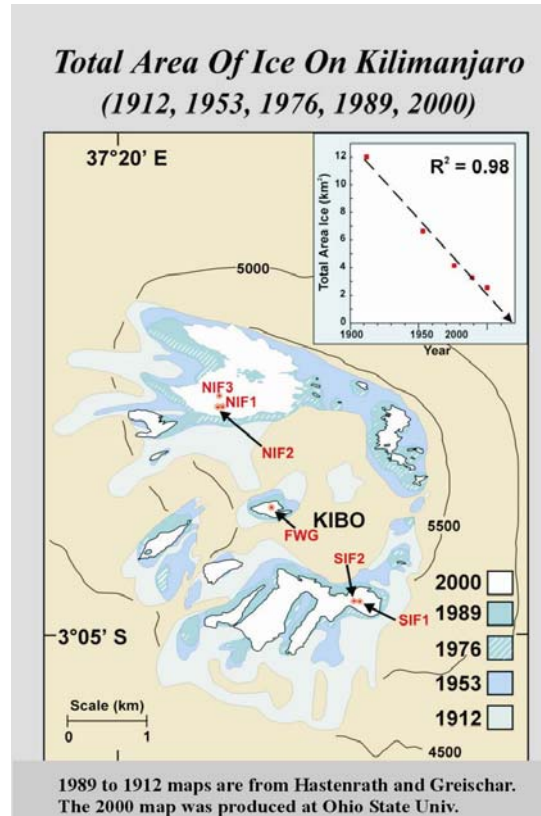
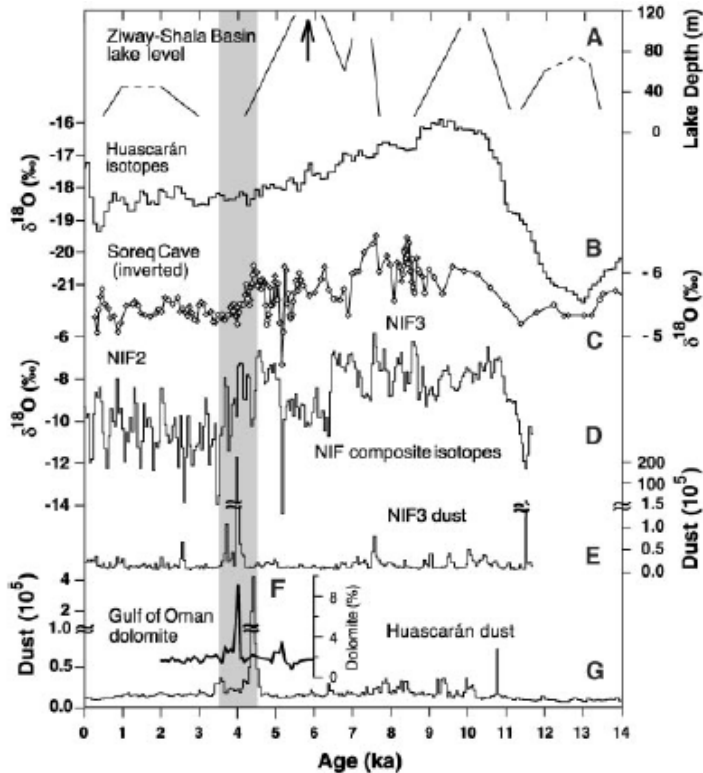


GISP2 Ice Core



Longevity Ices

Kilimanjaro Ice Core Climate Record



- Northern Ice Field (NIF) Glacier estimated to be 11,700 years old.
- Age based on $\delta^{18}\text{O}$ minima correlation to ^{14}C solar activity.
- $\delta^{18}\text{O}$, Na/F and dust proxies used to determine T and ppt record.
- Three major abrupt climate change events recorded in dust rich layers.
- Kilimanjaro Glaciers are estimated to disappear before 2050.



Kilimanjaro Glacial Record

Climate Change Impacts on Ecosystem?

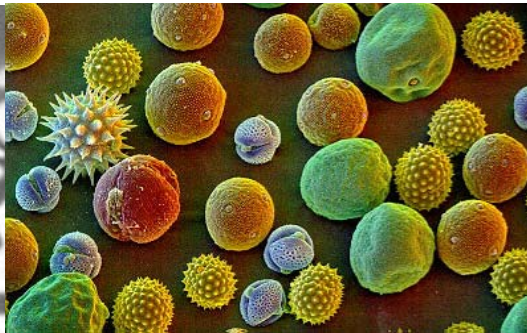
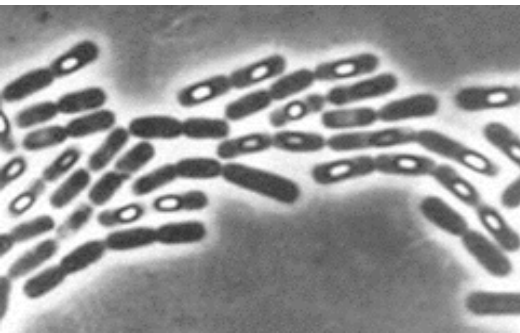
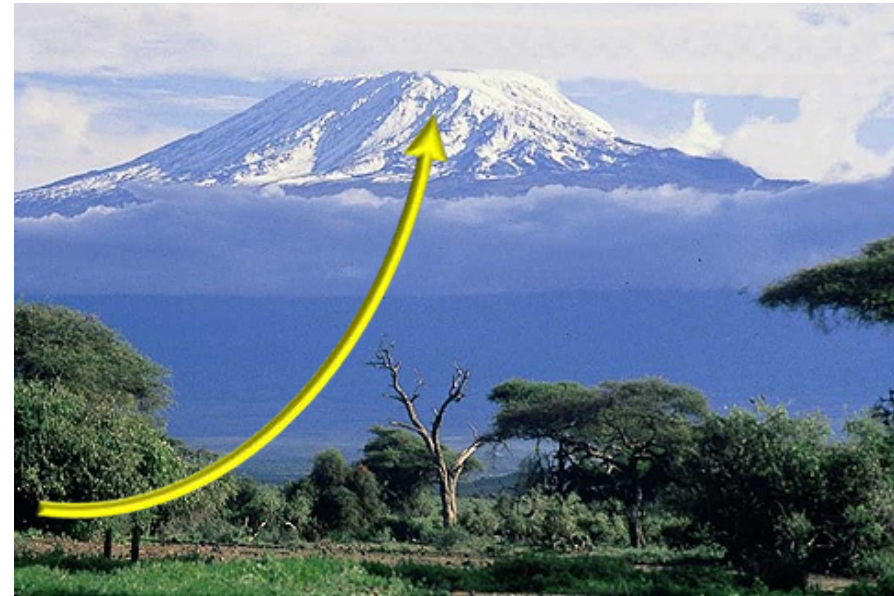


What is the impact of past climate change on ecosystem?

Measure microbial and pollen diversity as a function of time during past climate change.

Initial Assumptions

- Glaciers are ~12,000 years old.
- During droughts more dust gets carried to glaciers, forming layers.
- Microflora is attached to dust.





Soils from Surrounding Region



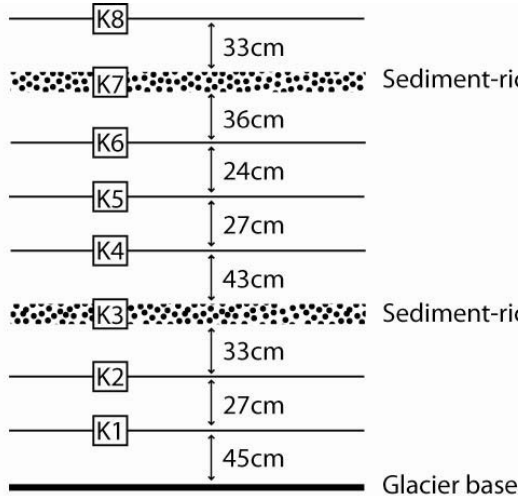
Ongoing work: Compare soils to dust from ice layers.



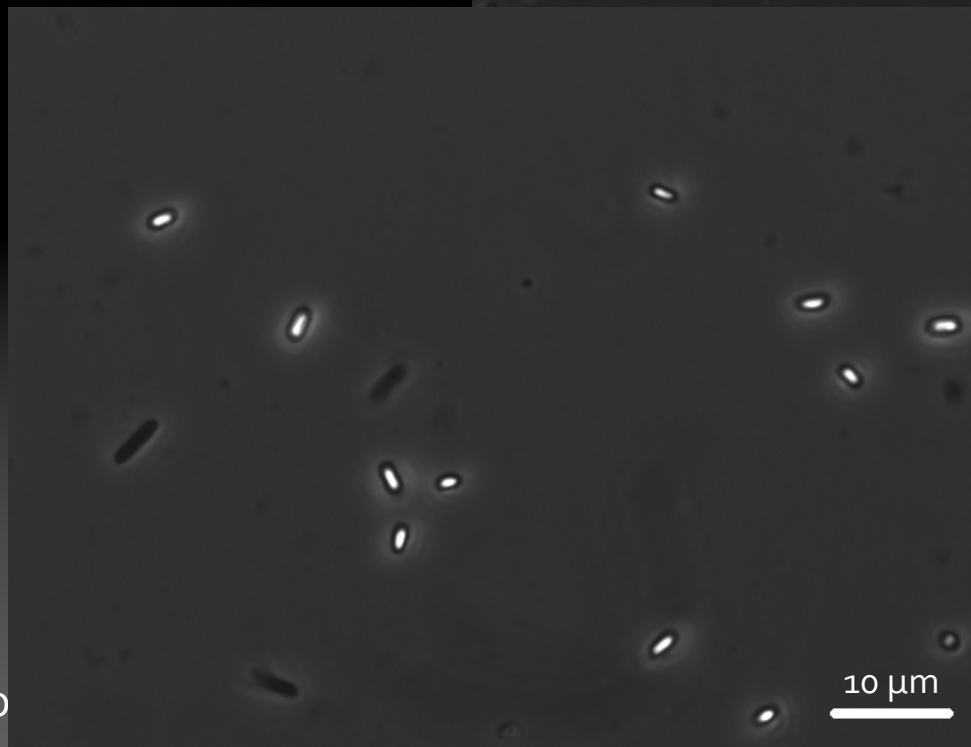
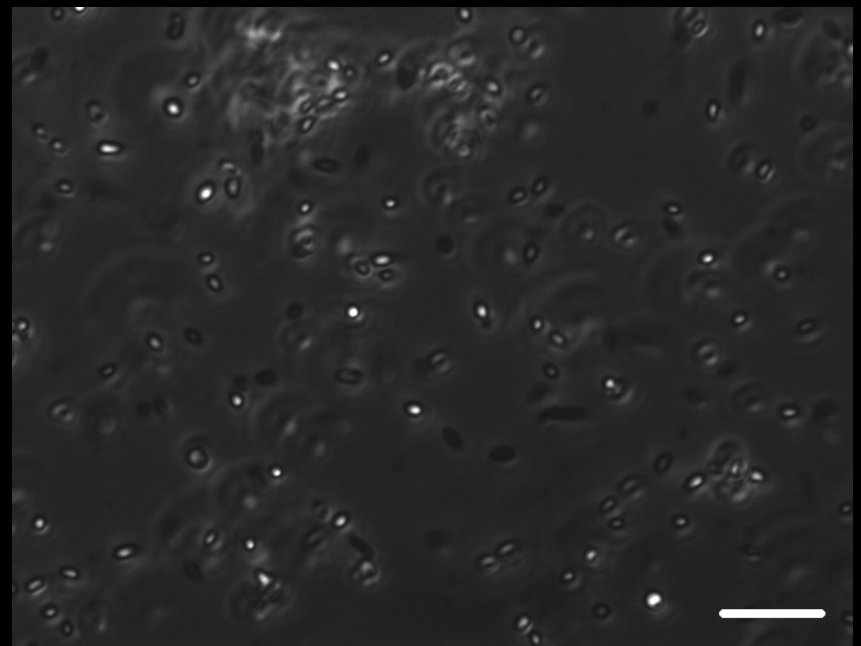
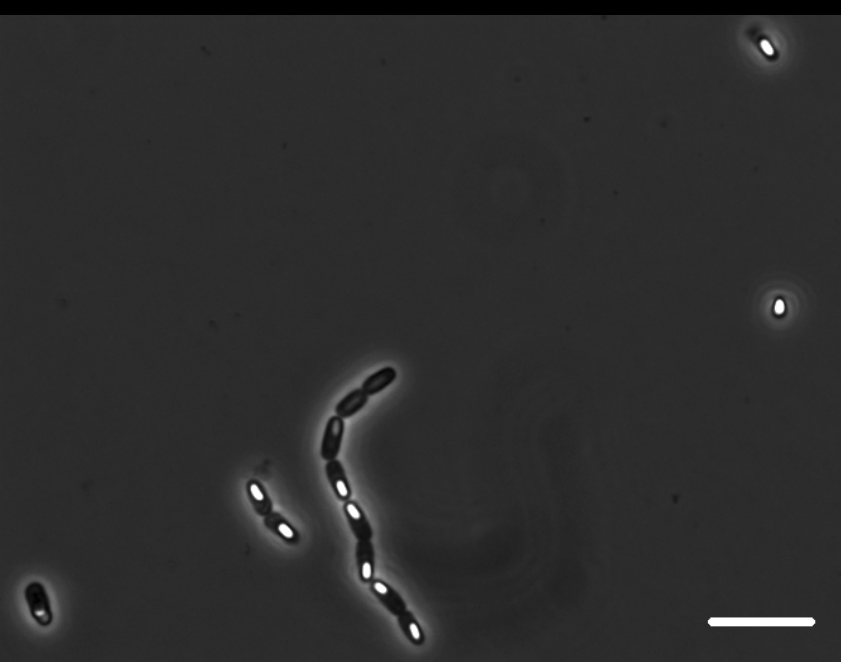
Age of NIF Kilimanjaro Glacier

^{14}C dates (AD)

1260-1320 (1290)
1350-1390



- This location of NIF does not contain Holocene ice.



Kilimanjaro

10 μm







A man and a woman are standing on either side of a large, rectangular stone sign in a lush, green forest. The man on the left is wearing a blue t-shirt, olive green cargo pants, a white cap with sunglasses, and a backpack. The woman on the right is wearing a teal t-shirt, light-colored cargo pants, and sunglasses on her head. The sign is made of stone and has yellow text on black rectangular backgrounds. The text on the sign reads: "NOTICE TO UMBWE ROUTE USERS THIS ROUTE IS FOR ASCENT ONLY YOUR DESCENT IS THROUGH MWEKA GATE". The background is filled with dense tropical vegetation and trees.

