Droplet Microfluidic Chip based qRT-PCR Amplification and Detection of Influenza Viruses

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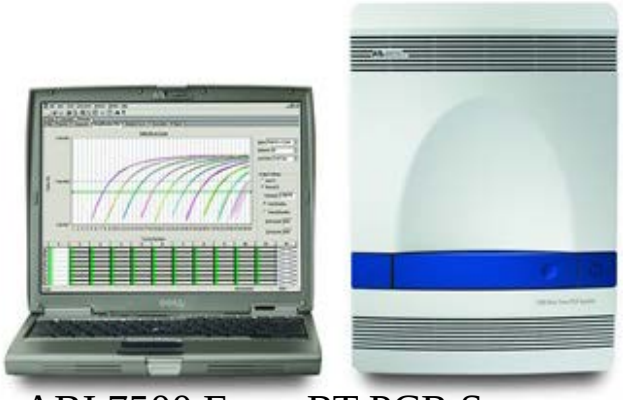
Presentation Outline

- **Introduction**
  - Polymerase Chain Reaction (PCR), rtPCR, RT-PCR and qRT-PCR nucleic acid amplification methods
  - Background: Droplet Microfluidics (DMF);
  - Droplet actuation methods: Dielectrophoresis (DEP) and Electrowetting (EW)
- **Micro-electrode architectures for chip based qRT-PCR and post amplification detection**
- **Design and optimization of micro-heater electrode structure**
- **qRT-PCR Device fabrication**
- **Experimental set-up and incorporation of the MIP interface**
- **Materials and methods**
- **Results**
  - Integrated DMF electrode architectures and post amplification detection of Influenza C virus using Molecular Beacon probe
  - Video illustrations of RT-PCR reaction on two integrated droplet microfluidic devices
  - Performance of chip based real-time, RT-PCR reaction for detection of Influenza viruses
  - Chip based real-time, quantitative RT-PCR reaction and detection of Influenza A, C viruses
- **Conclusions**

Polymerase Chain Reaction (PCR); Various PCR Techniques

- **PCR:** A biochemical method to amplify few copies of a DNA molecule, across several orders of magnitude to facilitate detection and characterization of the particular DNA sequence (End point detection).
- **RT-PCR:** PCR Method used to qualitatively detect gene expression through creation of complementary DNA (cDNA) transcripts from RNA (End point detection).
- **qRT-PCR:** A real-time, quantitative RT-PCR method where detection and quantification occurs during PCR cycling hence facilitating faster and more efficient quantification of the nucleic acid sample.

Early Model of PCR Machine (Gene Machine)



ABI 7500 Fast qRT-PCR System (Applied Biosystems)

QX200™ Droplet Digital PCR System (Bio-Rad)

- **Micro-chip based qRT-PCR systems:** Such micro qRT-PCR devices are currently in the development phase. These integrated microfluidic chips can utilize close-channel fluidics or, droplet based surface microfluidics to dispense, mix, transport and thermal cycle PCR reaction mixture, in ultra-low reaction volumes of few 100 nL to 20 µL.
- Micro-qRT-PCR devices can provide low cost, portable and multiplexed detection of nucleic acid samples

Advantages of Droplet Microfluidics (DMF)

Droplet Microfluidics:

- Droplet based open channel/surface microfluidic (SMF) technology (consist of patterned micro-electrodes/insulated top surfaces)
- Controlled dispensing of aqueous sample/reagent droplets (μL-pL volume)
- On-chip sample dispensing, manipulation (mixing/splitting) etc…
- Combinatorial Bio-chemistry and detection

Salient features of DMF devices:

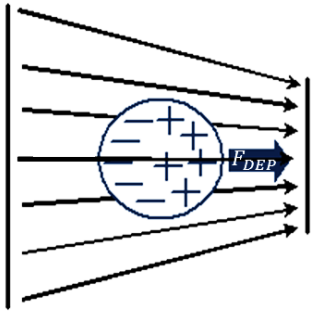
- Ultra low consumption of expensive bio- samples/reagents
- Rapid testing and high throughput screening
- Custom micro-fabrication of devices
- Ease of further integration of sensor technology (waveguides, CMOS/CCD sensor layers

