SAND2013-9243C

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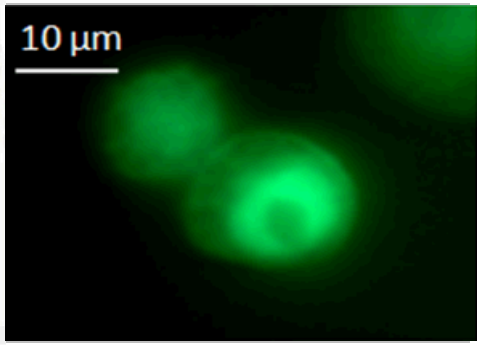

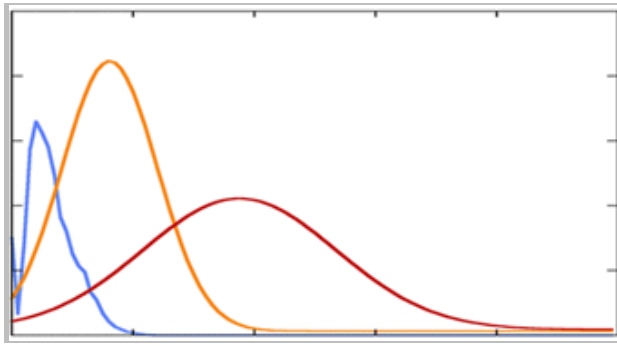

Novel Multiplexed Molecular Assay for the Simultaneous Detection of microRNA and Proteins at Single-cell Resolution

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U.S. DEPARTMENT OF ENERGY | NNSA

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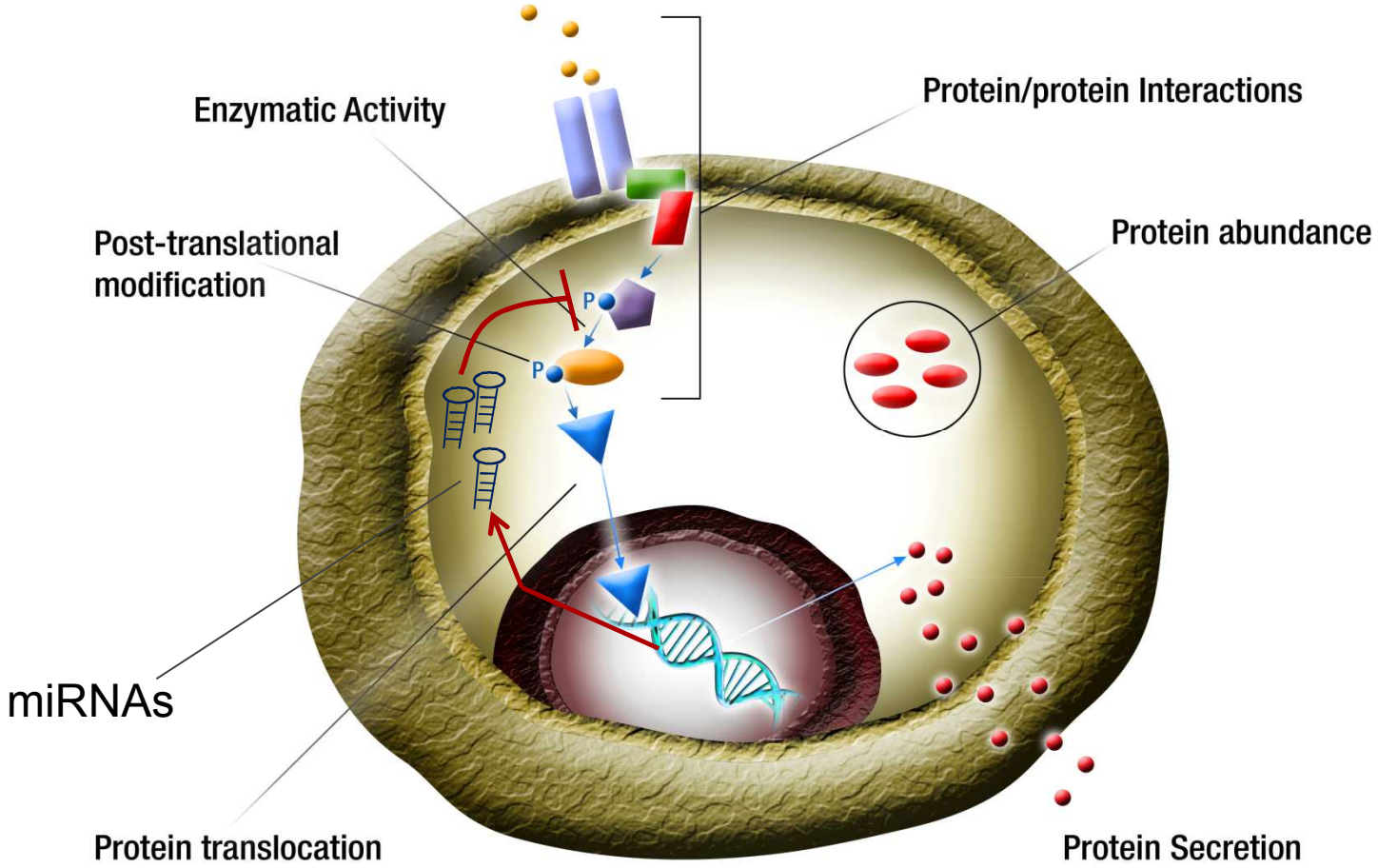
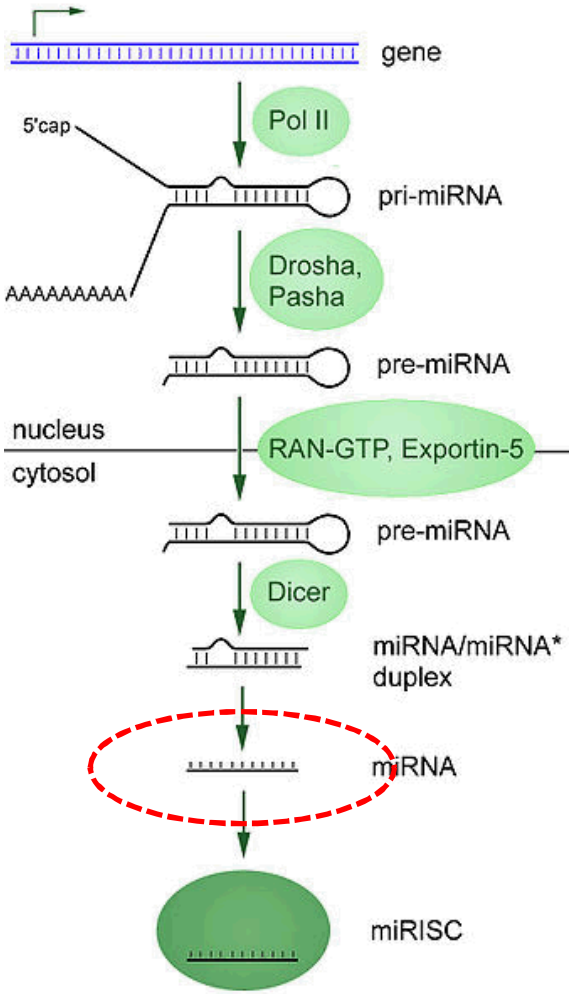
miRNA are released in response to cellular signals