**NOTICE TO
UMBWE ROUTE
USERS
THIS ROUTE IS
FOR ASCENT
ONLY
YOUR DESCENT
IS THROUGH
MWEKA GATE**





Colobus
Monkey









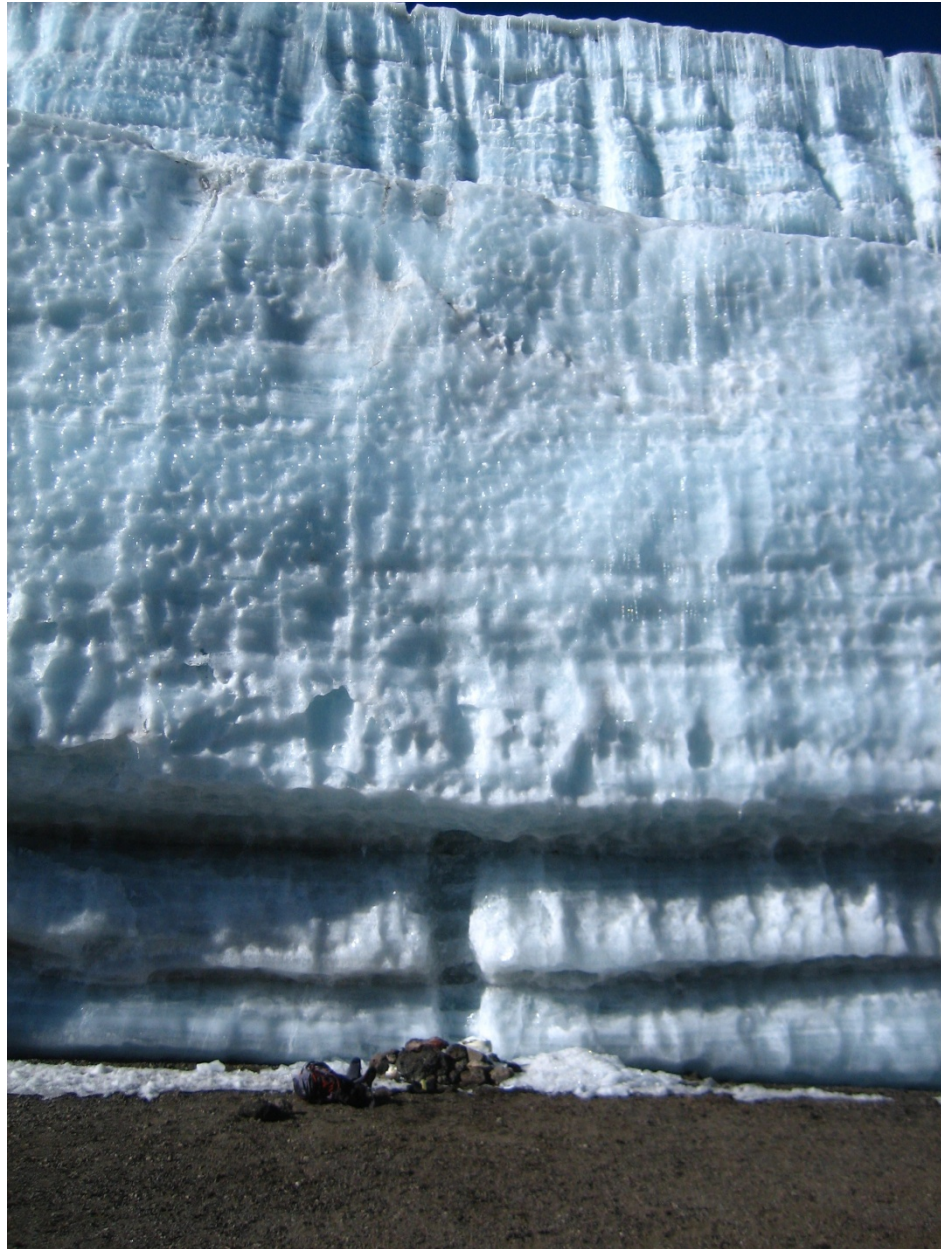




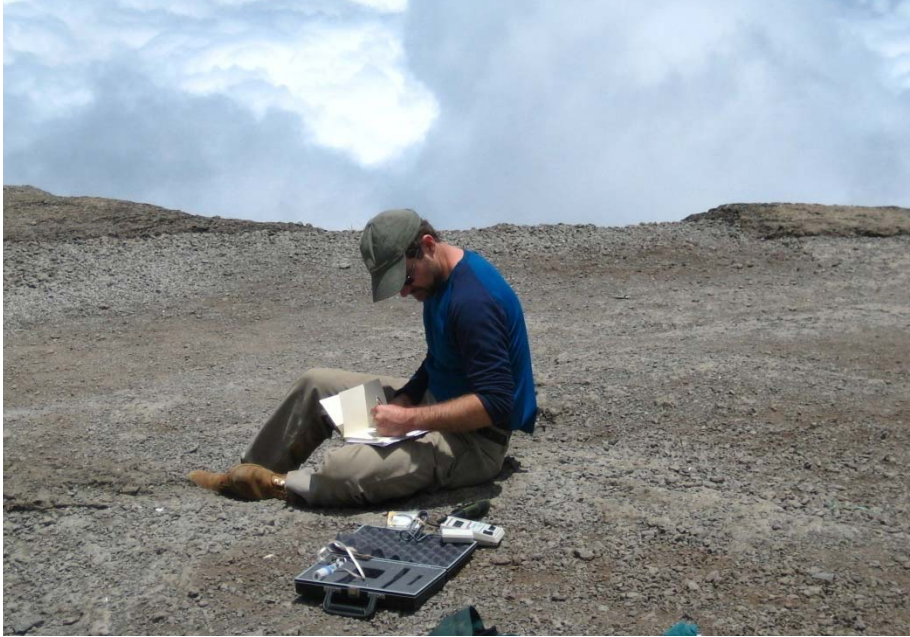




Sampling and Data Collection



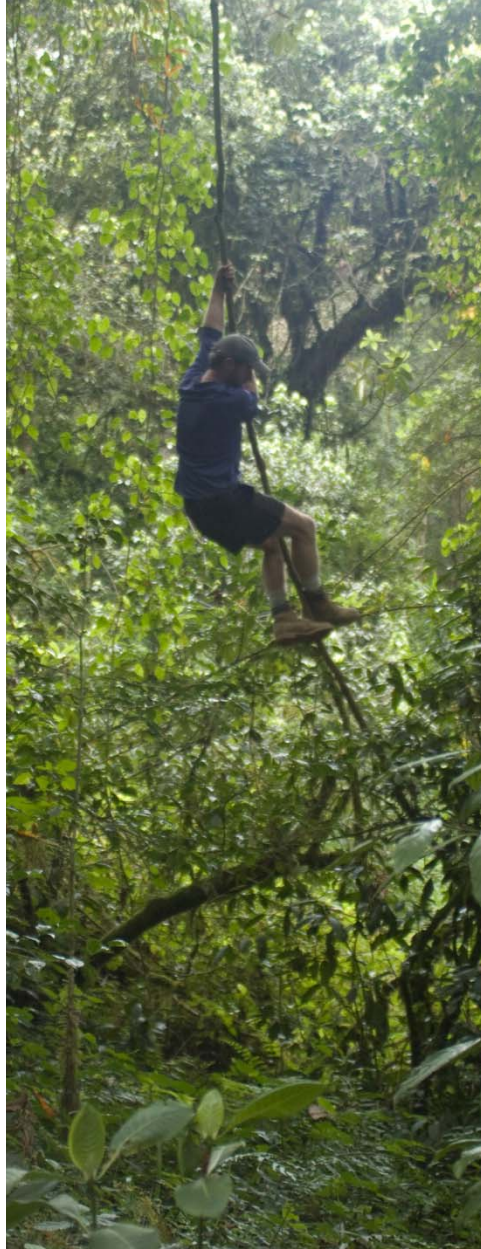
Sampling and Data Collection

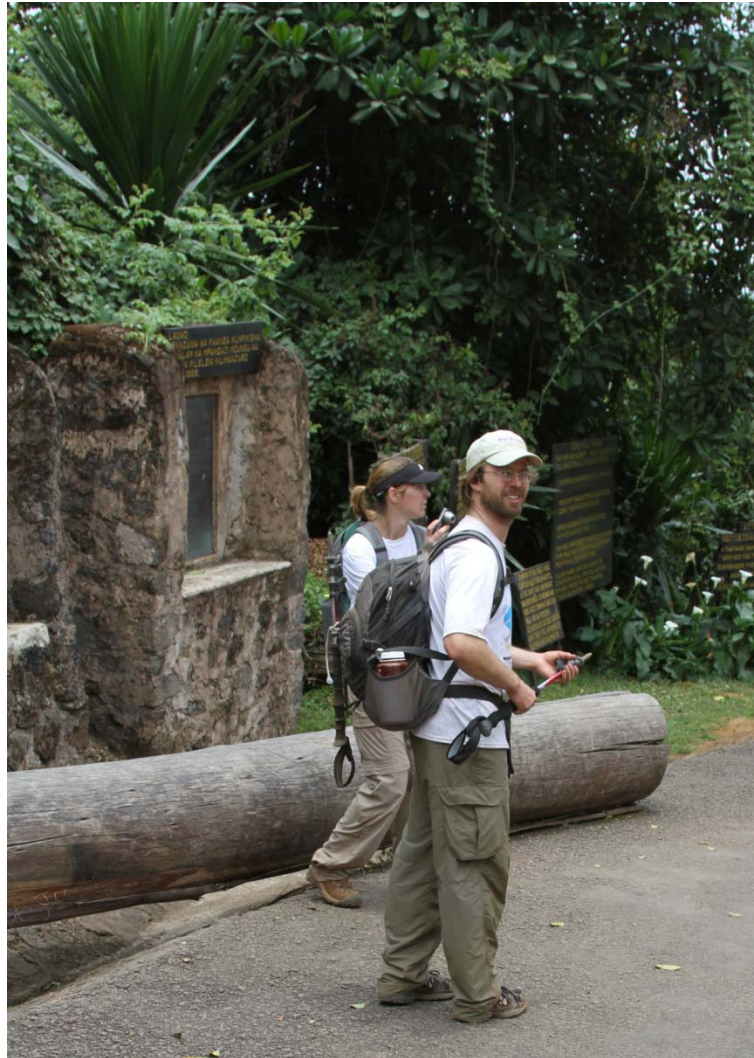


Sampling and Data Collection



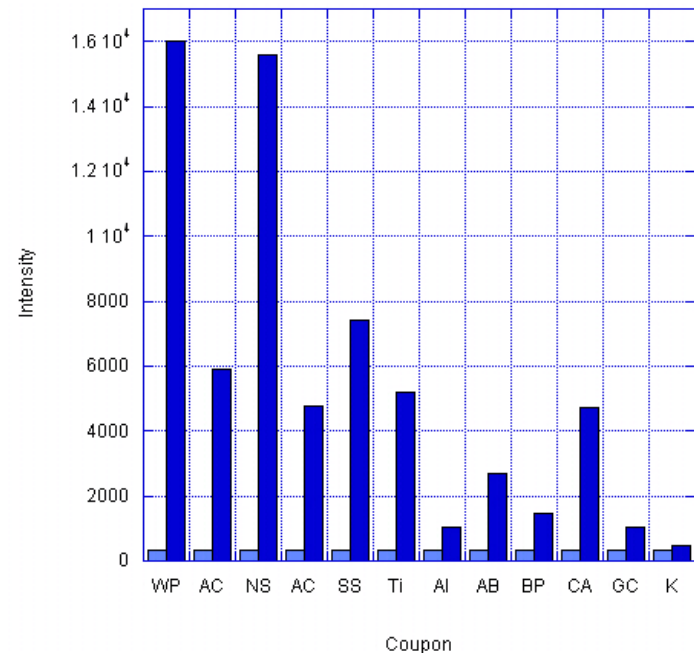
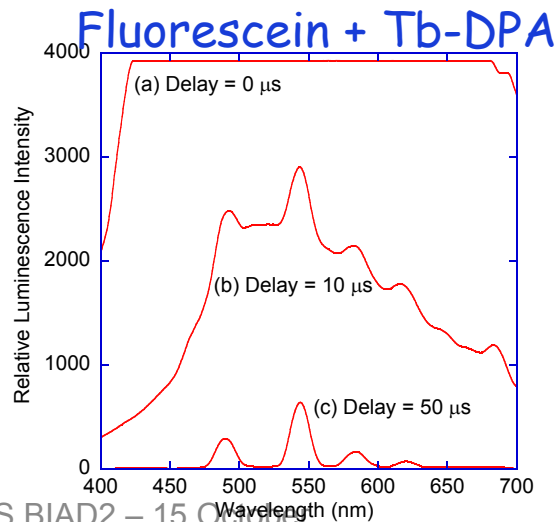
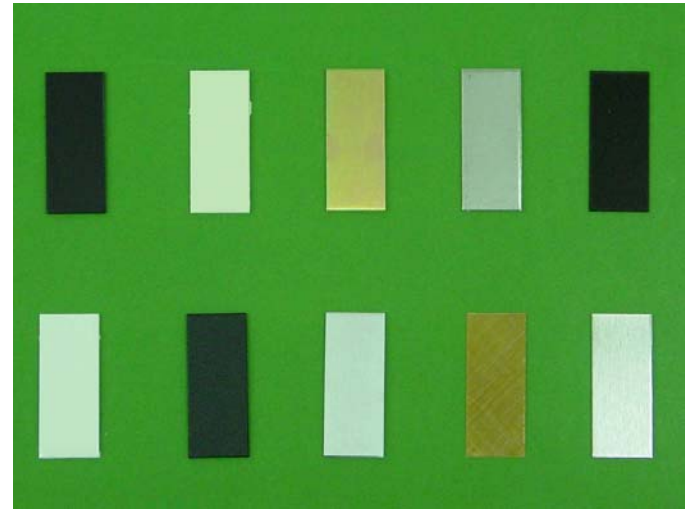
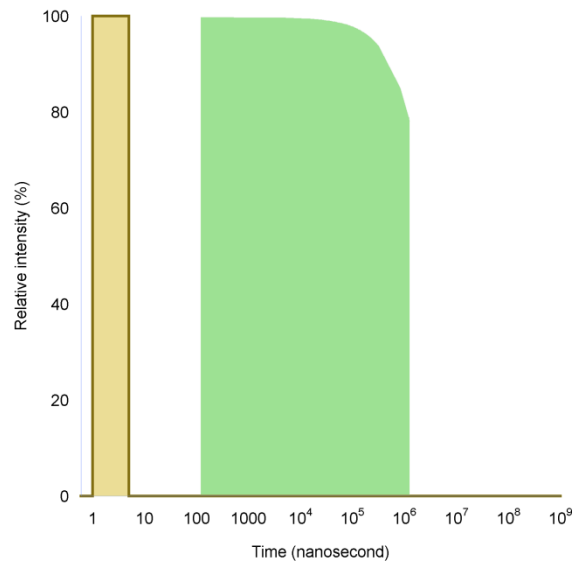


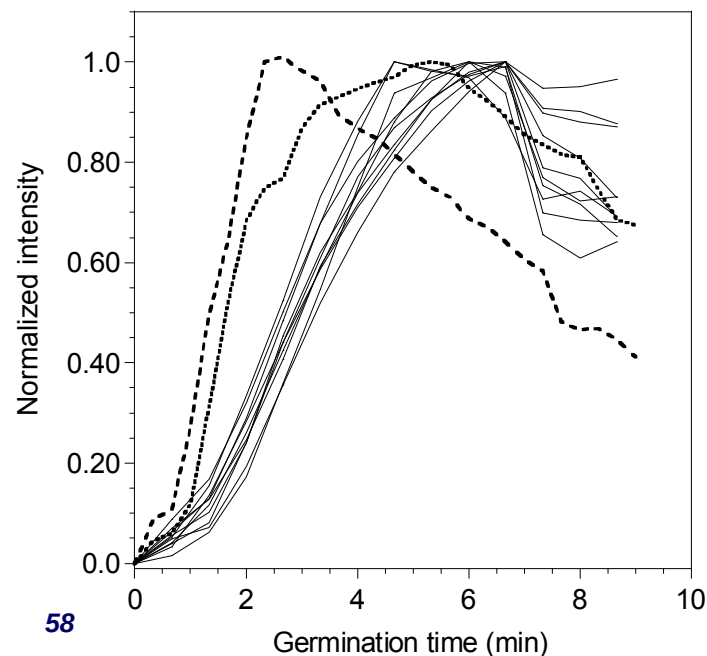
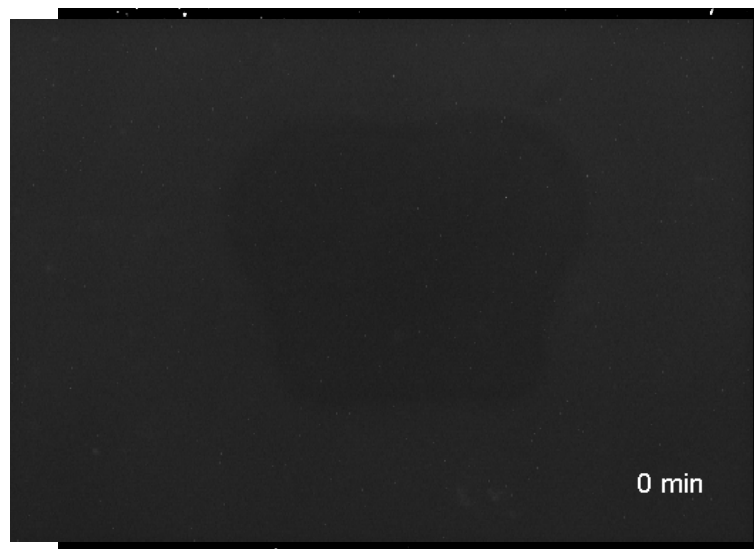
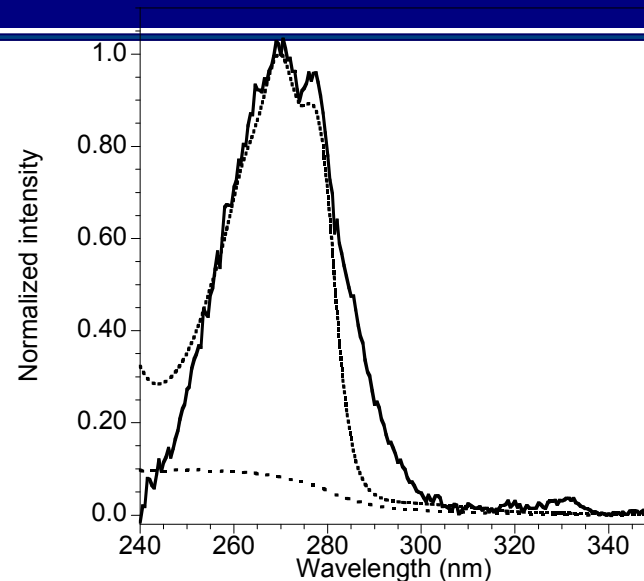
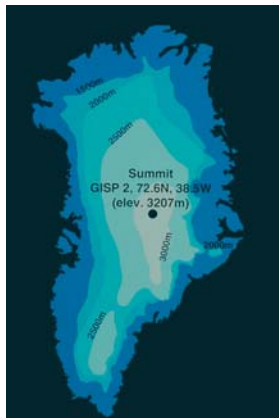