DMF: Popular Electro-actuation Methodologies

Key attributes of two popular DMF techniques Electrowetting (EW or EWOD)	Liquid Dielectrophoresis (L-DEP)
E- field based manipulation on patterned surfaces	E- field based manipulation on patterned surfaces
Precision droplet based	Precision droplet based
Low voltage (10 – 200 V_{pp}), low frequency (1 Hz – 1 kHz)	High voltage (200 V_{pp} – 500 V_{pp}), high frequency (50 kHz –1 MHz)
Interfacial forces at liquid-solid boundary under external E-field	Body forces (DEP) due to spatially non-uniform external E-field
Minimal sample dead volume	Minimal sample dead volume
Less sample adsorption, contamination issues (compared to close channel devices)	Less sample adsorption, contamination issues (compared to close channel and EW)
Easy on-chip hybrid integration	Easy on-chip hybrid integration
Low sample/reagent volume requirement	Low sample/reagent volume requirement
Slow and sequential dispensing (sec); superior for droplet mixing/transport/splitting	Rapid dispensing (msec), parallelism and scalability (100 nL to 10 pL); superior for rapid dispensing and multiplexed screening

Dielectrophoresis (DEP) and Liquid-DEP (L-DEP)[1, 2]

Spatially non-uniform Electric field

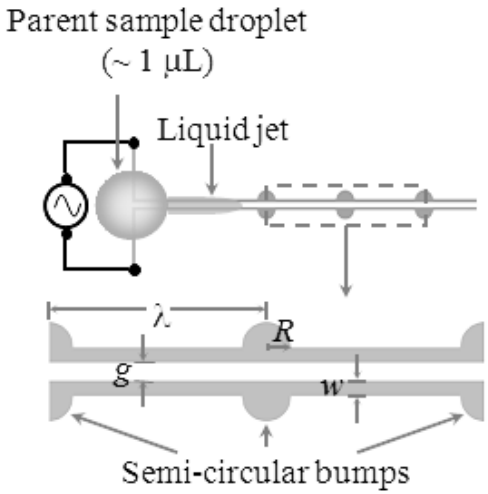


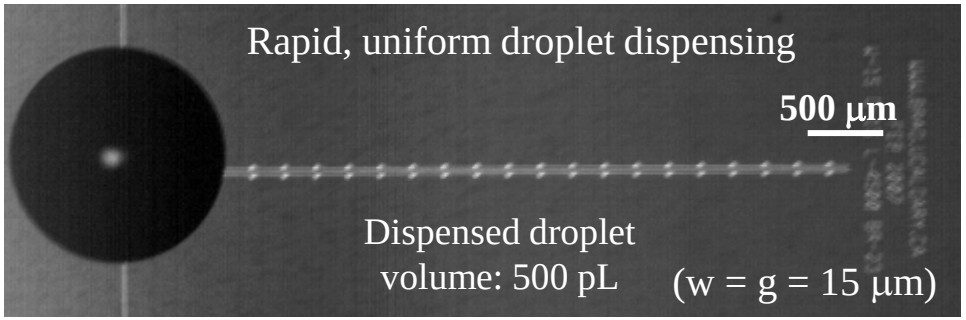
Pondermotive force on an electrically neutral, polarizable matter in spatially non-uniform electric field **(DEP)**

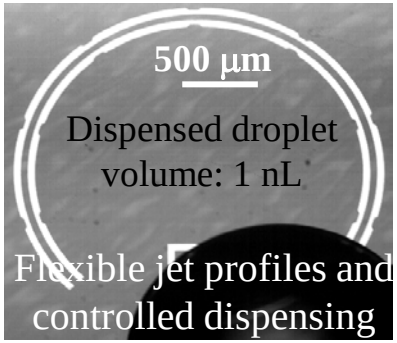
$$\bar{F}_{DEP} = \left(\bar{P}_{eff} \bullet \nabla\right)\bar{E}$$