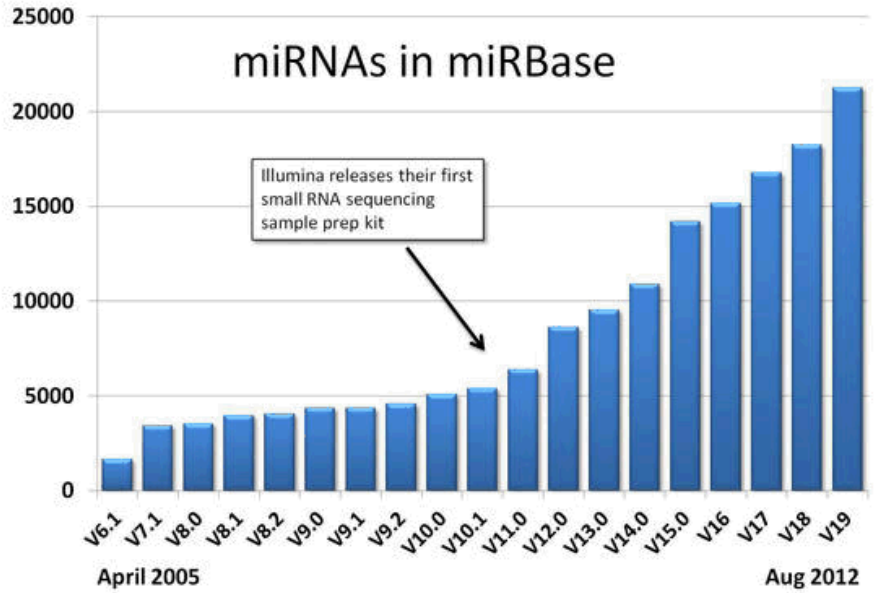
Wu and Singh, Curr. Opin. Biotechnol. 2012 Feb;23(1):83-8

microRNA biogenesis and processing



Repress gene expression via base-pairing with mRNA 3'UTR

microRNAs are subject of intense research and discovery



Potential biomarkers for:

- Alzheimers disease
- Multiple sclerosis
- Drug-induced liver injury
- Ulcerative colitis
- Cancer
- Rheumatoid arthritis

miRBase v.20 released in June, 2013, contains over 30,000 mature miRNA in 37 species, with 2555 unique human miRNAs (http://www.mirbase.org/)

miRNA biomarker discovery workflow

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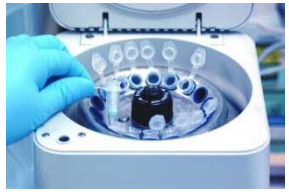
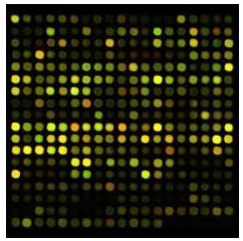
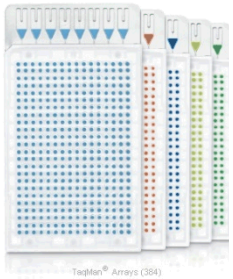
RNA isolation from sample
Reverse transcription



High throughput screening
(microarray, qPCR, RNA-seq)
~1000 miRNAs



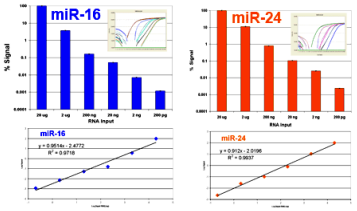


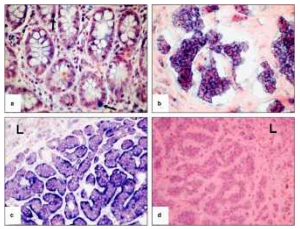
Validation of targets by qPCR
~ 100's

	1	2	3	4	5	6	7	8	9	10	11	12
A	m01	m02	m03	m04	m05	m06	m07	m08	m09	m10	m11	m12
B	m13	m14	m15	m16	m17	m18	m19	m20	m21	m22	m23	m24
C	m25	m26	m27	m28	m29	m30	m31	m32	m33	m34	m35	m36
D	m37	m38	m39	m40	m41	m42	m43	m44	m45	m46	m47	m48
E	m49	m50	m51	m52	m53	m54	m55	m56	m57	m58	m59	m60
F	m61	m62	m63	m64	m65	m66	m67	m68	m69	m70	m71	m72
G	m73	m74	m75	m76	m77	m78	m79	m80	m81	m82	m83	m84
H	m85	m86	m87	m88	HK1	HK2	HK3	HK4	RTC	RTC	PPC	PPC

Statistical analysis
~10's

Development of clinical diagnostic panel
(multiplex qPCR, ISH)





So what's the problem?

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-miRNAs are not yet proven as ideal clinical diagnostic biomarkers

- miRNA expression profiles are tissue-specific, and does not necessarily correlate with disease specificity
- Detection of miRNAs is costly, time-consuming, and variable depending on reagent and method
- No standard method of extraction and analysis – highly variable results depending on specimen source, processing, and contamination.

Need systems level interrogation to put miRNAs into context

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- Accurate determination of the level of miRNAs in a specific cell type or tissue is essential for linking miRNA expression to health and disease states
- Faster sample-to-answer time
- Lower the cost of multiplexed miRNA detection

Goal: to develop novel method for detecting miRNA in intact cells that preserve cellular context

miRNA 155 is the ideal choice for assay development

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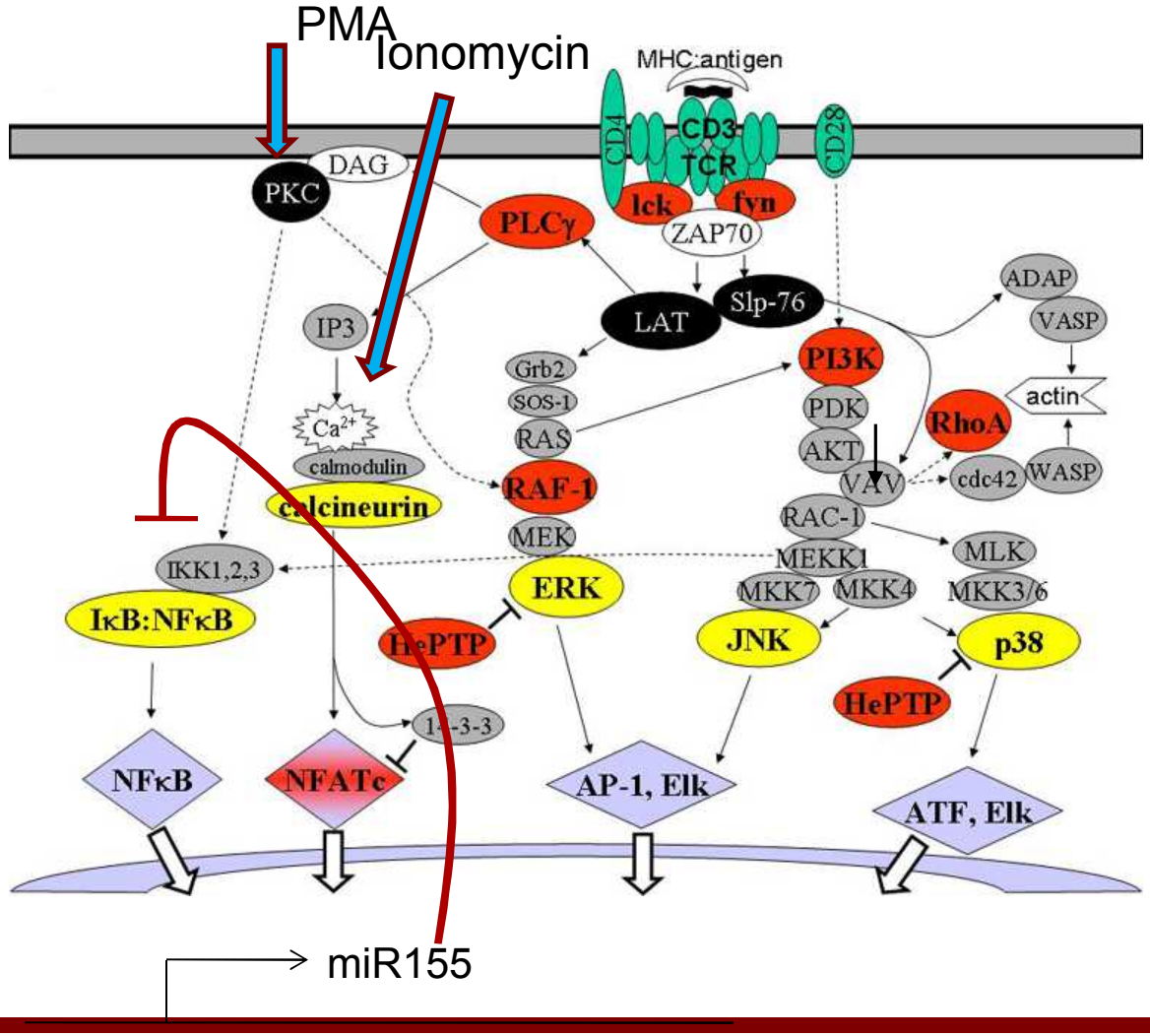
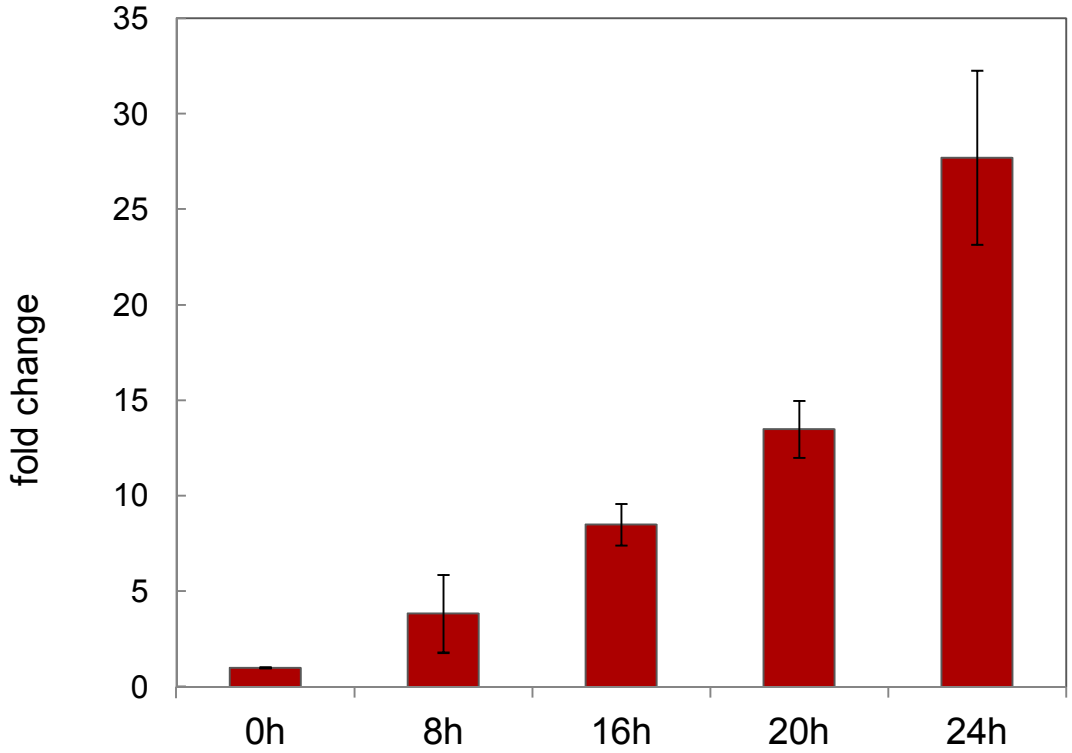
- miR155 is most well studied miRNA
- Only one miR155 gene, no significant sequence significance to other miRNAs
- miR155 is known to be expressed in activated cells of the immune system (T cell activation, B cell differentiation, Dendritic cells, macrophages)
- Known association with normal and transformed hematopoiesis (bic proto-oncogene)
- Overexpressed in human B-cell lymphomas and is target for therapy
- Known association with other cancers
- Significant body of knowledge of miR155 target pathways in multiple cell types