PARK HEADQUARTERS ALTITUDE 1970 M/6400 FT AMSL KILIMANJARO NATIONAL PARK MARANGU ROUTE.			
<u>PLACES.</u>	<u>E.T.A.</u>	<u>ALTITUDE.</u>	<u>VEG.ZONE.</u>
MANDARA	3HRS	2700M	FOREST
HOROMBO	5HRS	3720M	MOORLAND
KIBO	5HRS	4703M	ALPINE DESERT
GILMANS	5HRS	5685M	ALPINE DESERT
UHURU PEAK	1½HRS	5895M	ICE CAP

Lifetime Gated Detection



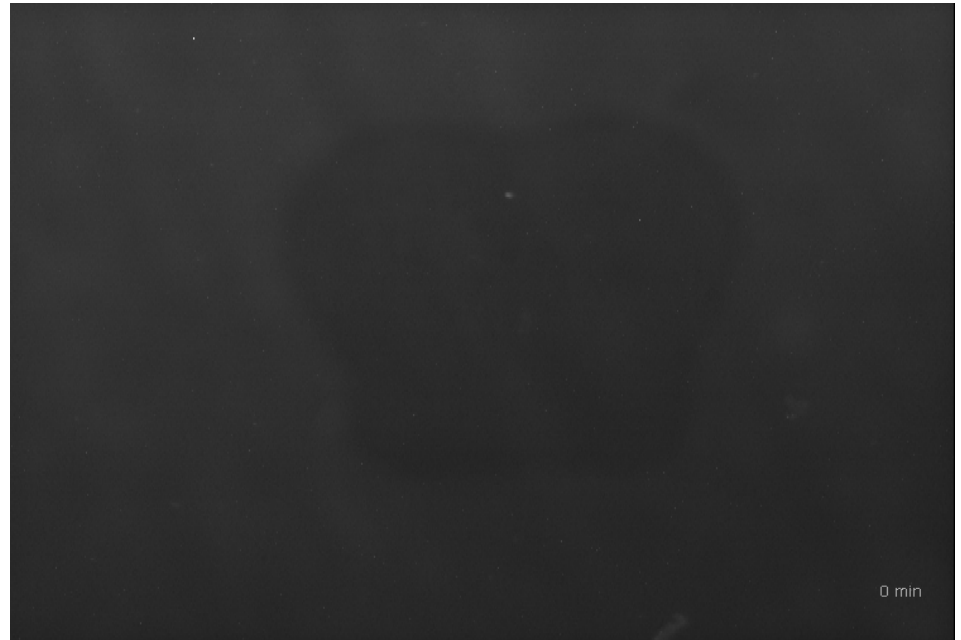




Atacama Desert, Chile

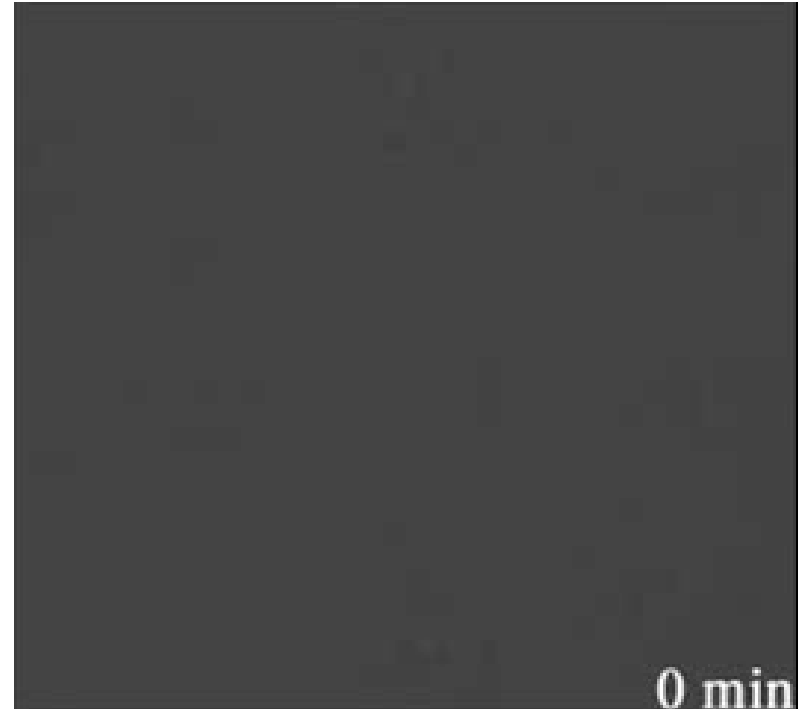


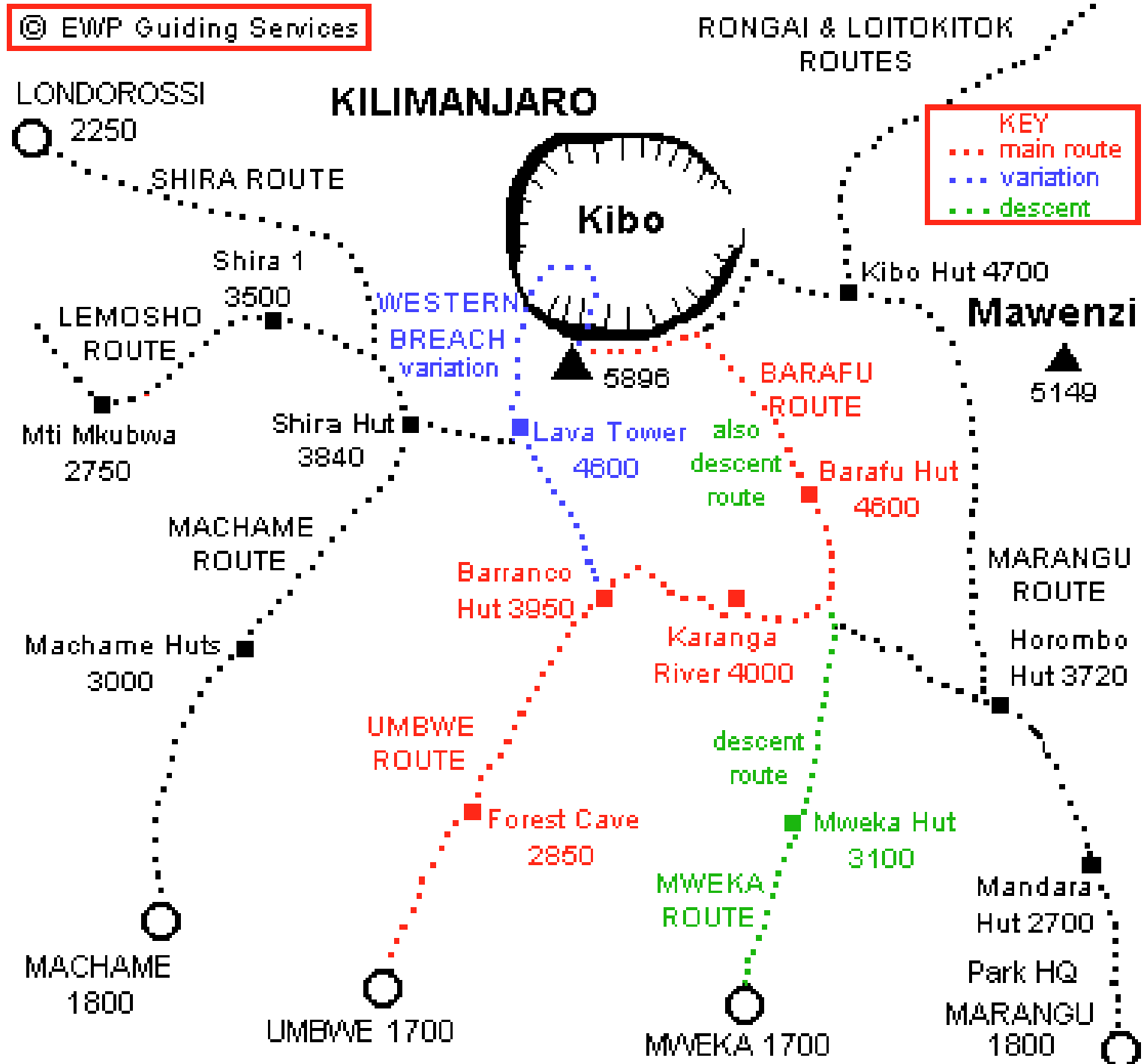
Atacama Desert (taken in Nov 2005)