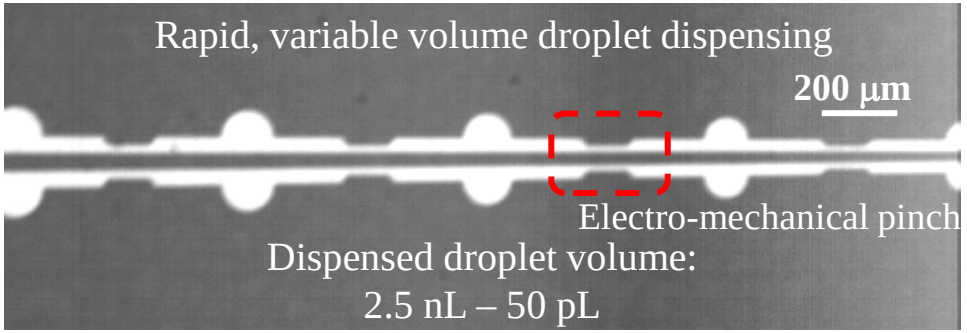
- **L-DEP:** Manipulation of dielectric fluids using spatially non-uniform electric field
- High frequency (f_{act}> 50 kHz) AC signal (V_{act}: 200-500 V_{pp}) required for L-DEP microfluidic applications
- High speed liquid actuation; actuation time: 10-20 msec; hemi-cylindrical liquid jet formation
- Jet break-up and droplet dispensing (in μL to pL range); **Rayleigh's Instability Criteria:**