Model system: miR155 in activated T cell lymphoma (Jurkat)



Adapted from Frontiers in Bioscience S2, 969-979, June 1, 2010

qPCR analysis of miR155 expression in Jurkat stimulated with PMA and Ionomycin



Novel assay to detect miRNA in intact cells

- Microfluidic sample preparation (~95% reduction in reagent cost)
- Flow Fluorescent *in situ* hybridization (flow-FISH)
- Flow cytometry – faster, single-cell resolution
- Locked Nucleic Acid (LNA) probes – higher specificity
- Rolling circle signal amplification – higher specificity, isothermal

Microfluidic sample preparation, imaging, and flow cytometry

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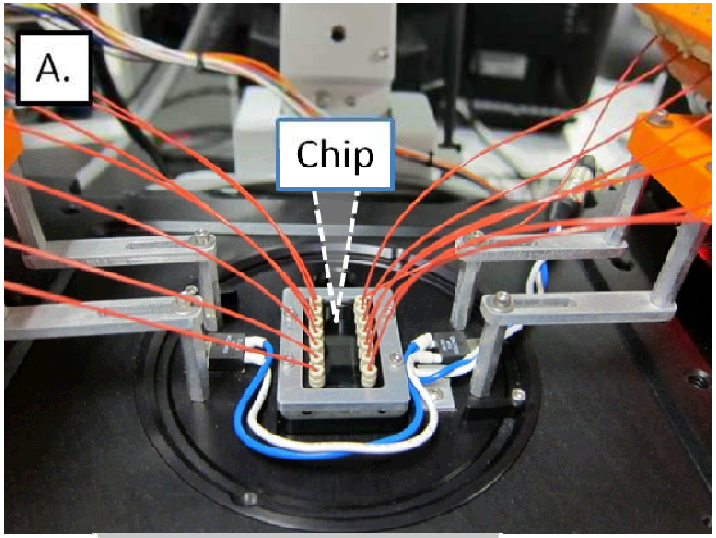
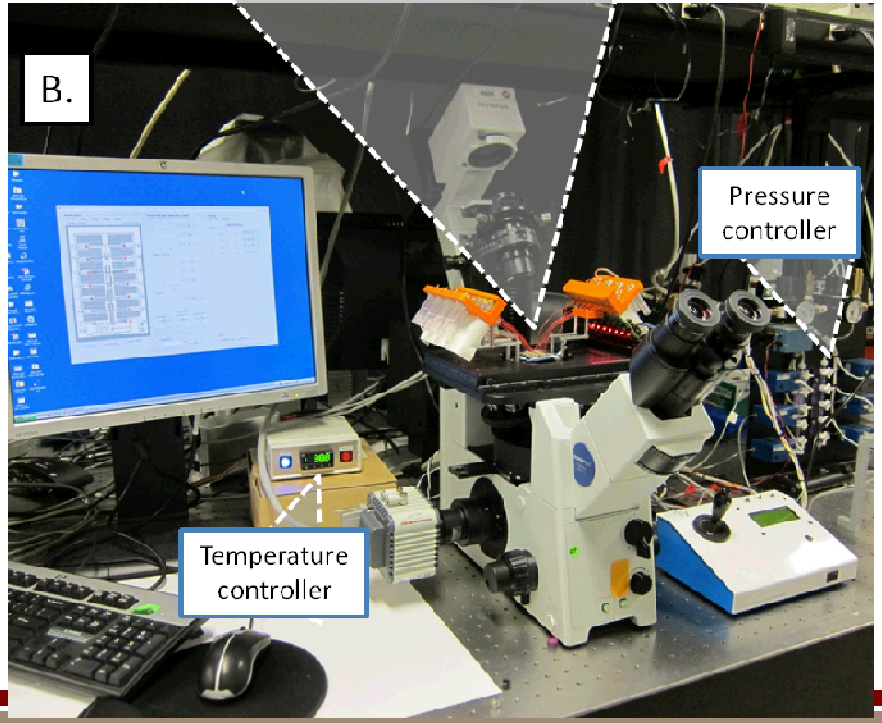
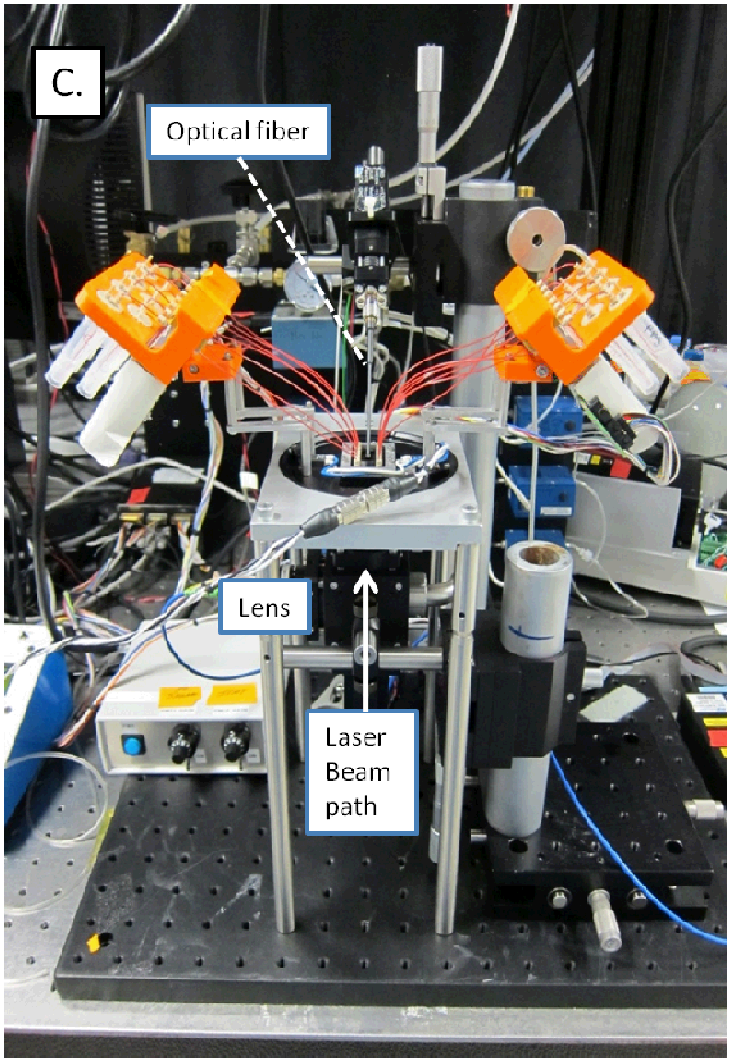
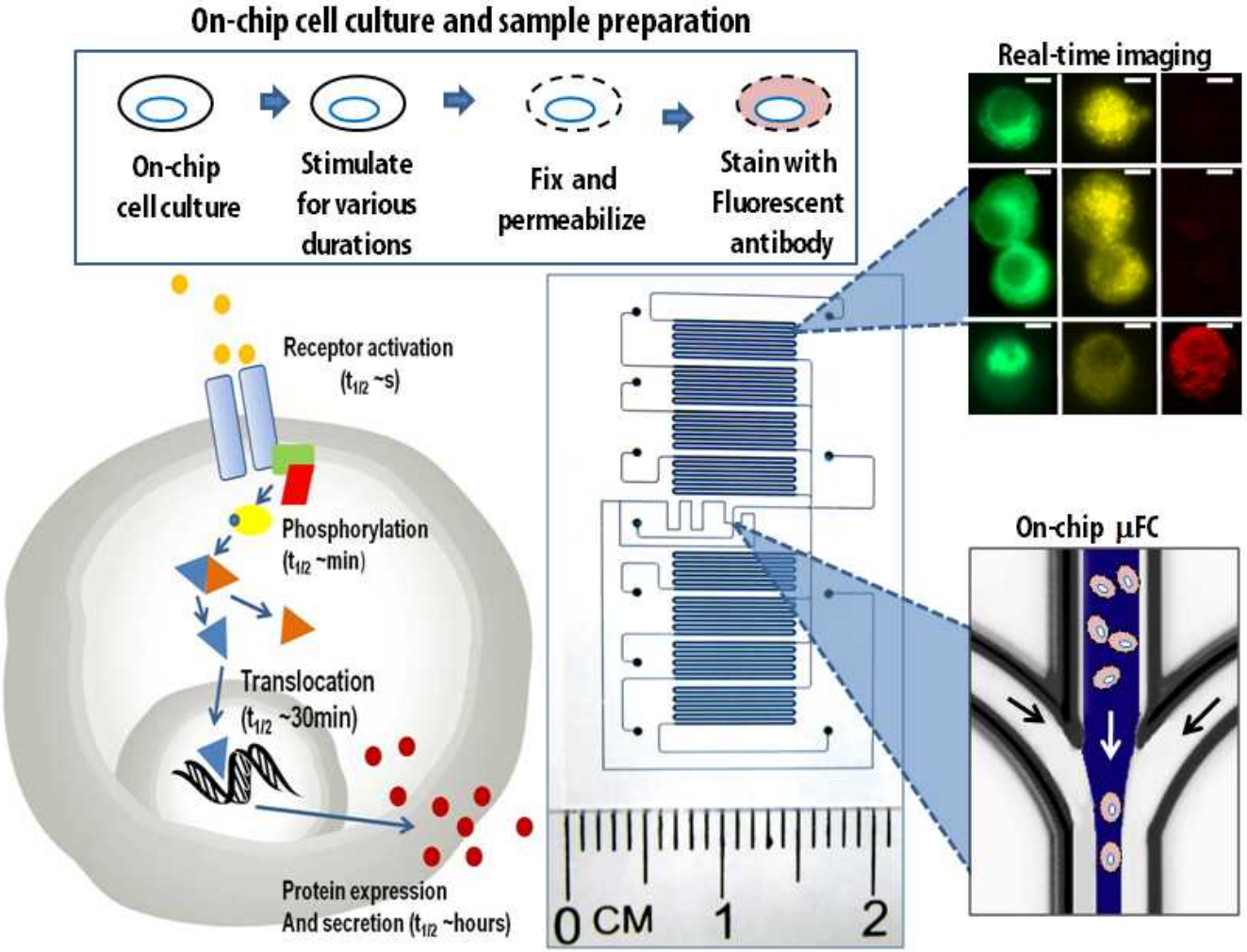