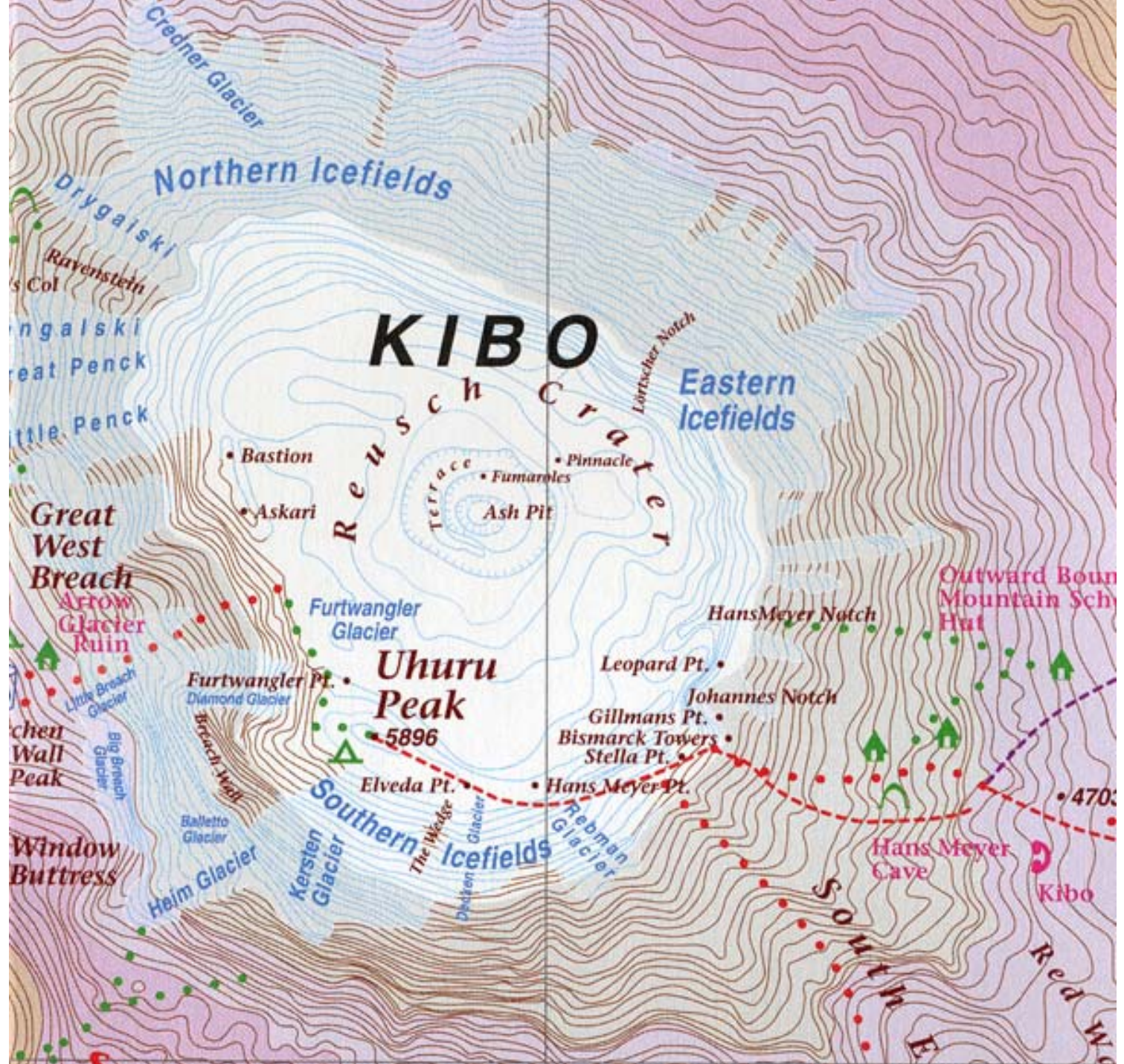


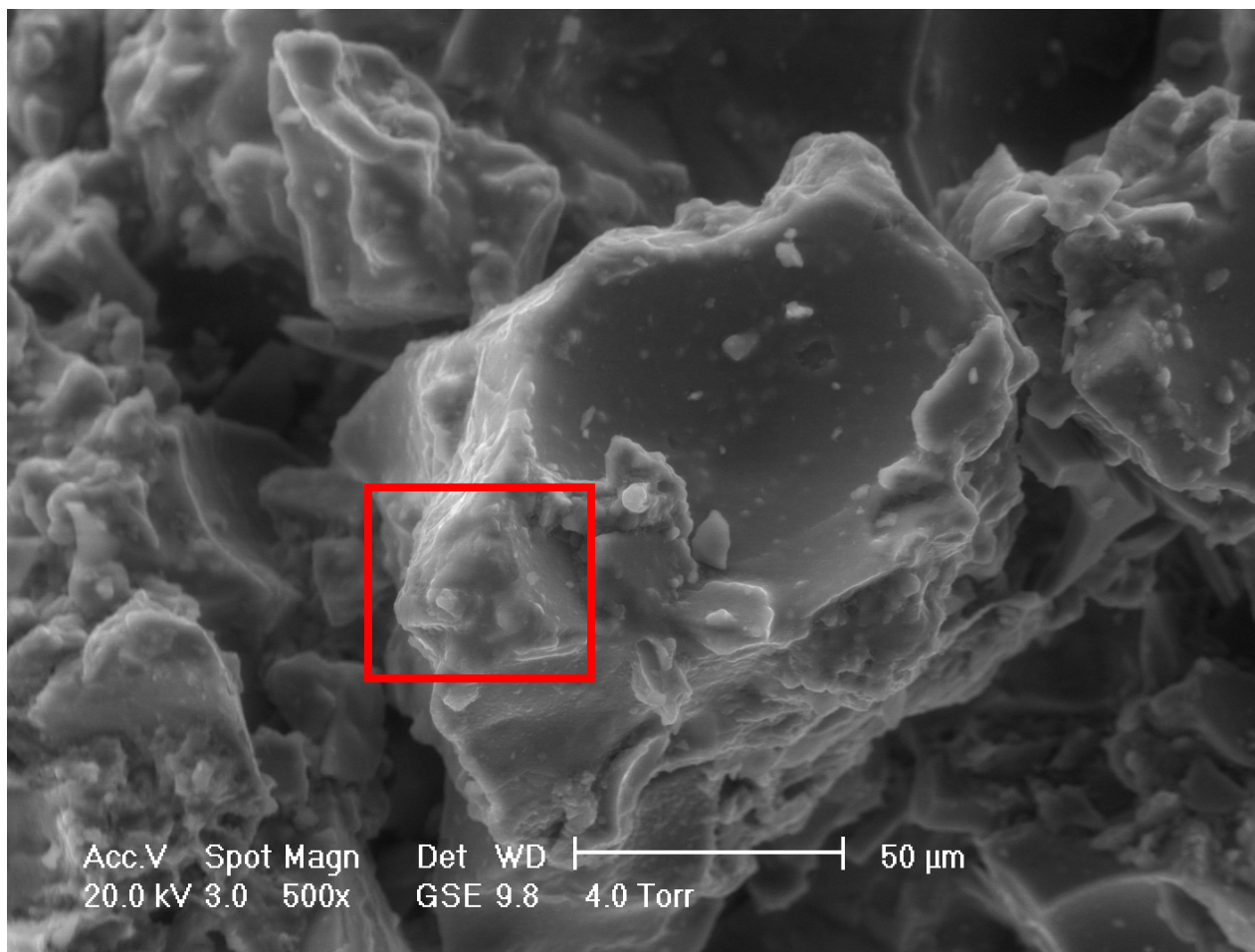


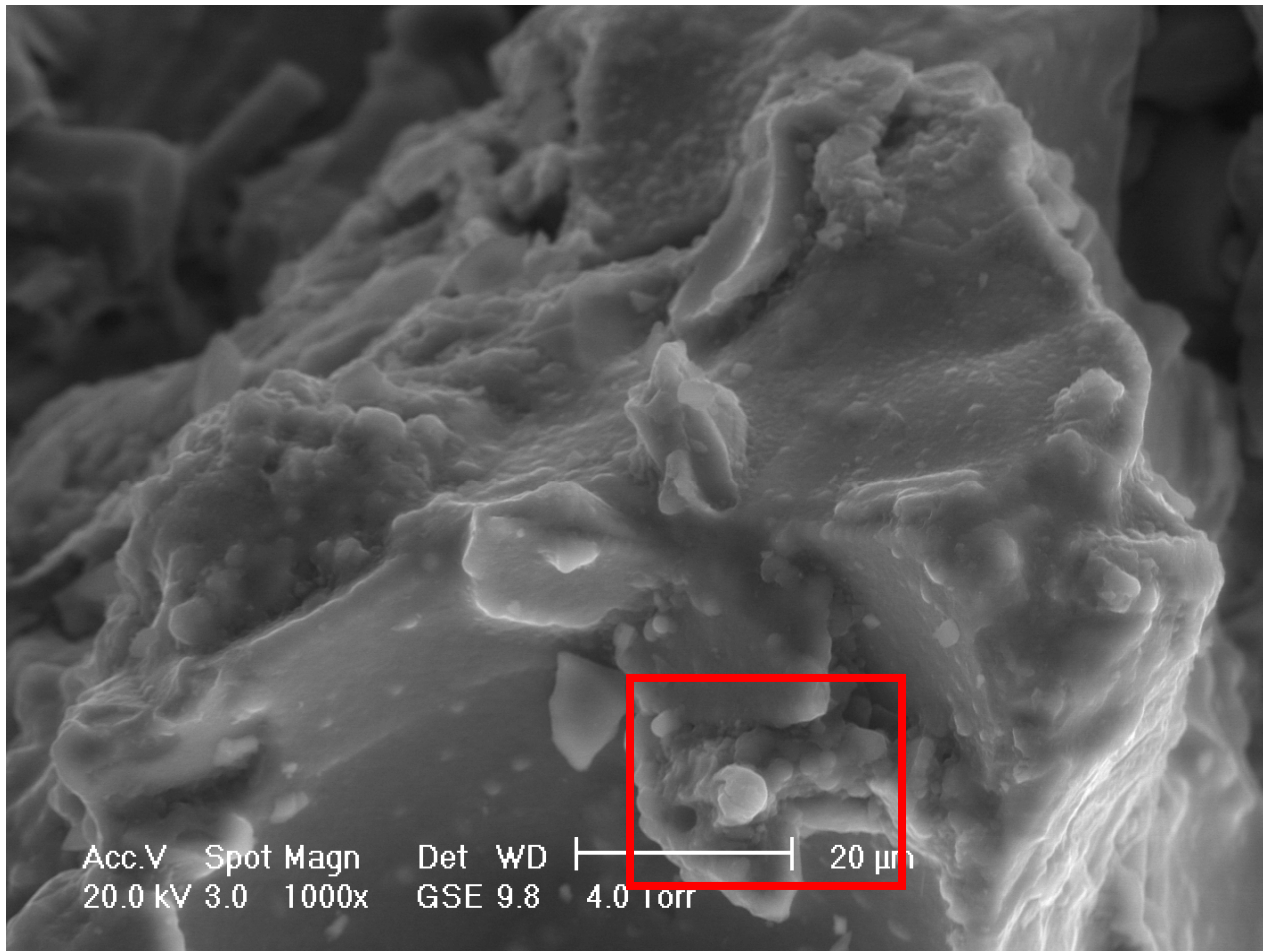
Kilimanjaro

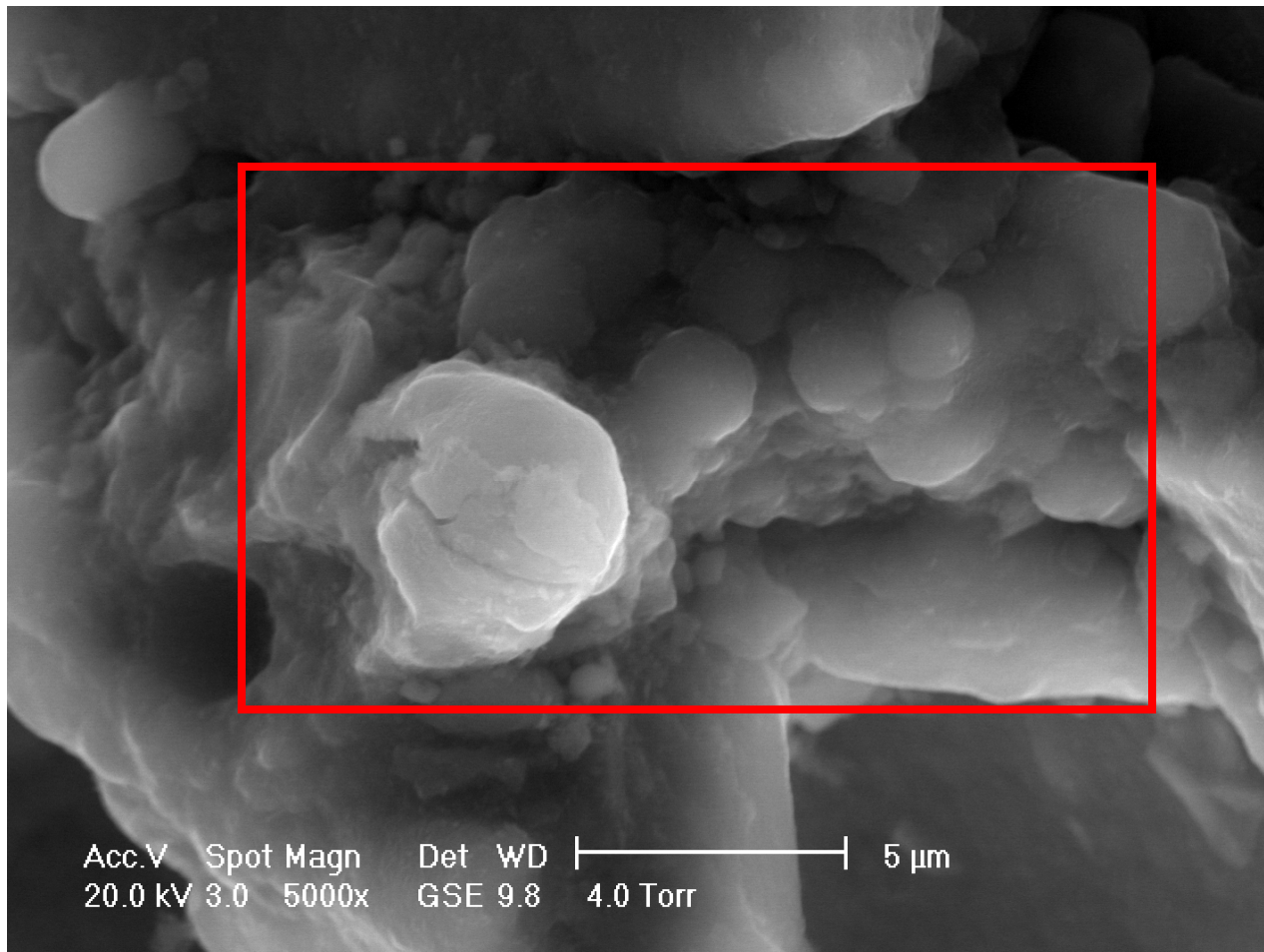


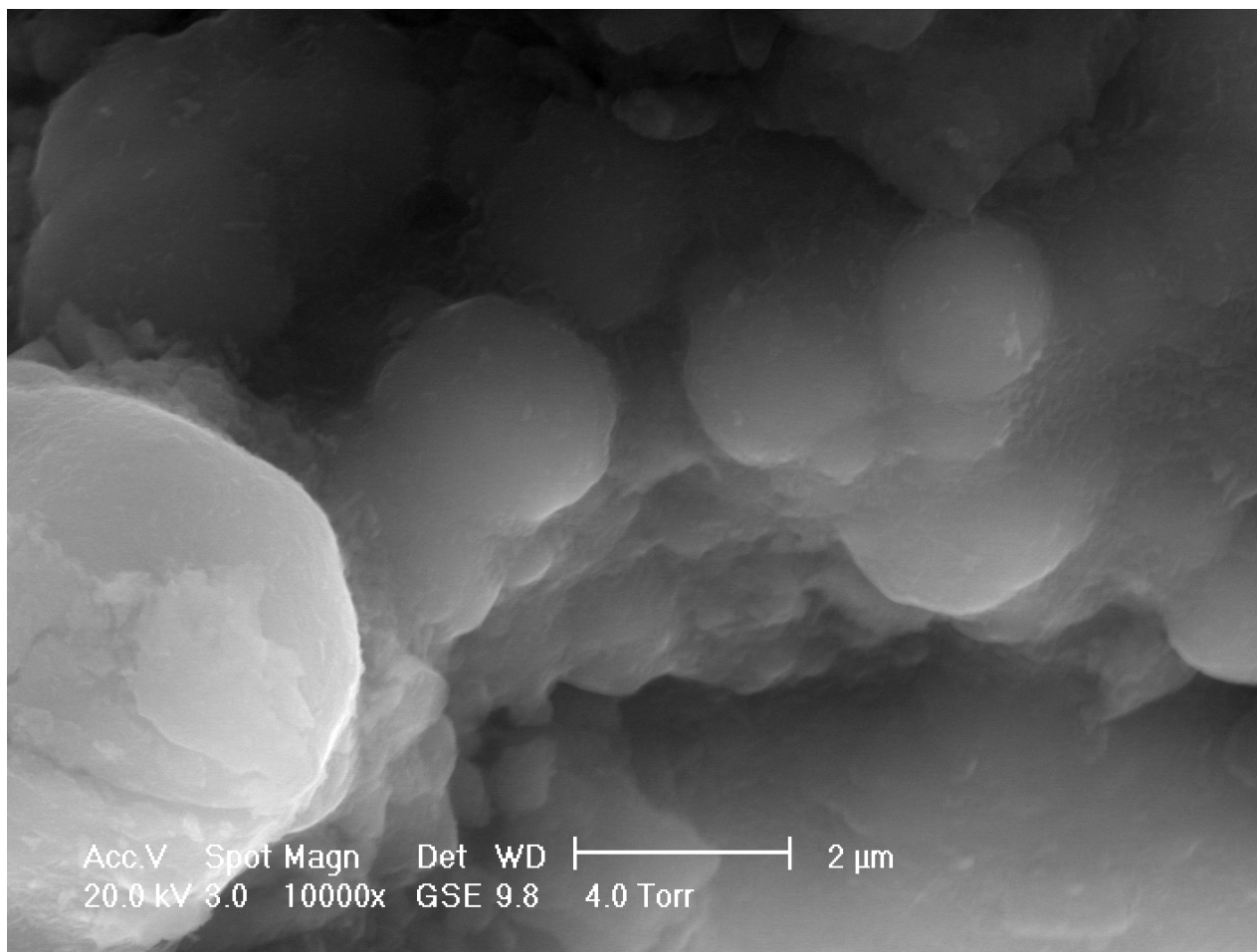


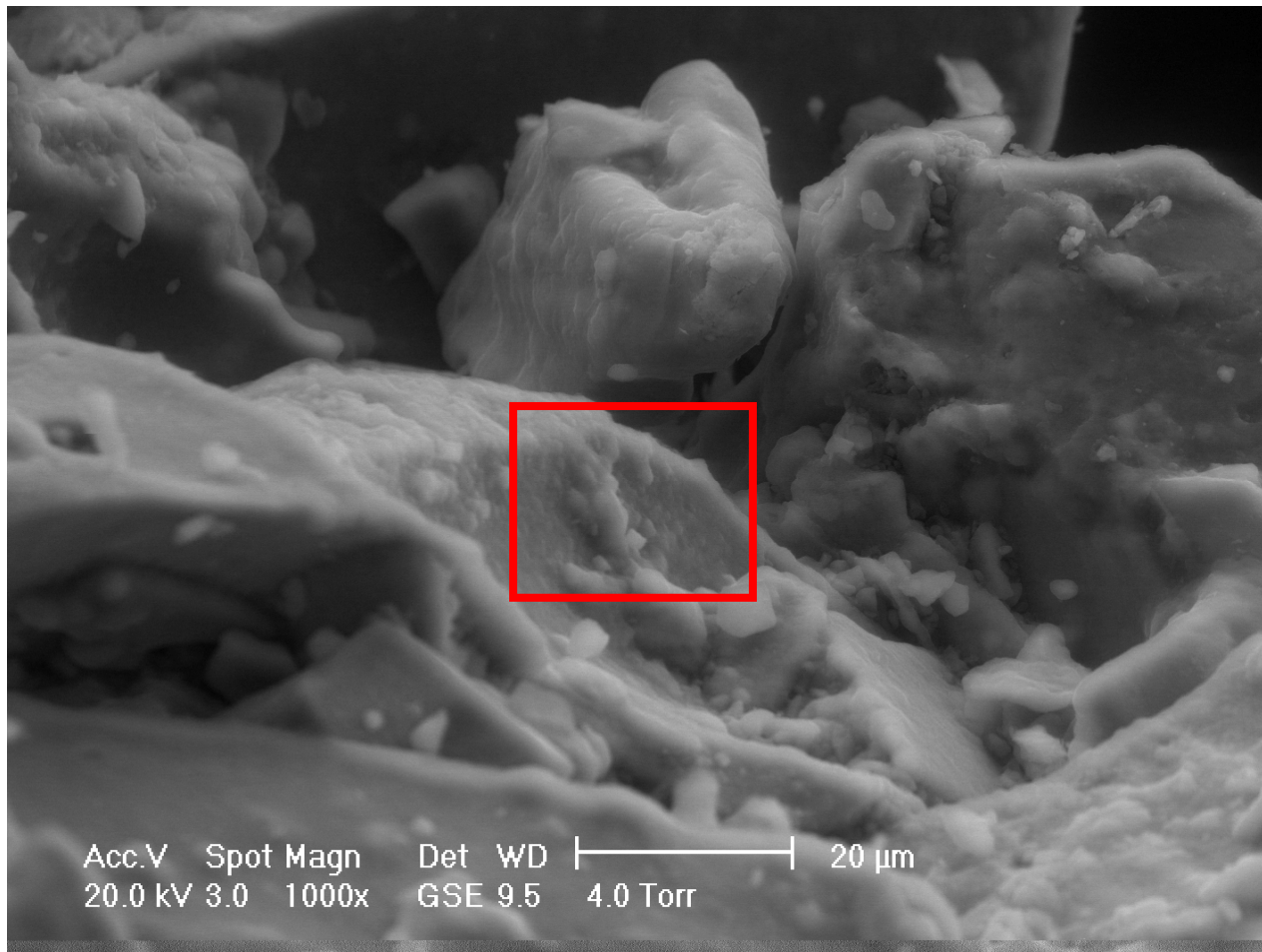


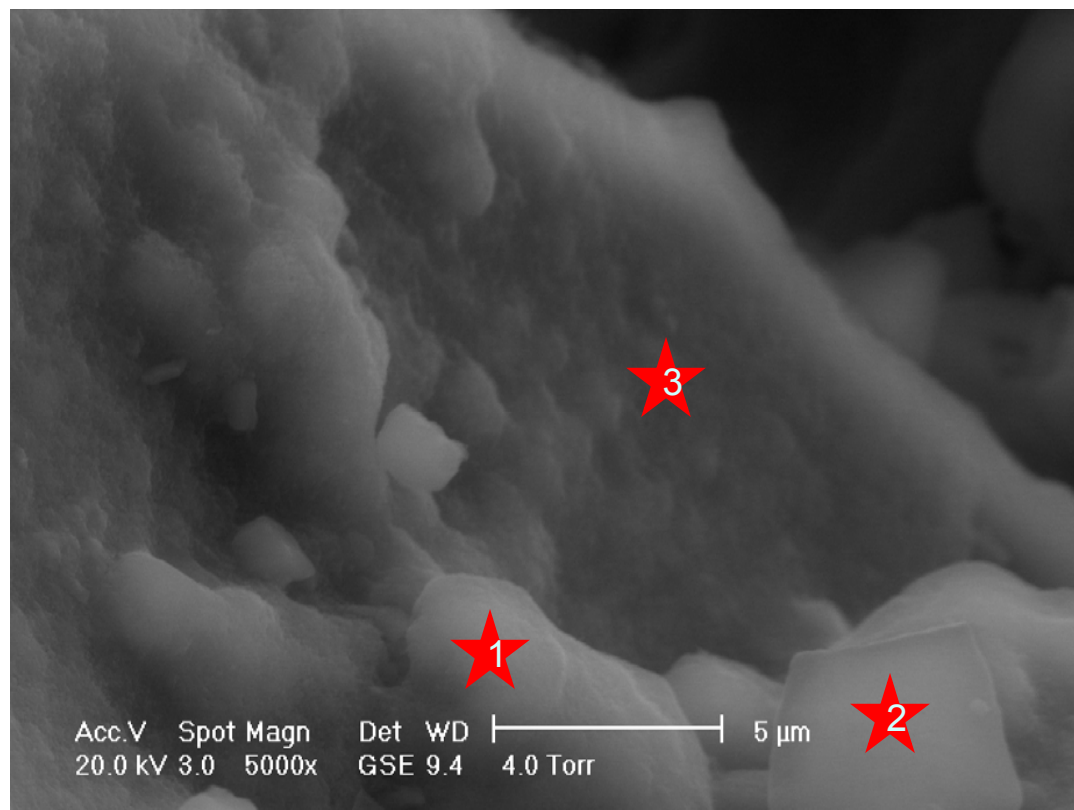




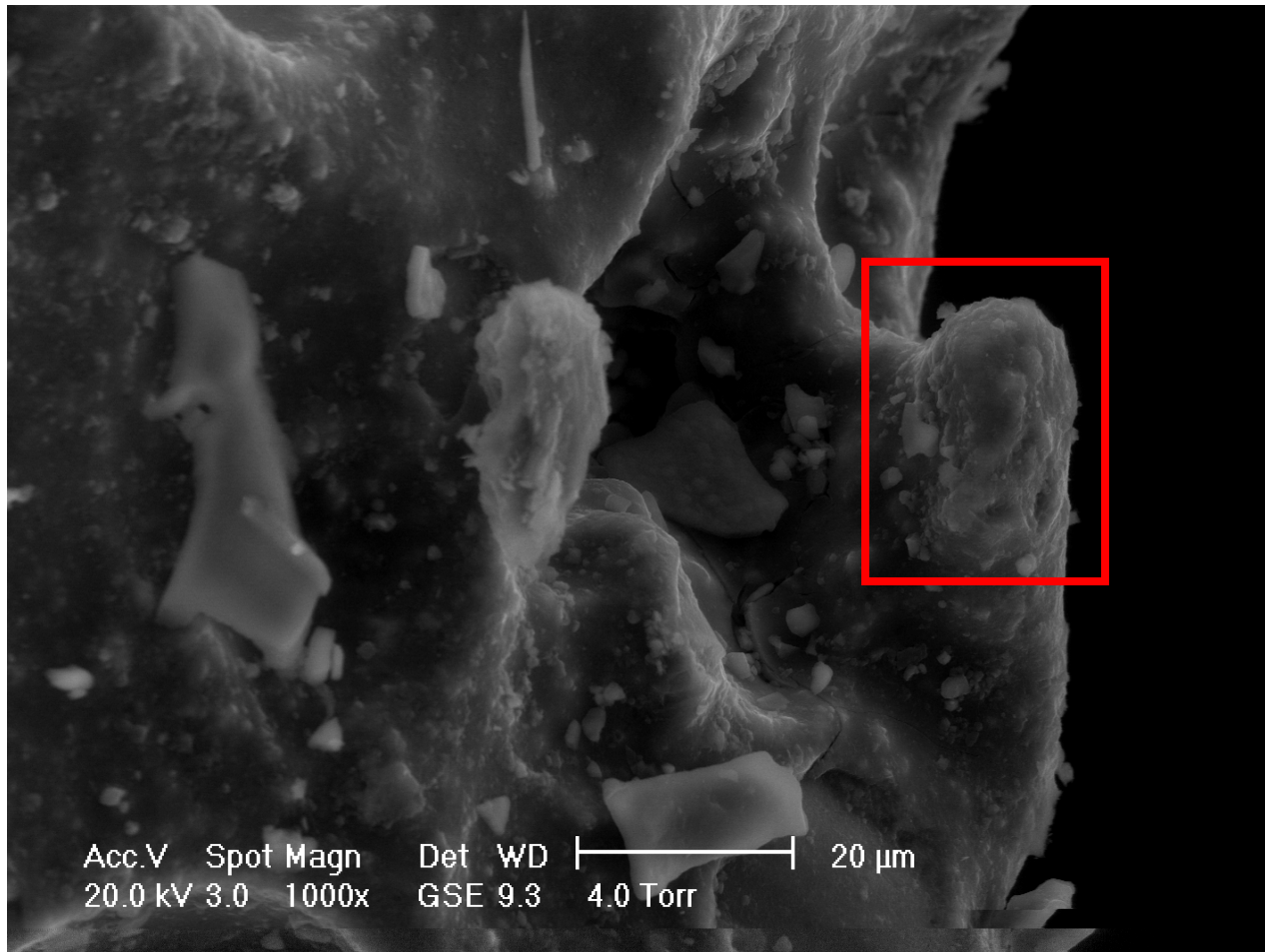


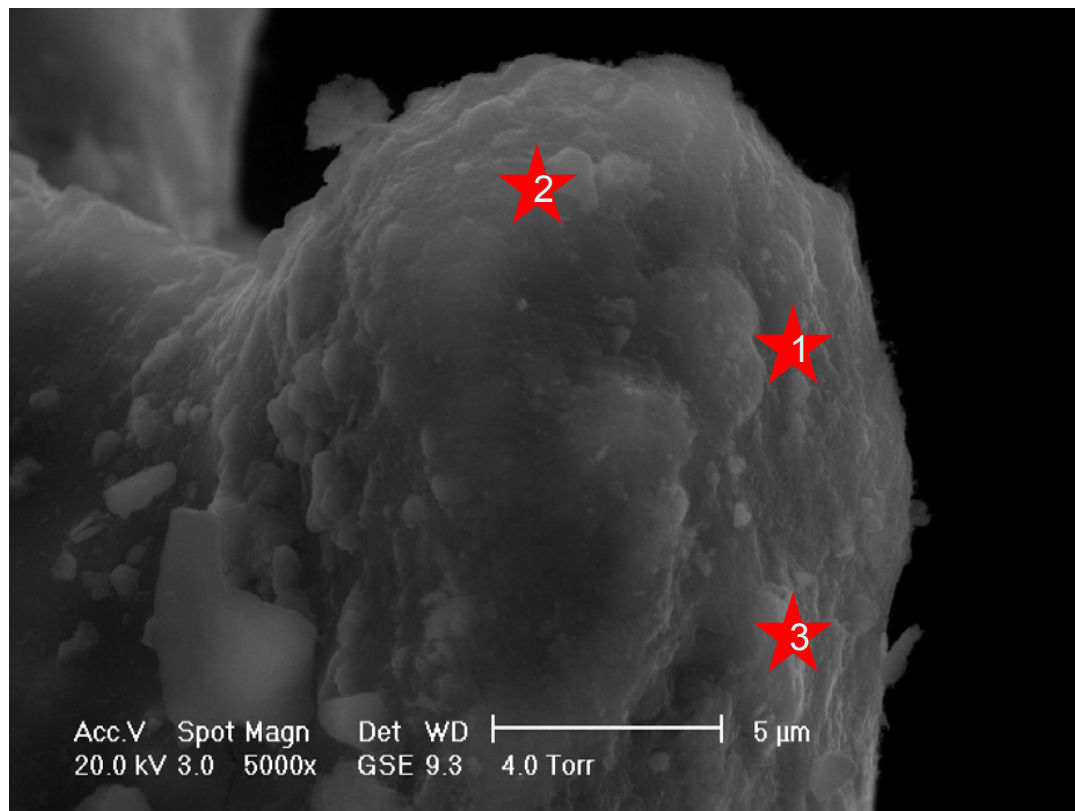






Elem	1. At %	2. At %	3. At %
-----	-----	-----	-----
C K	26.63	24.5	23.31
O K	54.5	55.26	54.67
NaK	0.98	0.99	1.77
MgK	0.51	0.37	0.46
AlK	6.98	8.18	7.46
SiK	8.64	9.12	10.14
P K	0.19	0.17	0.2
S K	0.1	0.12	0.1
ClK	0.1	0.08	0.09
K K	0.39	0.42	0.88
CaK	0.33	0.14	0.21
TiK	0.11	0.14	0.15
FeK	0.42	0.45	0.49