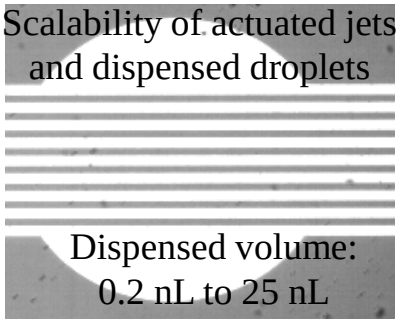
$$\lambda = 9(w + g/2)$$







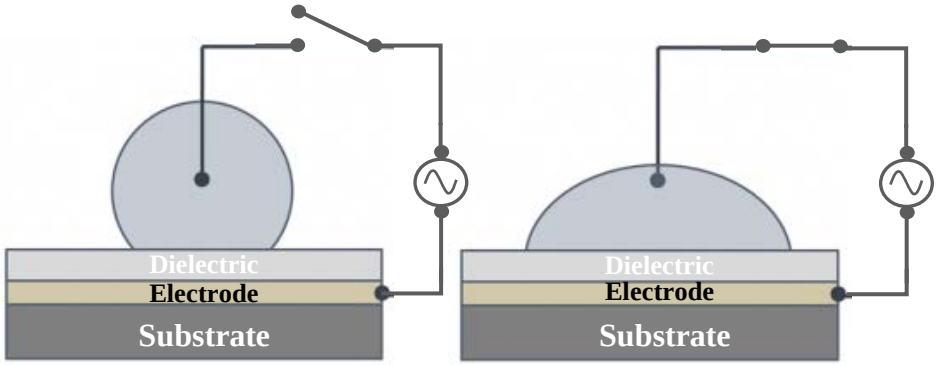


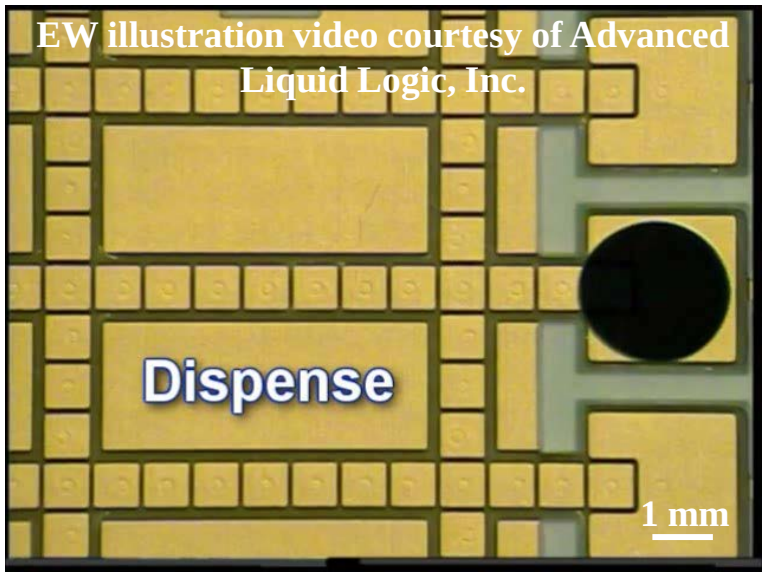


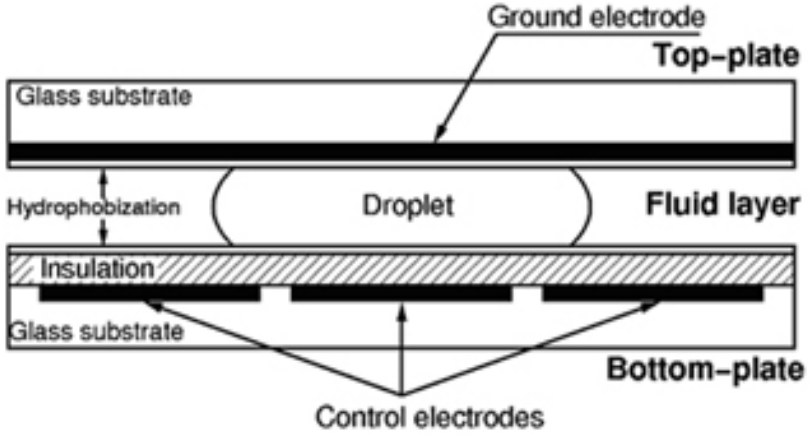
Droplet Manipulation Methodologies: Beyond Dispensing

Two and single surface Electrowetting (EW)[3, 4]

- Electrowetting (EW) refers to modulation of liquid contact angle as a result of an applied voltage (DC or, Low frequency AC voltage (50 – 200 V_{pp} at 10-90 Hz)
- EW can be leveraged to dispense, transport and to mix/split aqueous sample and reagent droplets sandwiched between an insulated, hydrophobic surface (bottom) and a top ground plate