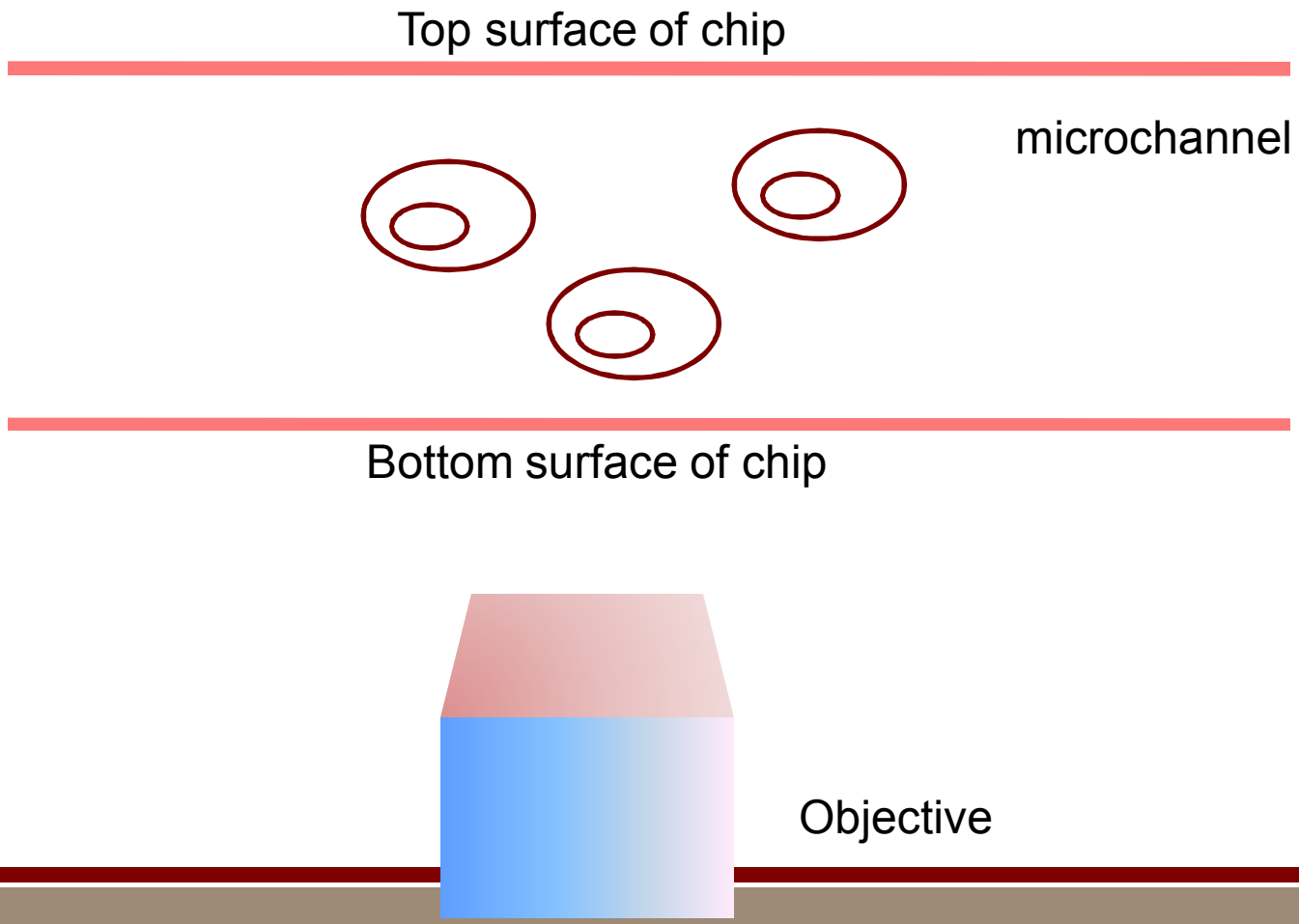


Wu et. al. PloS one 8 (1), e55044

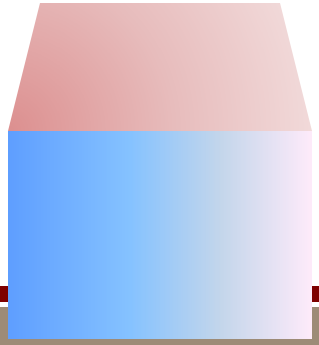
Microfluidic sample preparation



Typical microfluidic experiment



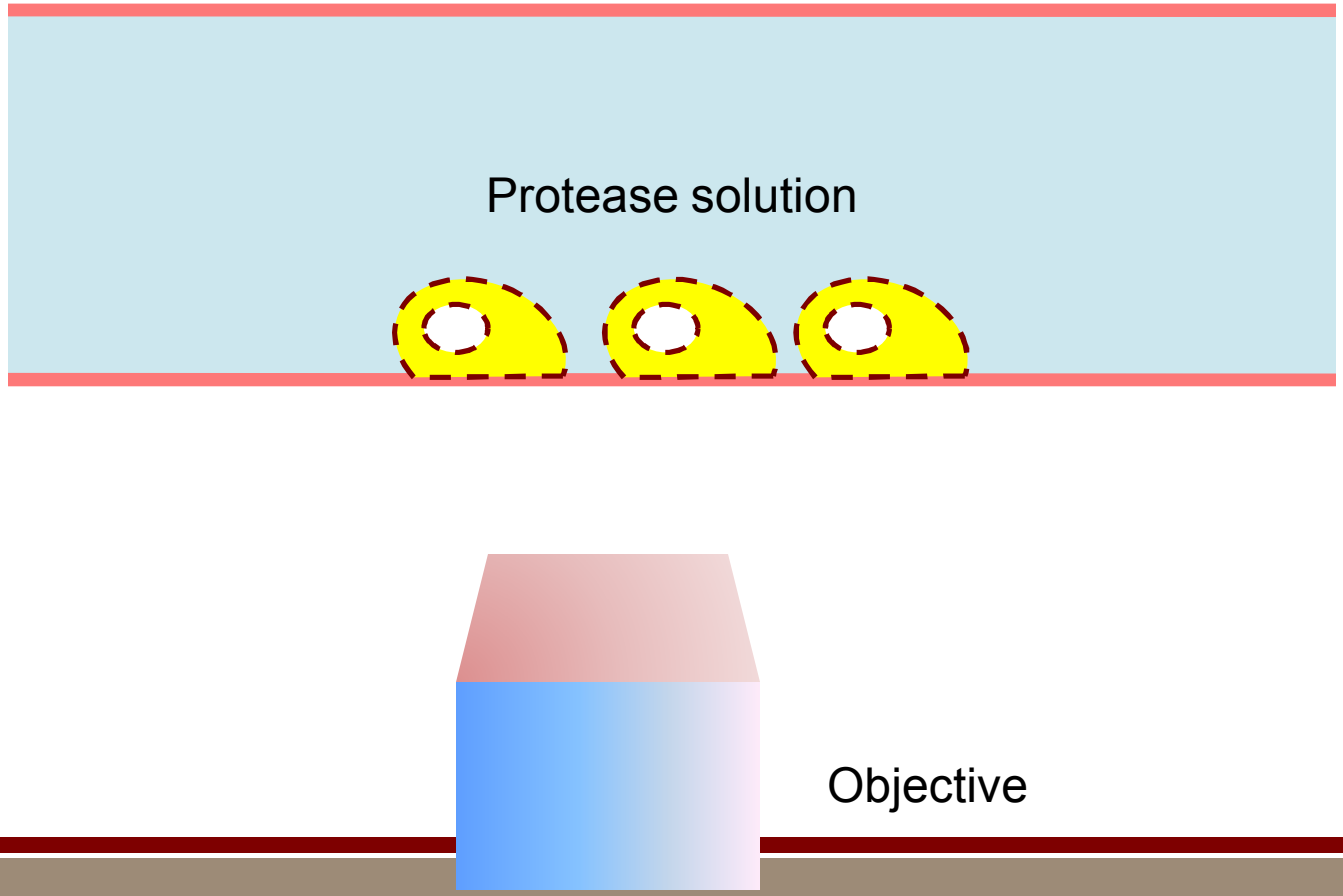
Typical microfluidic experiment



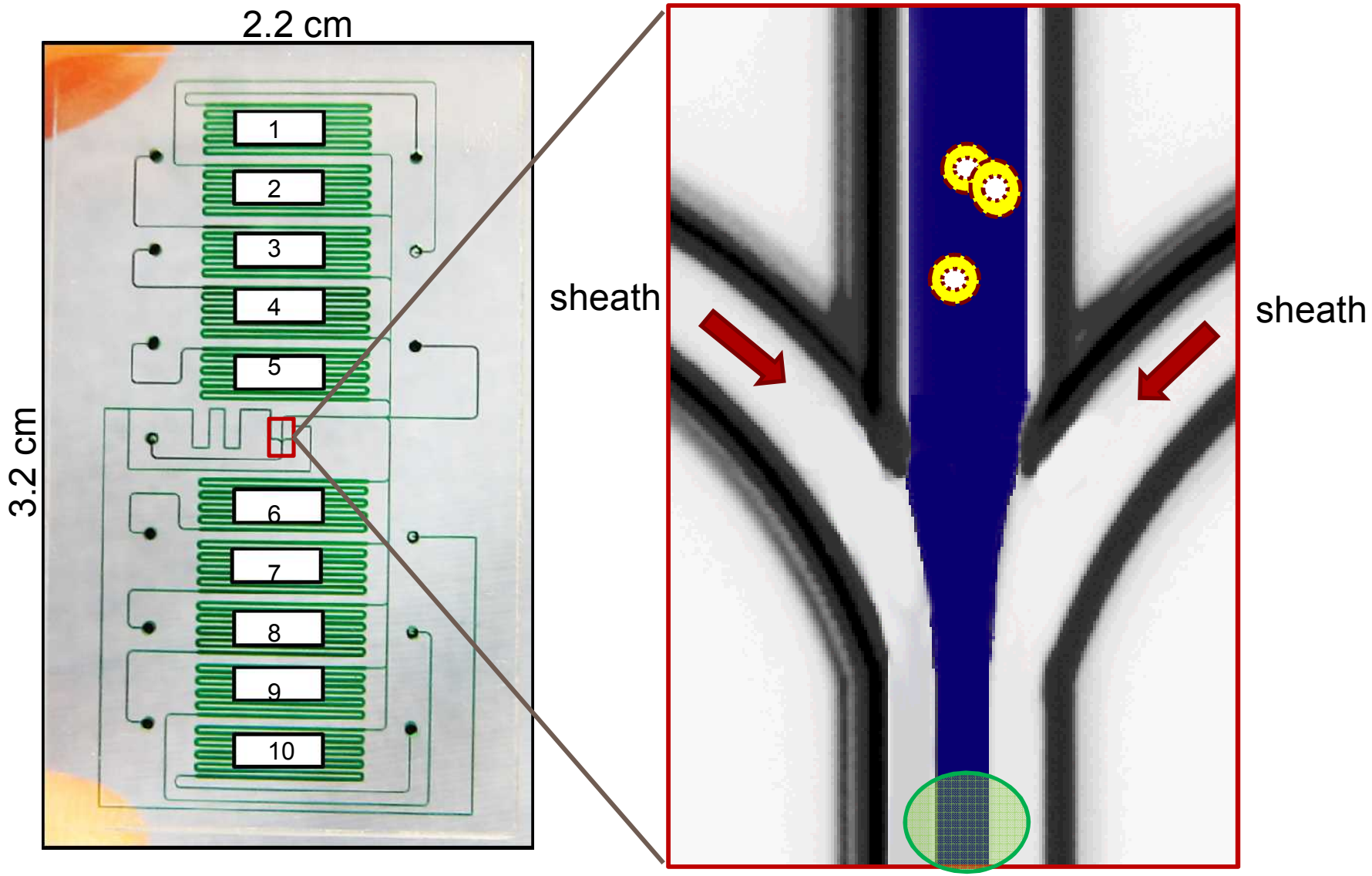
Take photographs of cells in the chip

Objective

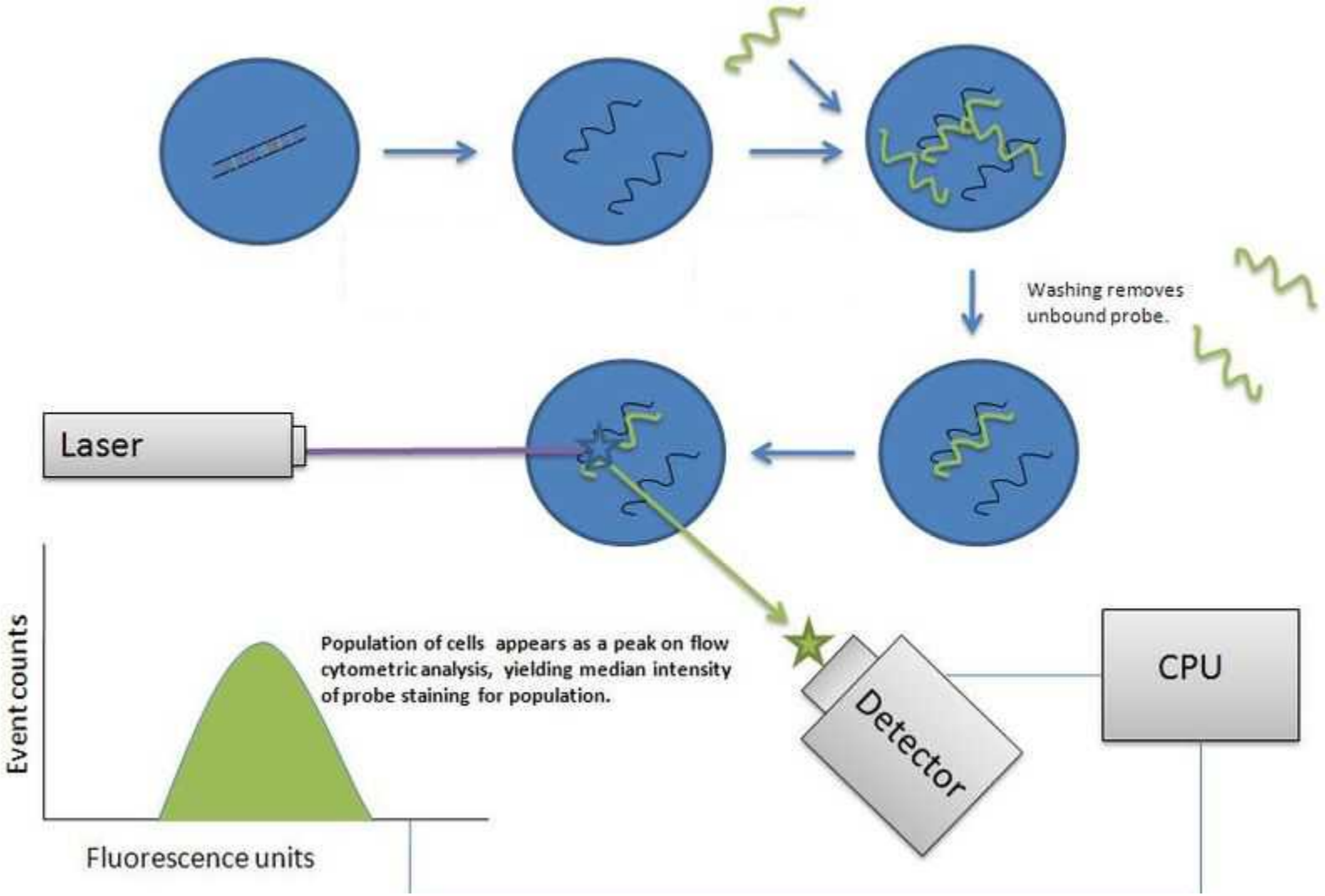
Typical microfluidic experiment