NiK	0.11	0.06	0.07



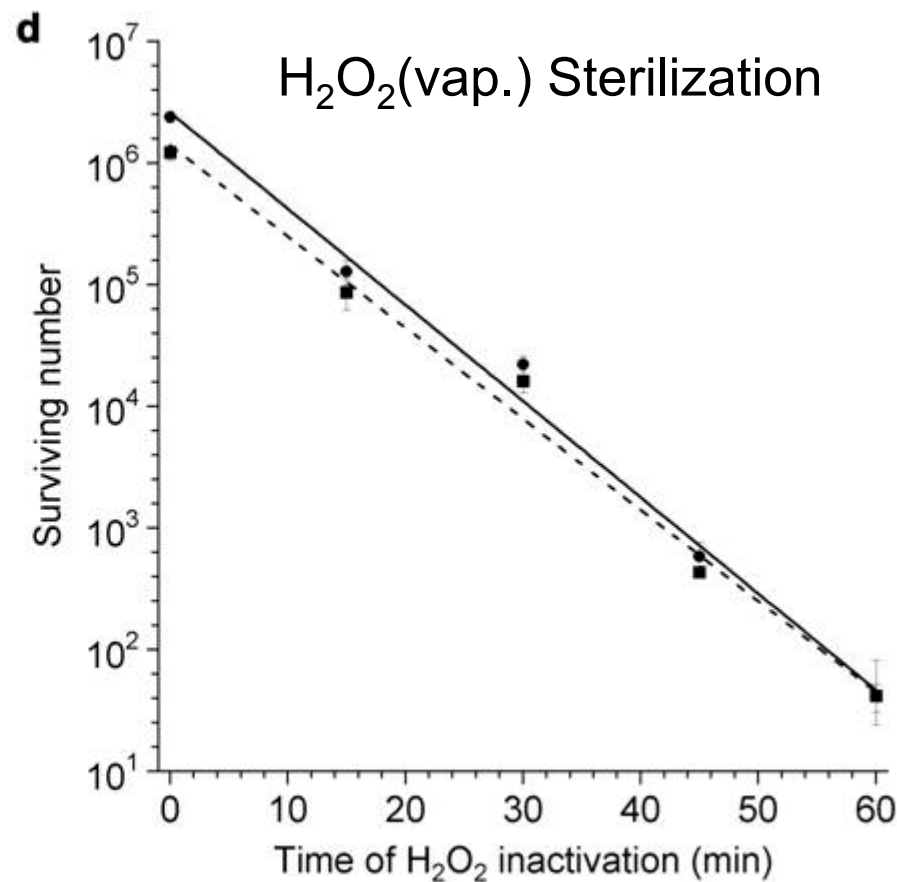
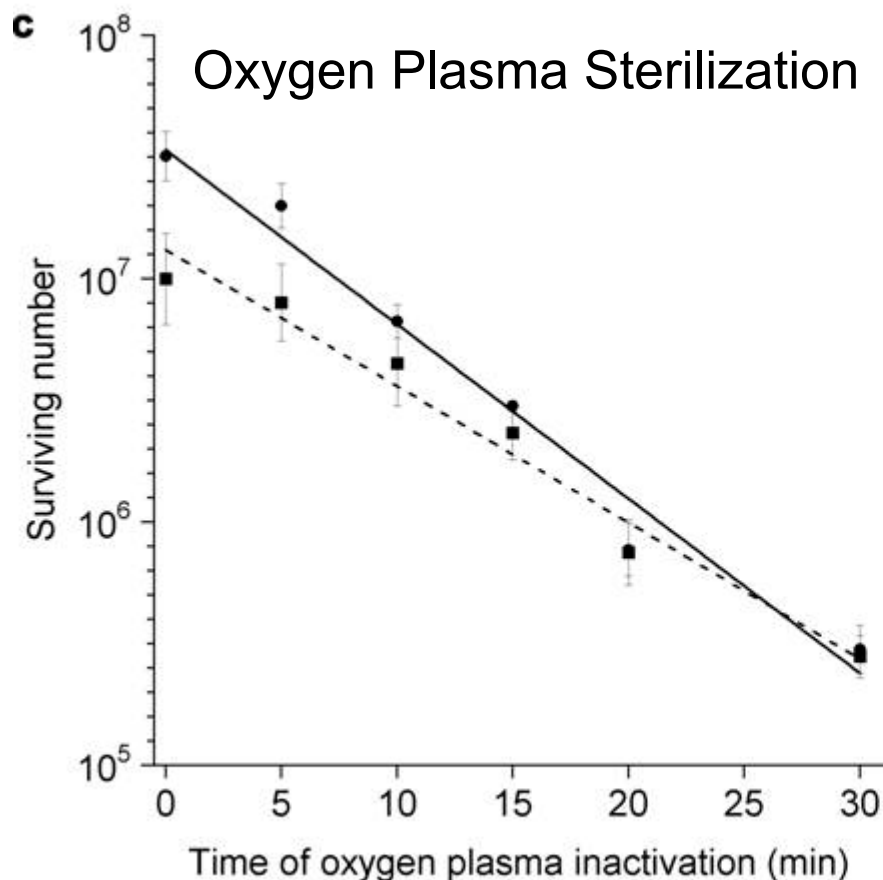


Elem	1. At %	2. At %	3. At %
-----	-----	-----	-----
C K	43.18	40.47	44.44
O K	44.44	46.12	43.39
NaK	0.77	0.63	0.49
MgK	0.3	0.25	0.3
AlK	2.8	3.44	3.14
SiK	7.3	8.04	7.34
P K	0.16	0.08	0.12
S K	0.13	0.04	0.1
ClK	0.07	0.04	0.04
K K	0.37	0.43	0.25
CaK	0.17	0.14	0.1
TiK	0.09	0.06	0.06
FeK	0.22	0.23	0.18
NiK	0.02	0.02	0.05



Application to Monitor Sterilization Processes

Bacillus spores on dry surfaces



PHASE
CONTRAST

MALACHITE
GREEN

DETECTION
TARGET

Refractive index

Uptake of stain

OBSERVABLE

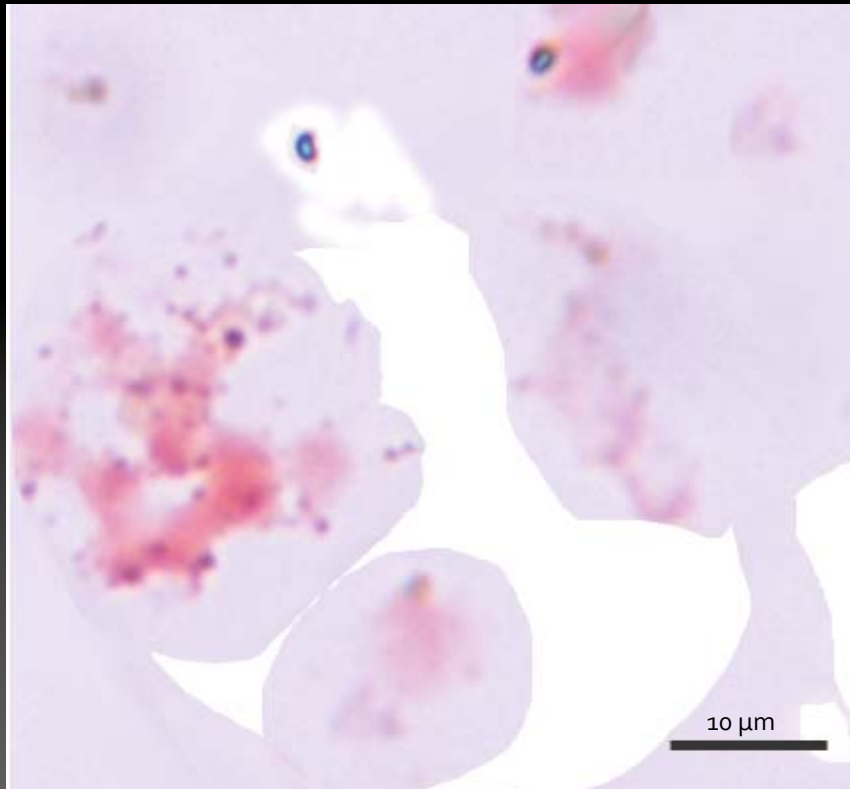
Spores: phase bright
Vegetative cells: phase dark