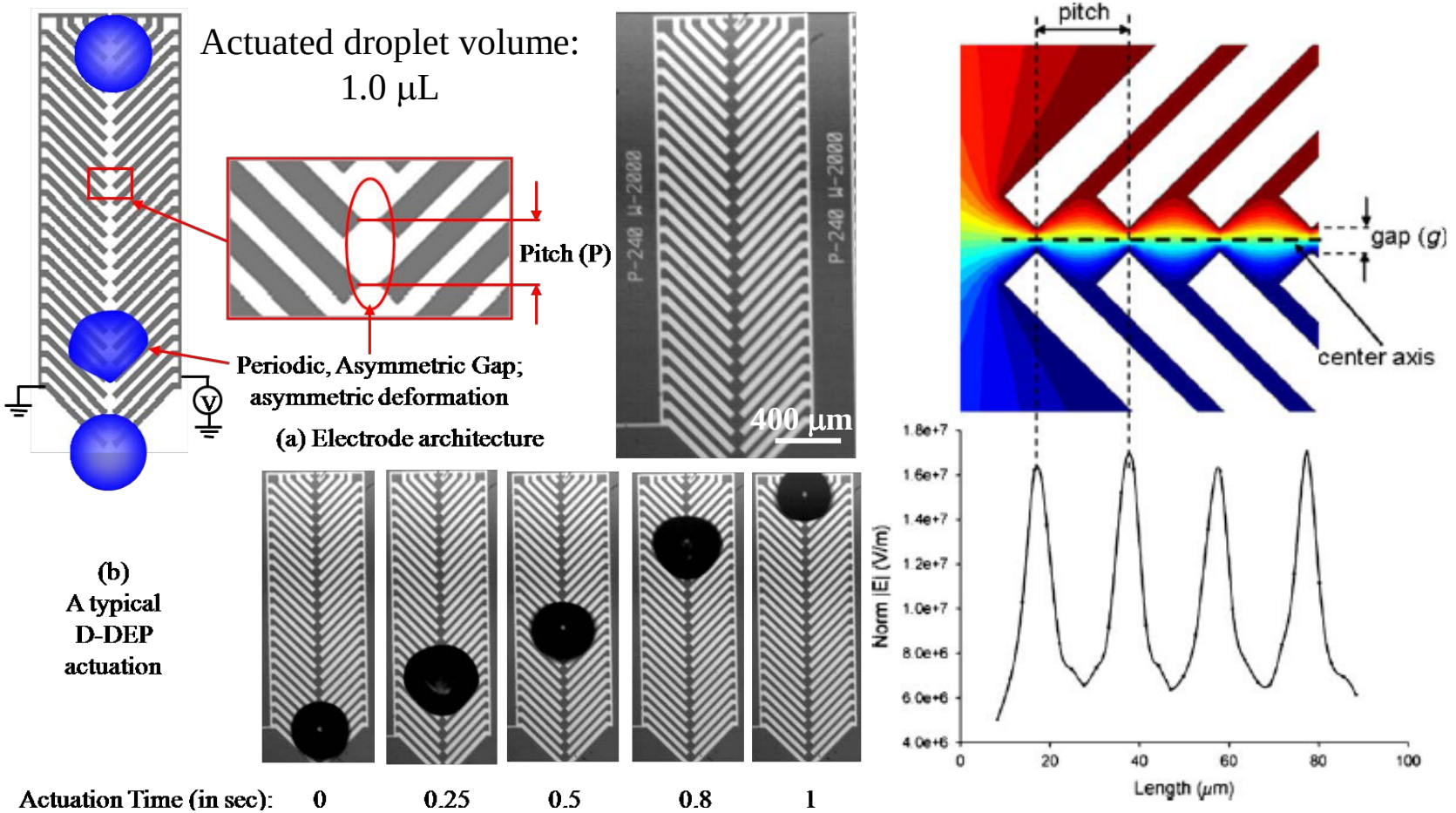


Topology of an Electrowetting DMF device

- Single surface EW often requires super hydrophobic coatings to manipulate droplets on top of passivated electrode structures without the need for a top ground plate

Droplet Manipulation Methodologies: Droplet DEP (D-DEP)

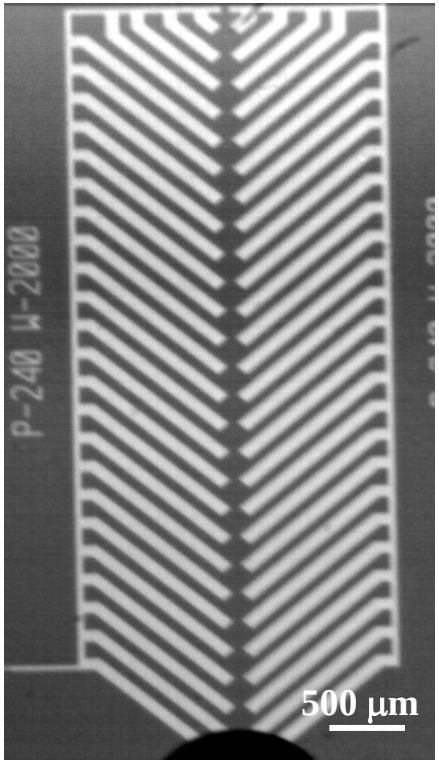
- **Electrostatic droplet actuation (or, D-DEP)[5, 6]:** D-DEP utilizes herringbone shaped electrode architecture to create a unidirectional droplet motion using a combination of EW and DEP effect.
- Low frequency AC voltage (50 – 100 V_{pp} at 30-90 Hz) applied across the herringbone shaped electrodes.