10 chamber microfluidic chip



Flow-FISH method

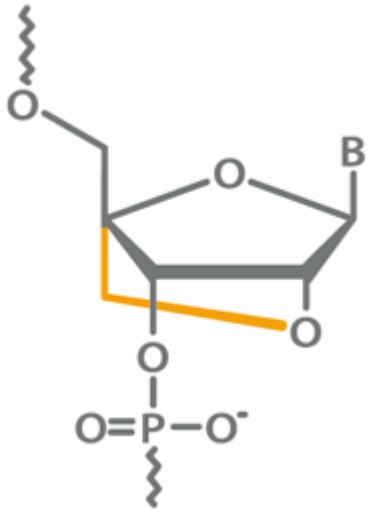


Nathan Corbett, 2008

Challenges:

- Tiny size of mature miRNA (~22nt)
- Low signal to noise ratio
- No known existing protocol for miRNA detection for flow cytometry

Use Locked Nucleic Acid probes to detect miRNA



LNA - The ribose ring is connected by a methylene bridge (orange) between the 2'-O and 4'-C atoms, "locking" the ribose ring in the ideal conformation for Watson-Crick binding.
Oligonucleotide probes with LNA form much more stable complementary duplexes.

miR155: uuaaugcuaaucgugauaggggu

LNA Probe: DIG-acccctatcacgattagcattaa-DIG

Scrambled LNA Probe: DIG-gtgtaacacgtctatacgccca-DIG

Special considerations when working with miRNA:

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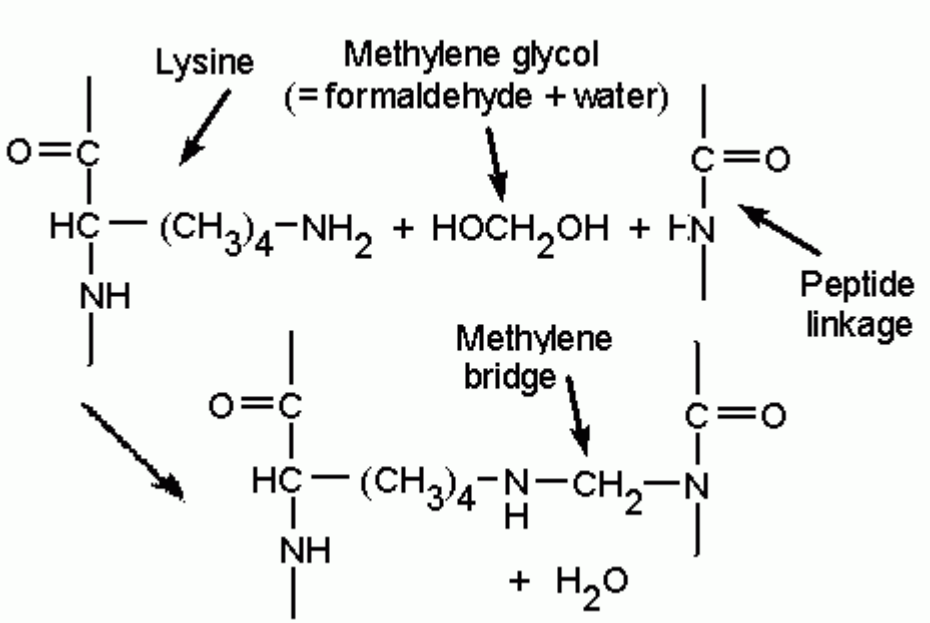
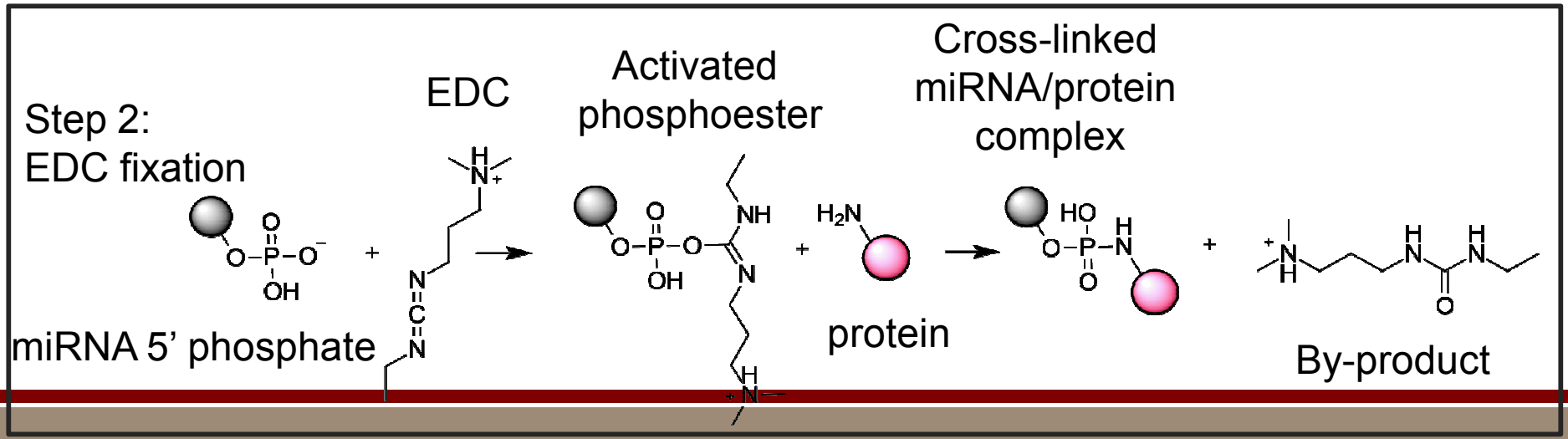
Double fixation required