Spores: Green
Vegetative cells: Pink

CONCLUSION

0 – 10² spores/mL

Presence of spores



PHASE
CONTRAST

MALACHITE
GREEN

SYTO-9/PI

DETECTION
TARGET

Refractive index

Uptake of stain

Membrane integrity

OBSERVABLE

Spores: phase bright
Vegetative cells: phase dark

Spores: Green
Vegetative cells: Pink

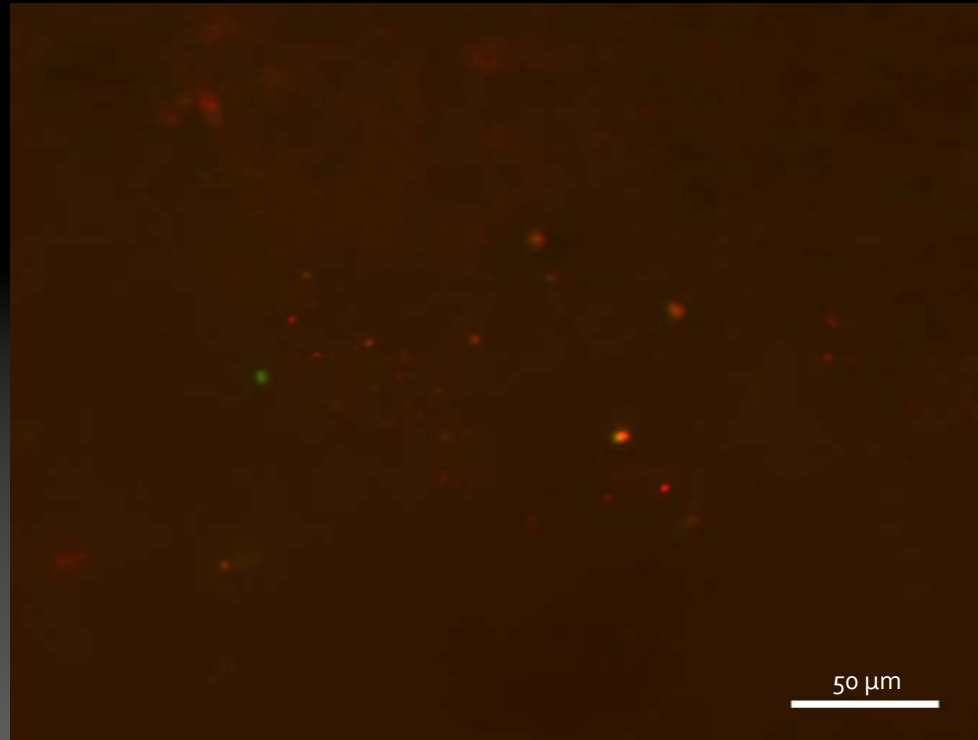
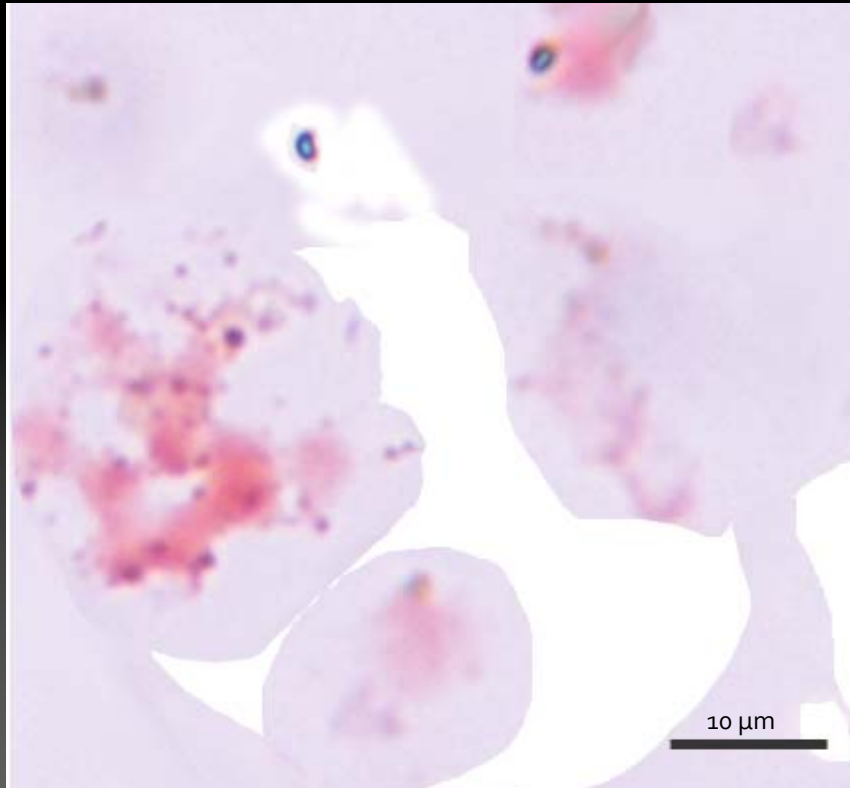
Live cells: Green
Dead cells: Red

CONCLUSION

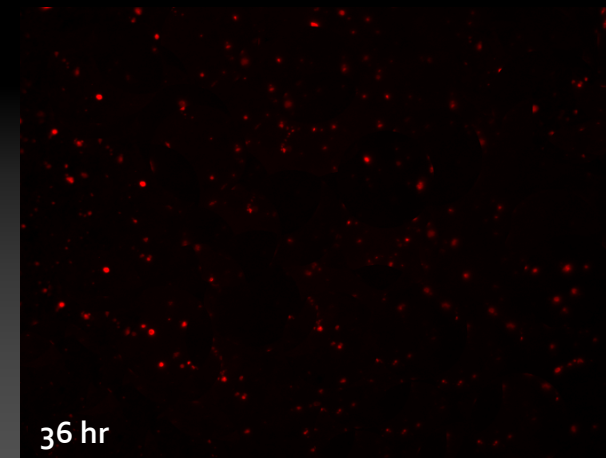
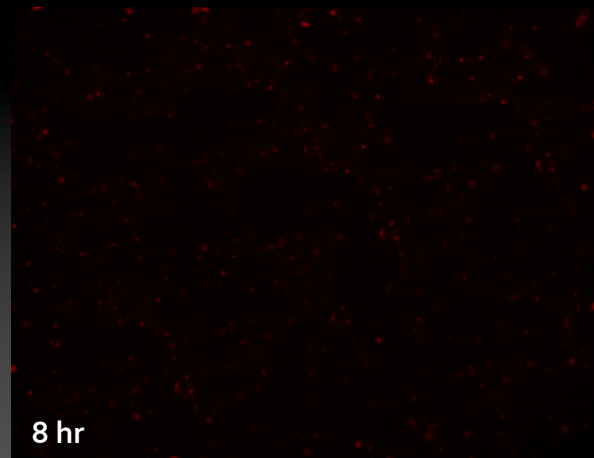
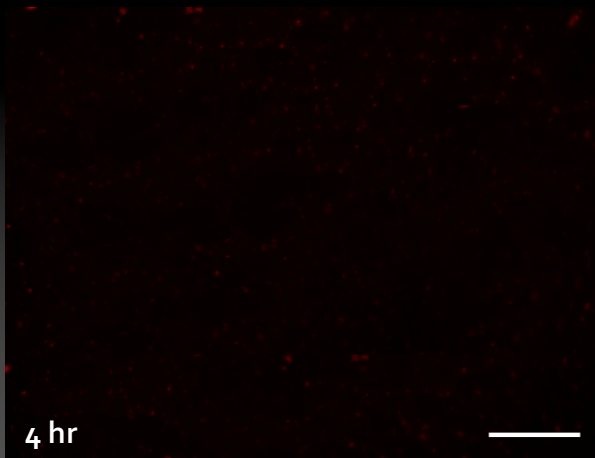
0 – 10² spores/mL

Presence of spores

< 0.01 live cells/mL
< 0.1 cells/mL



	PHASE CONTRAST	MALACHITE GREEN	SYTO-9/PI	TETRAZOLIUM CHLORIDE	DiBAC ₄ (3)
DETECTION TARGET	Refractive index	Uptake of stain	Membrane integrity	Respiratory potential	Membrane potential
OBSERVABLE	Spores: phase bright Vegetative cells: phase dark	Spores: Green Vegetative cells: Pink	Live cells: Green Dead cells: Red	Dehydrogenase activity: Red	Active cells: Green
CONCLUSION	0 – 10 ² spores/mL	Presence of spores	<0.01 live cells/mL < 0.1 cells/mL	Presence of dehydrogenase activity and membrane potential upon incubation with TSB	



Kilimanjaro samples incubated with 1/5 TSB at 37°C

PHASE CONTRAST

MALACHITE GREEN

SYTO-9/PI

TETRAZOLIUM CHLORIDE

DiBAC₄(3)

DETECTION
TARGET

Refractive index

Uptake of stain

Membrane integrity

Respiratory potential

Membrane potential

OBSERVABLE

Spores: phase bright

Spores: Green

Live cells: Green

Dehydrogenase activity: Red

Active cells: Green

Vegetative cells: phase dark

Vegetative cells: Pink

Dead cells: Red

CONCLUSION

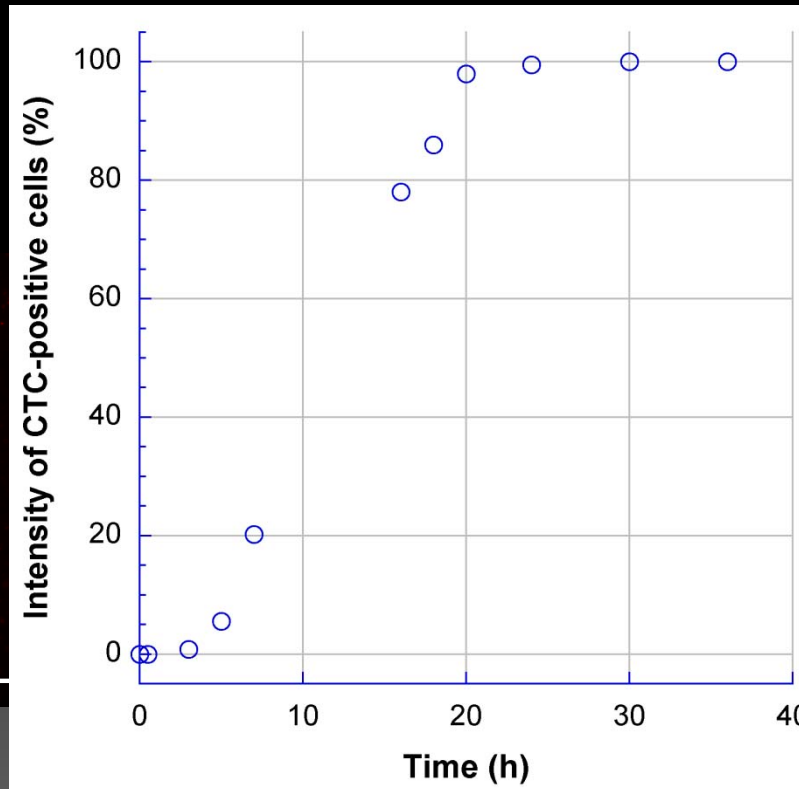
0 – 10² spores/mL

Presence of spores

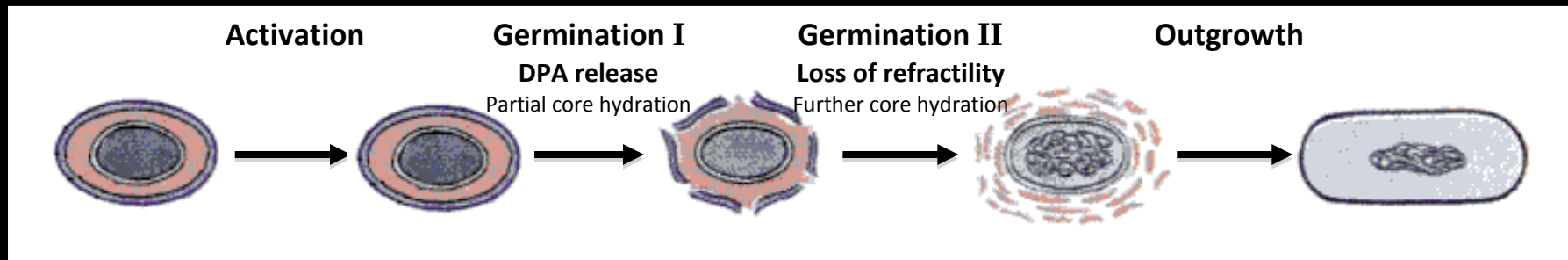
<0.01 live cells/mL

< 0.1 cells/mL

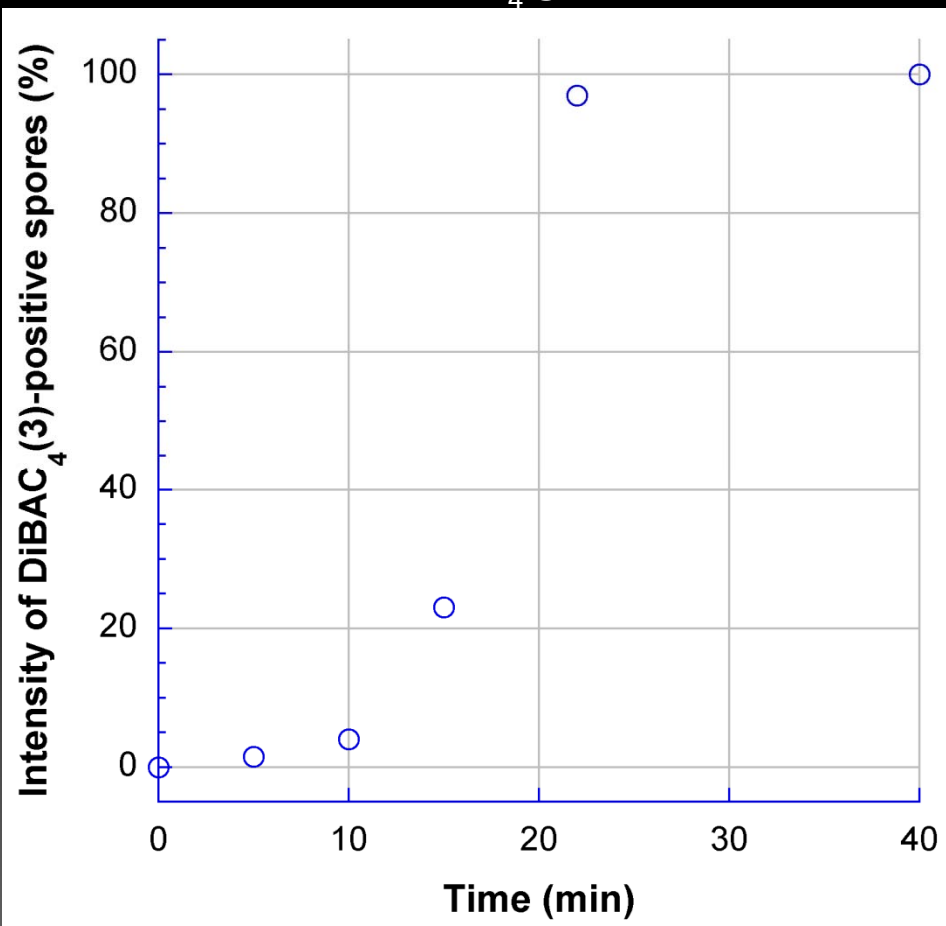
Presence of dehydrogenase activity and
membrane potential upon incubation with TSB