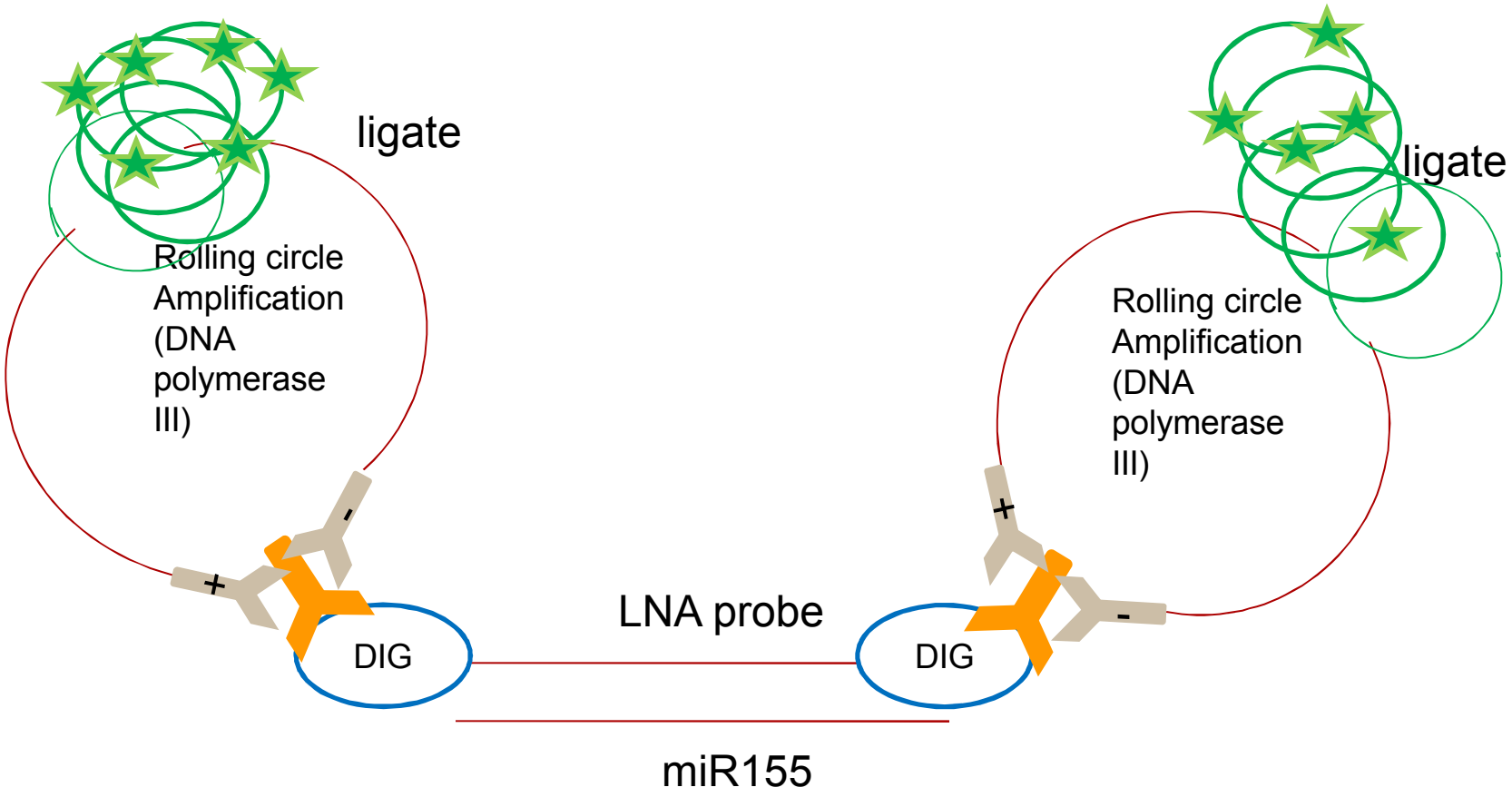
Step 1:
Formaldehyde fixation



Step 2:
EDC fixation



Use Rolling Circle Amplifcation (RCA) to Amplify miRNA signal in single cells



LNA flow-FISH profiling of miR155 expression

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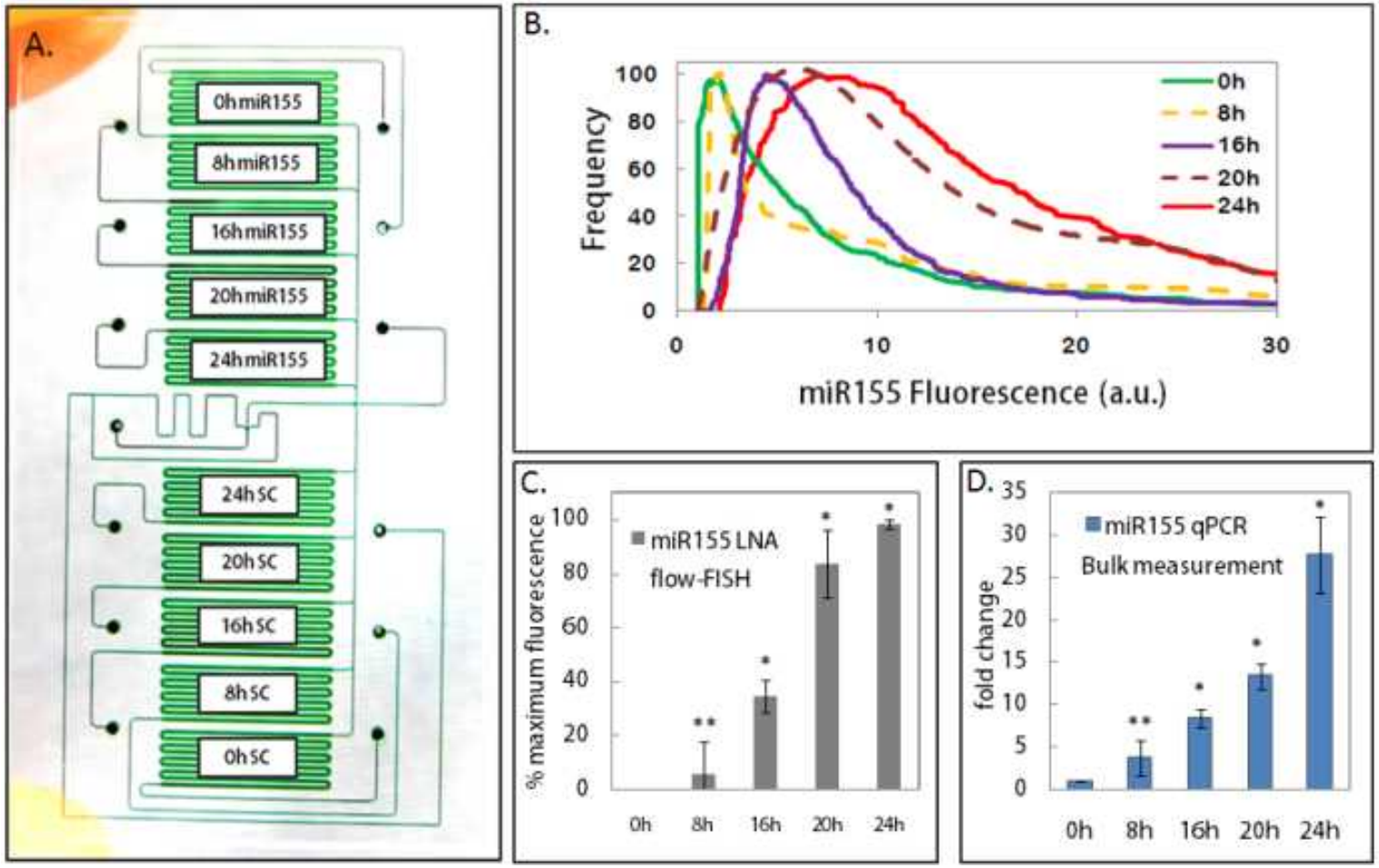