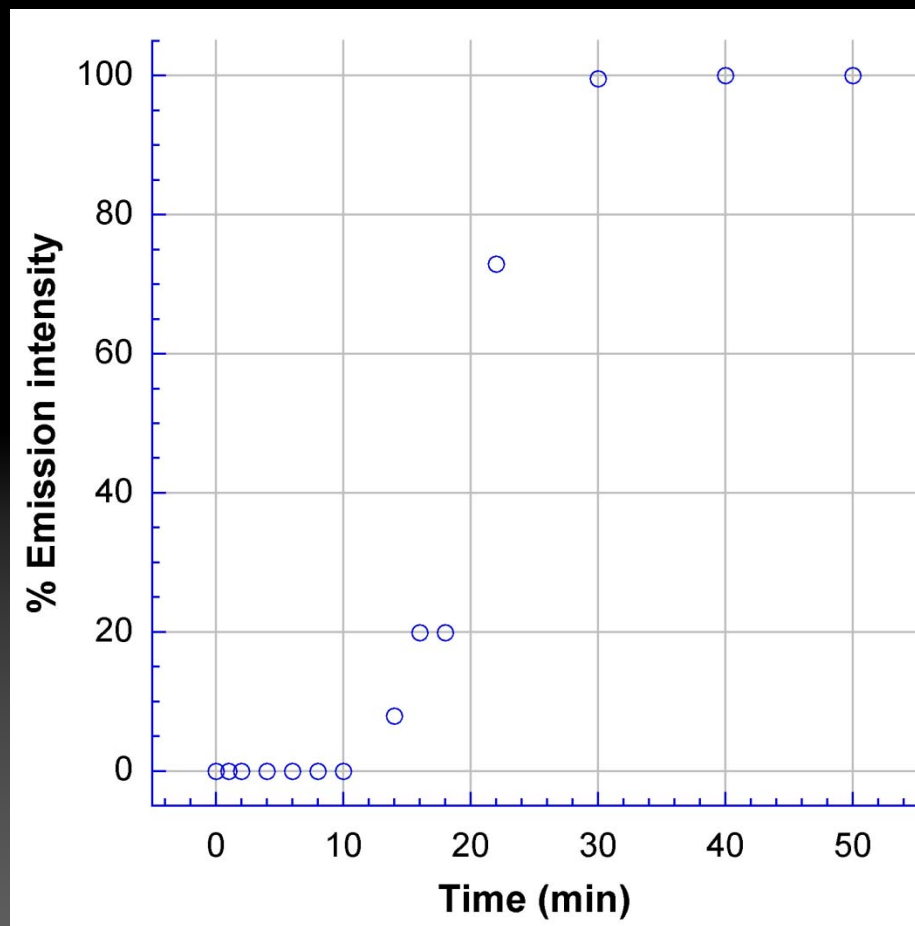
Kilimanjaro samples incubated with 1/5 TSB at 37°C



DiBAC₄(3)

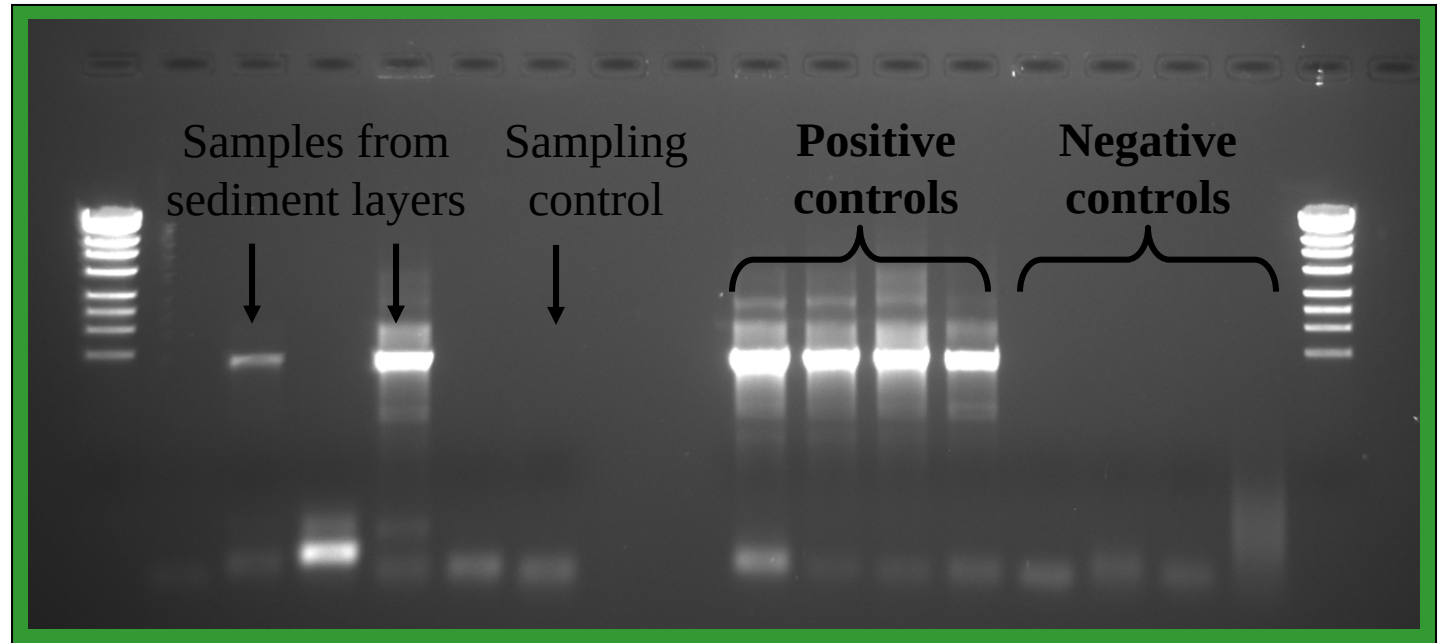


MicroEVA



Microbial Diversity

PCR



Evaluate rate of microbial diversity decline and recovery with specific past climate change events.



- Chemistry, Methods, Instrumentation for viability assessment
 - Bacillus, Clostridium as biological indicators for sterility assurance.
 - Air (MEM), water, surface hygiene monitoring.
 - Bacterial spore resistance, longevity, and characteristics of ancient viable microbes
 - Microbial survival and growth in extreme environments
 - Applications in Planetary Protection, Astrobiology and Biological Defense
 - Microbial survival and growth in extreme environments
 - Atacama, Lake Vida, Greenland, Kilimanjaro
- Lessons for life detection
 - limit for life vs limit of detection
 - spatial and temporal heterogeneity
- Kilimanjaro Story
 - Add new carbon dates,
 - Add description of nearest clone matches
 - Add new pictures, popular culture Hemingway, Gore, Thompson
 - Impact of climate change on ecosystem,
 - Closest analog to ancient Martian lakes and Europa ocean on earth
 - cold, dry, windy, and protected by only a thin atmosphere.
 - catalog the bacteria and algae that live there
 - look for extremophiles that can survive the harsh ultraviolet light at 20,000 feet
 - Science in situ versus return
 - design science mission strategies for planetary exploration and search for life in the Solar System
- Add references to slides
- Add context of Mars exploration
 - Follow the water, special regions and habitability
 - Biomarkers to cells
 - Second origin or panspermia (lithoinoculation, discuss feasibility data for each step of transit)

Kilimanjaro

Climate change impacts on Soil Quality and Ecosystem





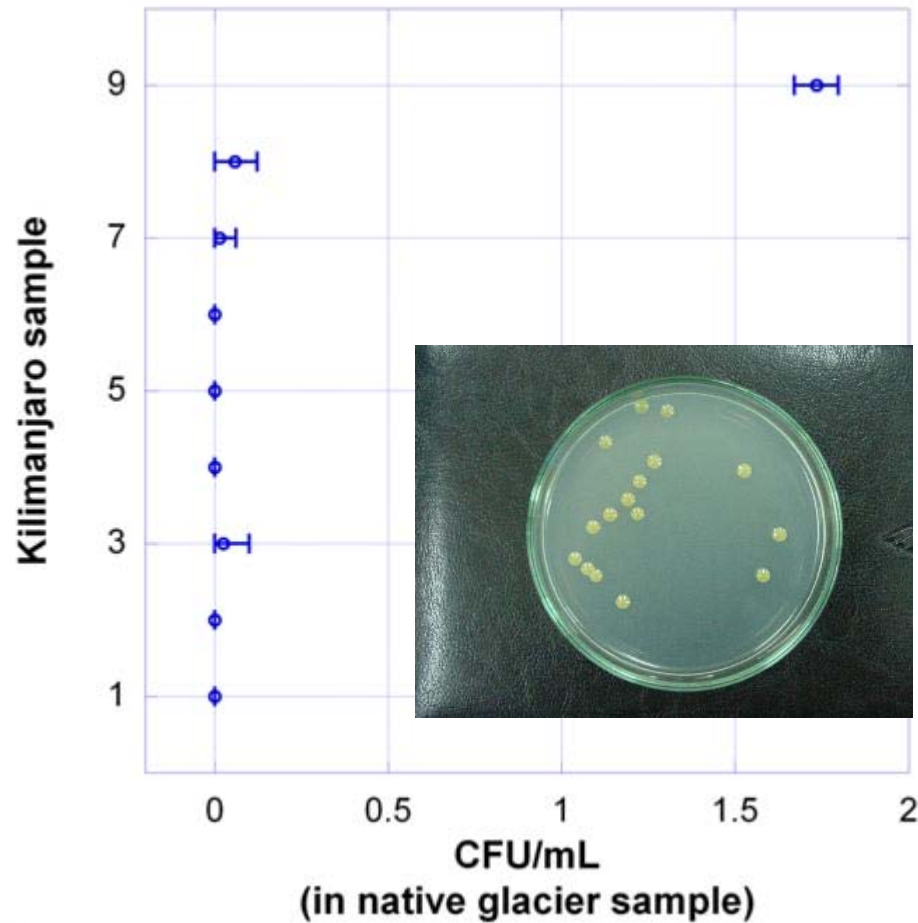
Analysis of the closest relatives on the basis of 16S rDNA sequences

Phylogenetic affiliation	Clone	Top matches, source ^a	GenBank accession no.	Nucleotide identity (%) ^b
Proteobacteria, Γ -subclass	K323G02	uncultured gamma, Lake Tanganyika anoxic hypolimnion, Tanzania	DQ463708	98.69%
Proteobacteria, B-subclass	K711B09	uncultured bacterium, fresh snow at 6350m in the Everest region	EF190150	97.15%
		uncultured bacterium, west antarctic ice sheet, KIS sediment	EU030494	97.00%
Proteobacteria, A-subclass	K712B08	uncultured bacterium, indoor dust	AM697086	95.18%
Proteobacteria, A-subclass	K713B07	uncultured bacterium, surface sample from Gulkana glacier, Alaska	AB464938	97.88%
Proteobacteria, A-subclass	K713G11	uncultured bacterium, mattress dust	FM874375	99.23%
		<i>Roseomonas aquatica</i> , drinking water	AM231587	98.85%
Proteobacteria, A-subclass	K713A05	alphaproteobacterium 34619, hospital water	AF288301	99.09%
		uncultured bacterium, water from the Arctic ocean	EU919762	98.63%
Proteobacteria, A-subclass	K711A11	alphaproteobacterium 34619, hospital water	AF288301	94.96%
		uncultured bacterium, contaminated sediments, Tennessee	DQ404891	95.58%
Bacterioidetes	K711A10	Flexibacteraceae bacterium VUG-C4, Victoria upper glacier, Antarctica	EU159489	95.37%
		Bacterioidetes bacterium, soil from La Gorce mountains, Antarctica	DQ351728	95.29%
Actinobacteria	K322A02/ K711C06	Cryobacterium sp. ZS1-15, marine sediments from the Antarctic ocean	FJ889650	99.41% / 99.63%
		Cryobacterium sp. DR8, water and soil from Crater Lake, Antarctica	FJ464984	99.34% / 99.56%
Actinobacteria	K713E06	Antarctic bacterium, water and soil from Crater Lake, Antarctica	FJ464978	97.52%
Actinobacteria	K712C01	uncultured bacterium, surface sample from Gulkana glacier, Alaska	AB464943	99.49%
		Antarctic bacterium, Collins glacier, Antarctica	EU636015	99.33%
Actinobacteria	K713H01	Cryobacterium sp. ZS1-15, marine sediments from the Antarctic ocean	FJ889650	97.79%
		Cryobacterium sp. DR8, water and soil from Crater Lake, Antarctica	FJ464984	97.71%
Actinobacteria	K712A07	uncultured actinobacterium, vicinity of a uranium mine, Bulgaria	FM877676	97.61%
		uncultured actinobacterium, soil from the Atacama Desert, Chile	EF016798	95.88%
Actinobacteria	K711E08	<i>Aeromicrobium panaciterrae</i> , soil from a ginseng field, South Korea	AB245387	97.55%

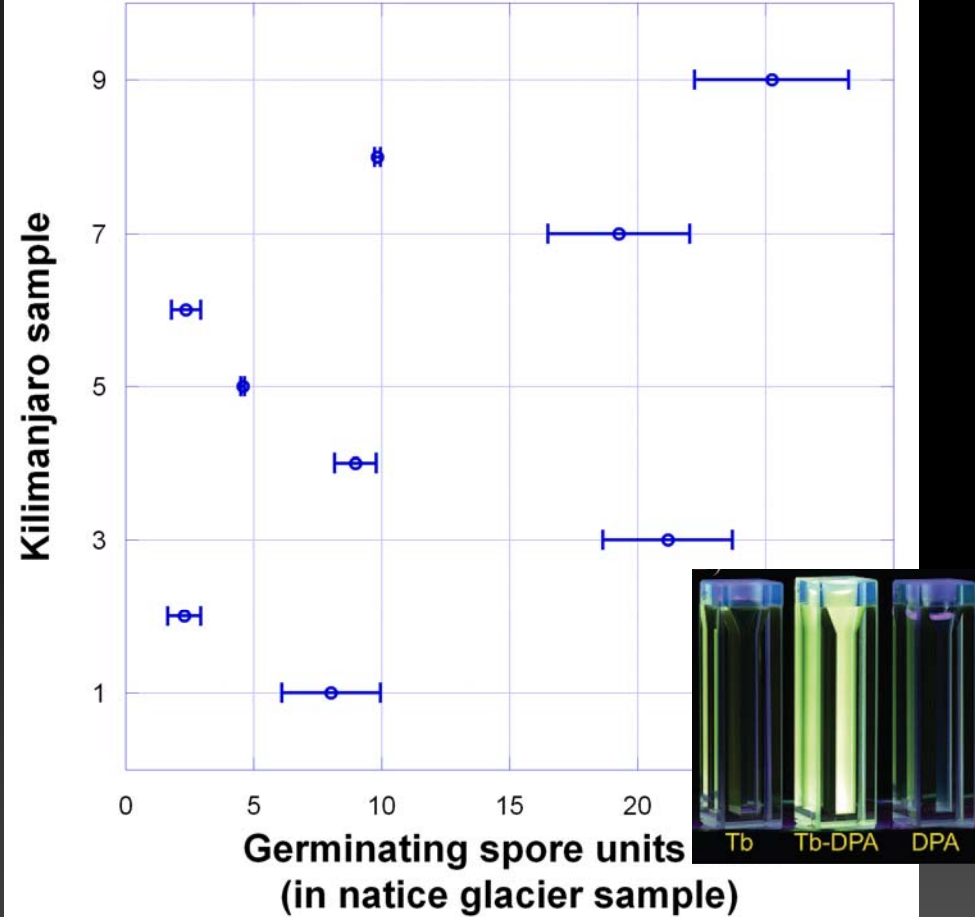
^a Top match as determined by the BLAST method using the Genbank database [Altschul, S.F., 1990].

^b Nucleotide identity as determined by the sequence similarity function in ARB.

Culturing



Germinable Spores



DETECTION
TARGET

OBSERVABLE

CONCLUSION

PHASE
CONTRAST

MALACHITE
GREEN

SYTO-9/PI

TETRAZOLIUM
CHLORIDE

DiBAC₄(3)

Refractive index

Uptake of stain

Membrane integrity

Respiratory potential

Membrane potential

Spores: phase bright
Vegetative cells: phase dark

Spores: Green
Vegetative cells: Pink

Live cells: Green
Dead cells: Red

Dehydrogenase activity: Red

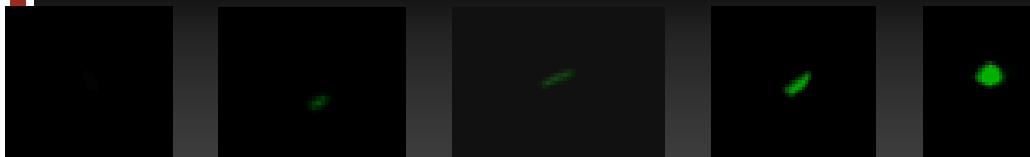
Active cells: Green

0 – 10² spores/mL

Presence of spores

<0.01 live cells/mL
< 0.1 cells/mL

Presence of dehydrogenase activity and
membrane potential upon incubation with TSB



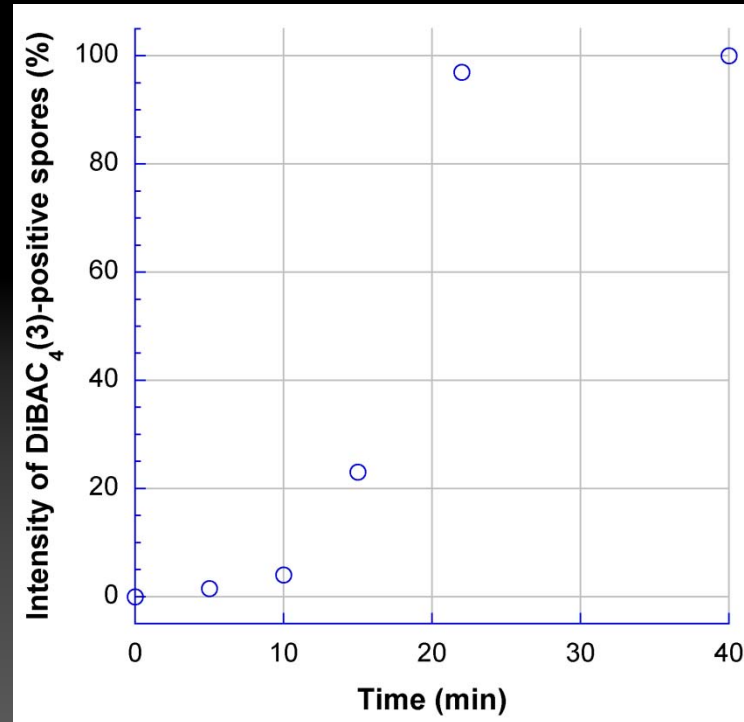
0 min

5 min

10 min

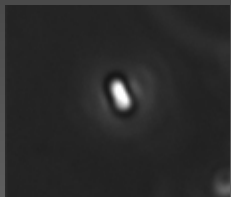
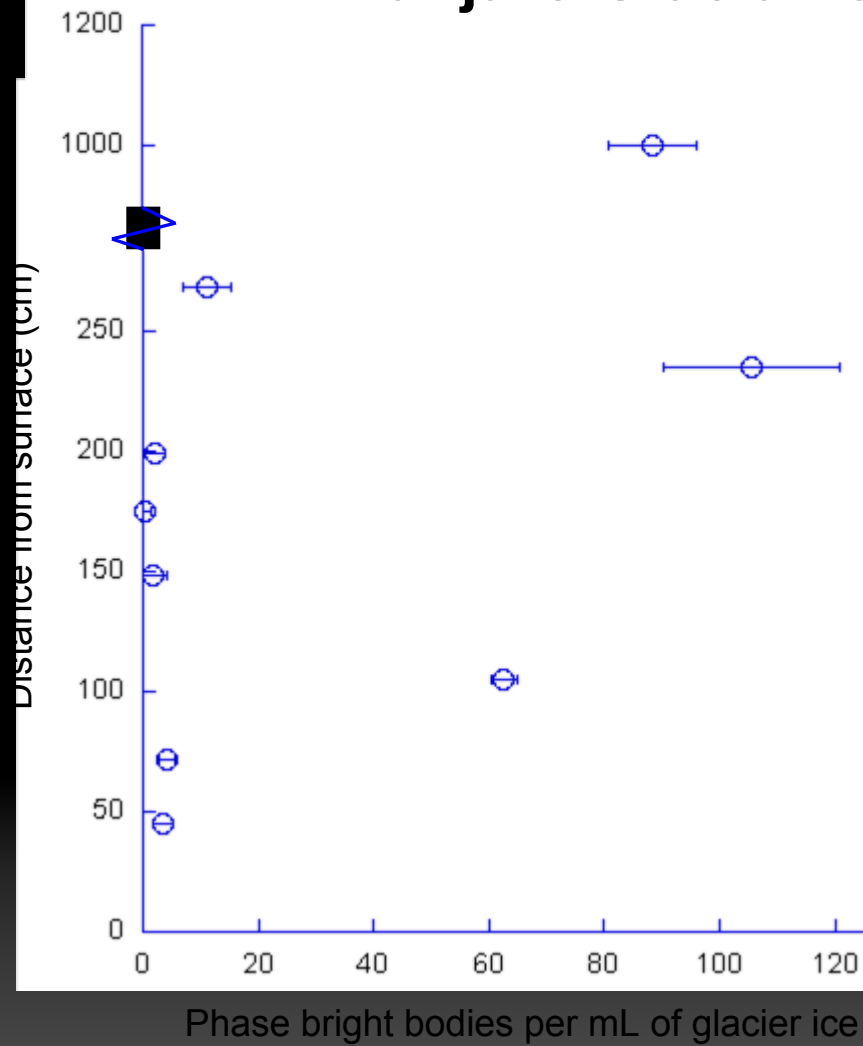
15 min

22 min



Kilimanjaro samples incubated with 10 mM L-alanine and AGFK at 37°C

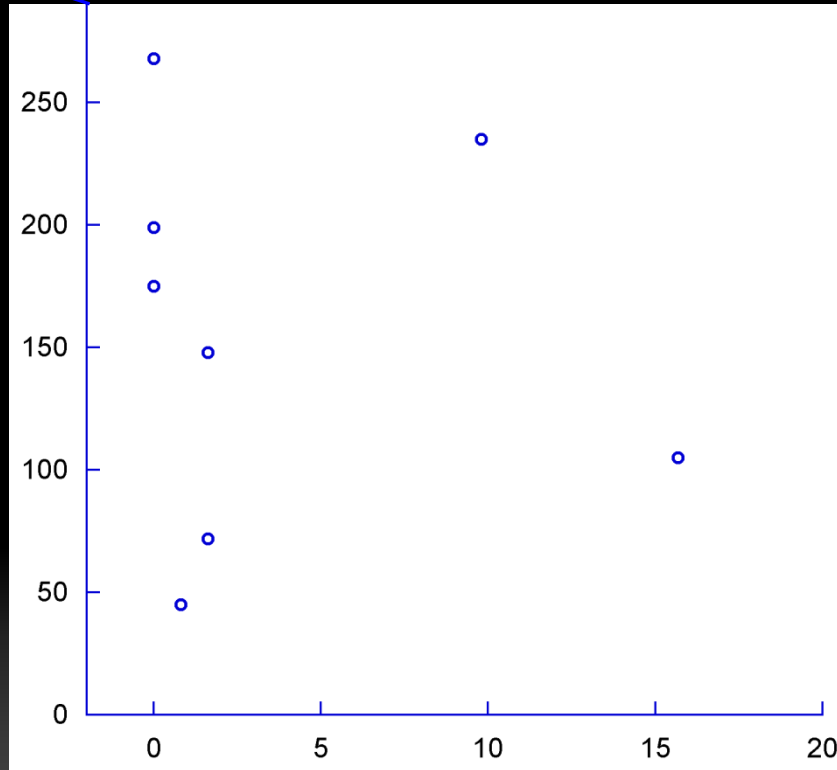
Kilimanjaro Glacial Ice E



Kinnamanjaro Glacier Ice Core



Distance from surface (cm)



Malachite green stained cells per mL of glacier ice

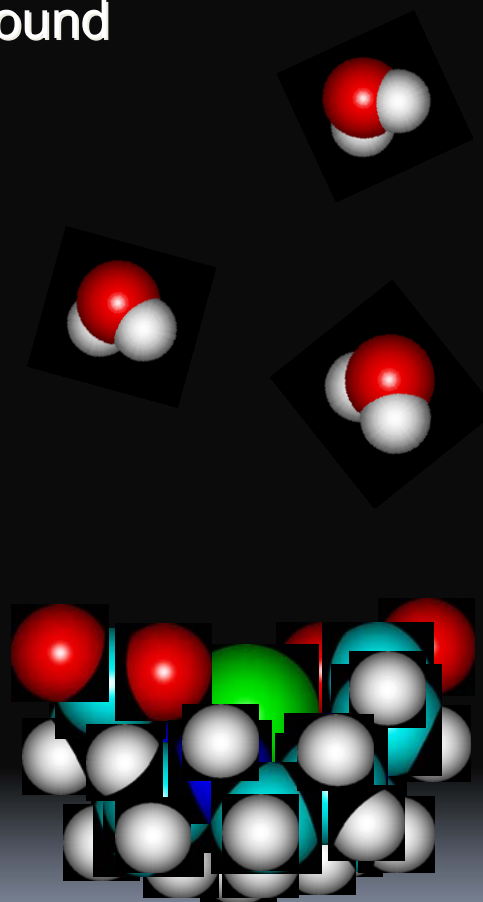




Summary of Ligand-Induced Effects

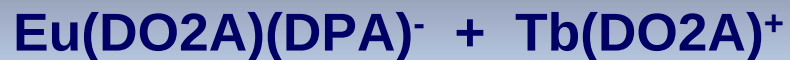
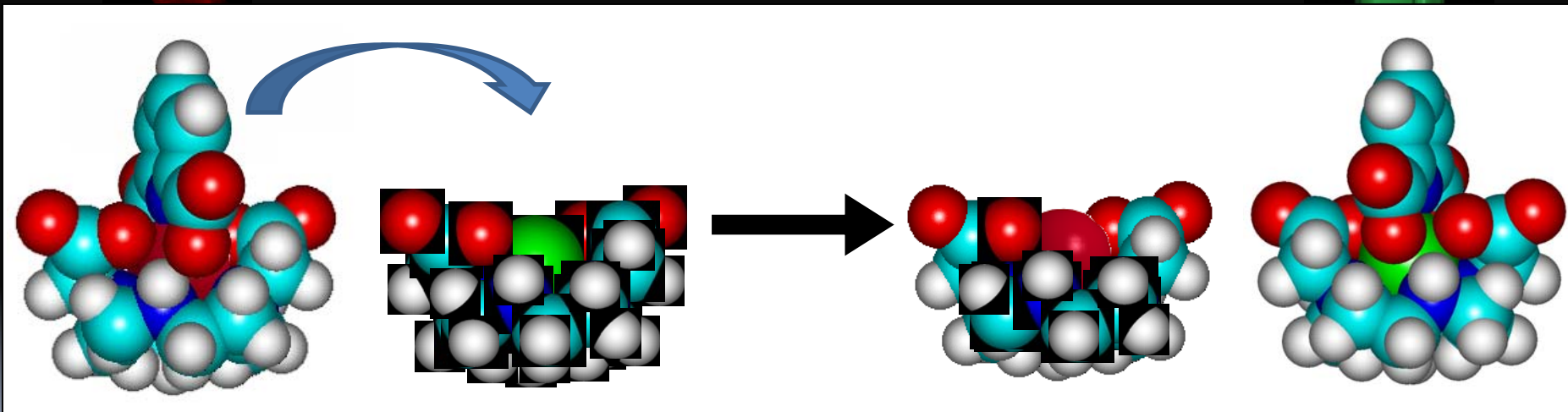
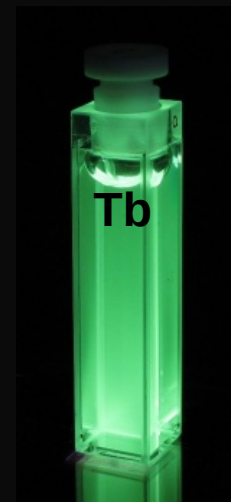
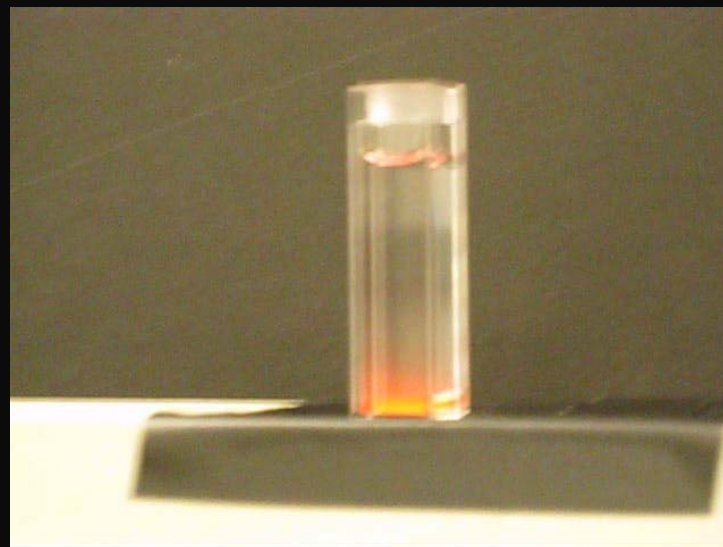
- Ligand Enhancement
 - Binding affinity always increases when ligand is bound
 - Independent of ligand/analyte charge, denticity
 - Due to polarizing effect of ligand!
- Induced Gadolinium Break
 - Ionization energy of Ln^{3+} determines extent of Å-environment
 - Effect of ligand on lanthanide is governed by Ln^{3+} polarizability!
 - Terbium is most affected due to half-shell effect!

These effects can be used to optimize lanthanide receptor site design!



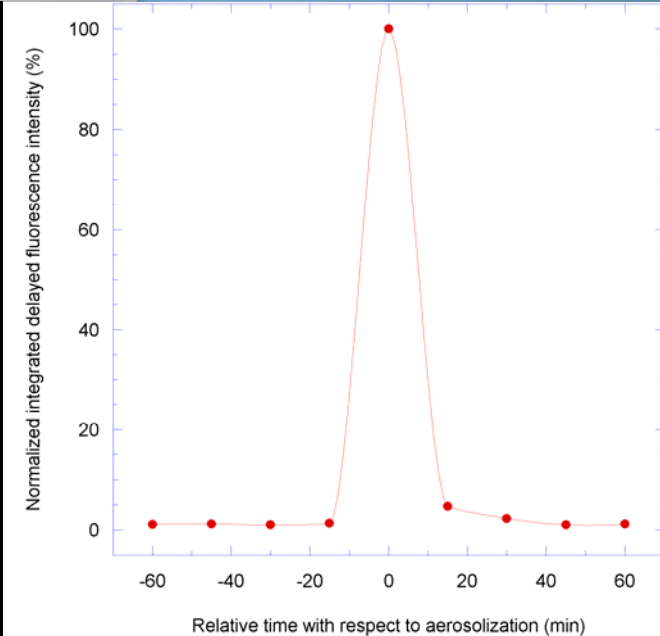
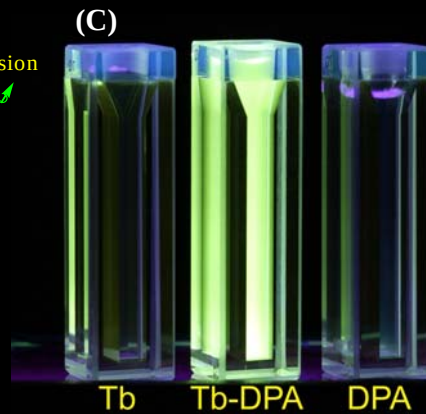
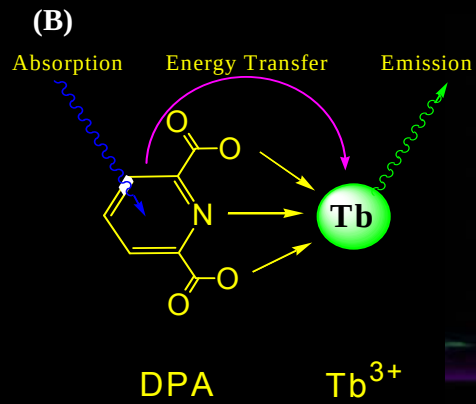
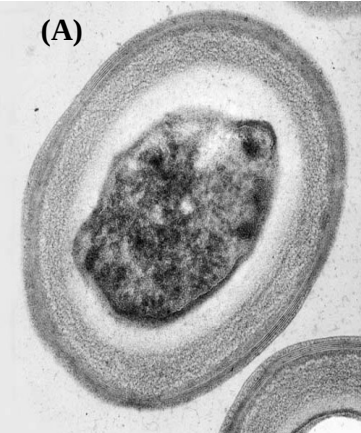
$\text{Ln}(\text{DO2A})^+$

The Terbium Advantage



Tb-DPA Luminescence

Anthrax Smoke Detector



1. Yung, P.T., Lester, E.D., Ponce, A., *Biotech. Bioeng.*, 2007, 98(4), 864-71.
2. Lester, E.D.; Ponce, A., *IEEE Eng. Med. Bio.*, 2004, 23(1), 130-135.