Multiplex with protein immunostaining

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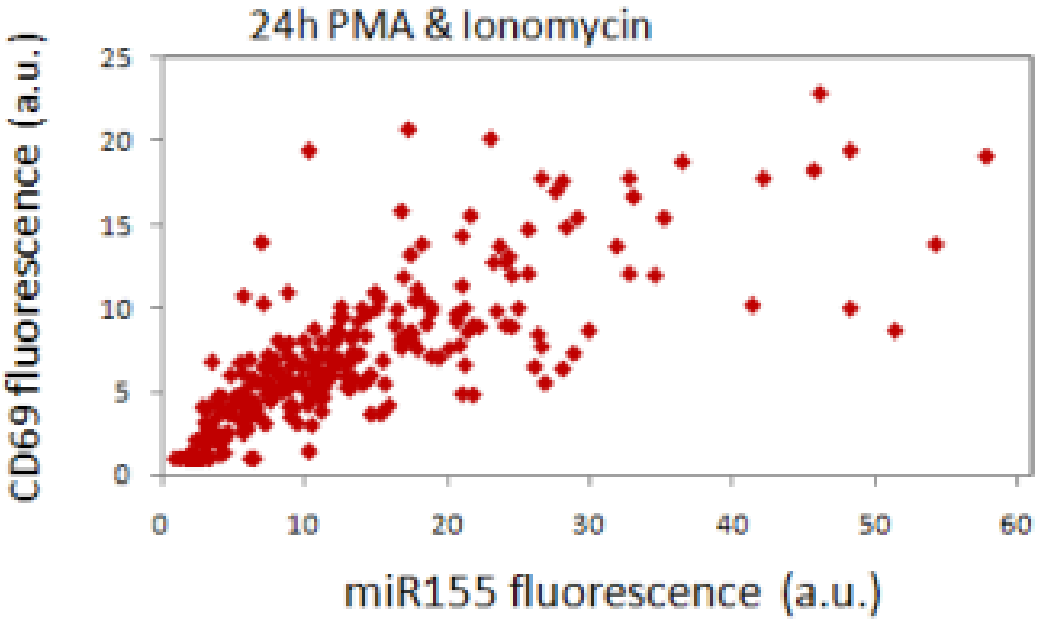
Multiplex LNA Flow-FISH with surface identification protein marker. Useful for detection of miRNAs in a heterogeneous cell population such as PBMC.

Challenge: EDC fixation is necessary for miRNA detection, but destroys protein epitopes and protein fluorescent dye (PE)

Solution: use Qdot for CD69 protein detection to multiplex with LNA Flow-FISH

miRNA detection at single cell resolution shows heterogeneity in the population



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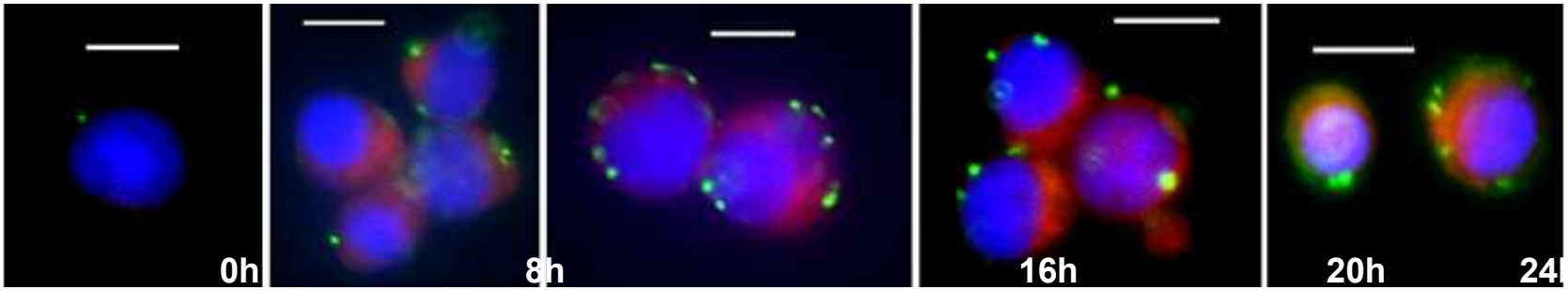
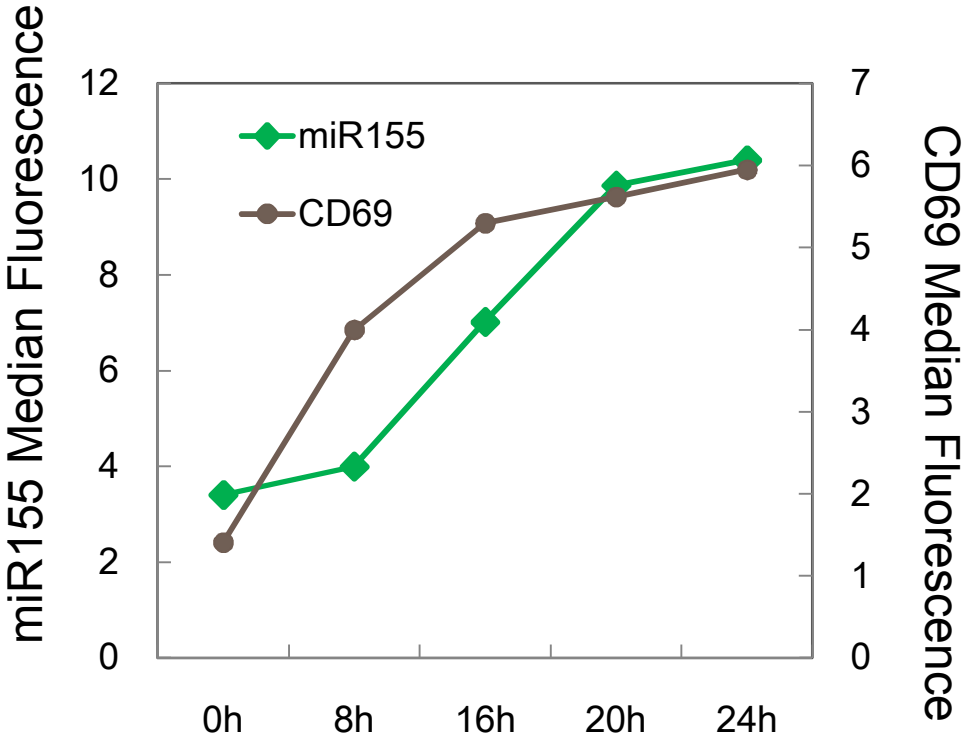
Multiplexing Results



All scale bars = 10 µm
Blue = nucleus
Red = CD69 surface protein
Green = miR155



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