D-DEP electrode architectures

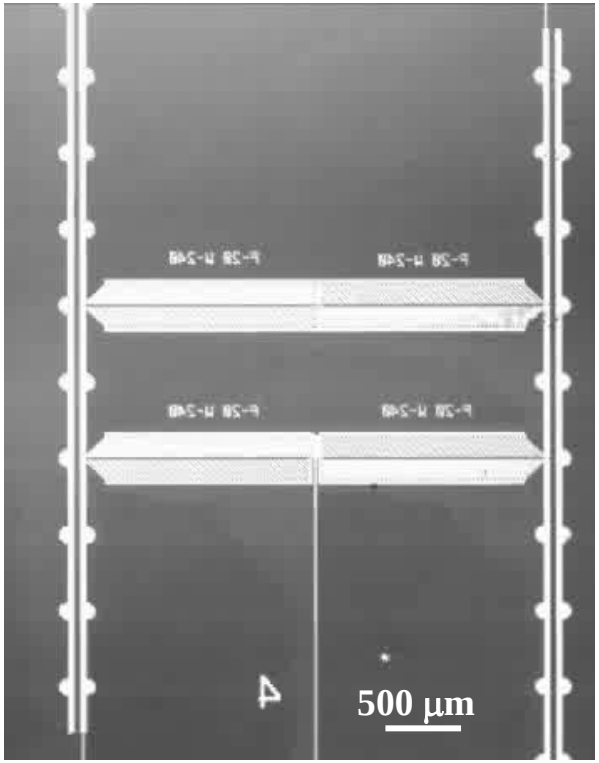
Scalability and versatility of D-DEP based droplet manipulation

Electrostatic droplet actuation (D-DEP)



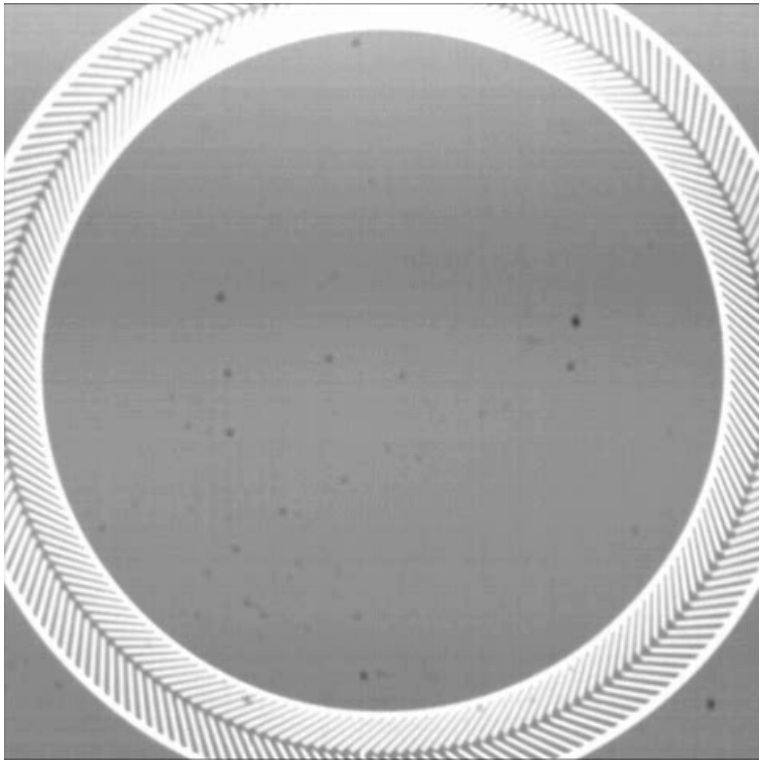
Actuated droplet volume: 1.0 µL

1x1 integrated L-DEP-D-DEP scheme



Actuated droplet volume: 1.0 nL

Circular droplet transport scheme

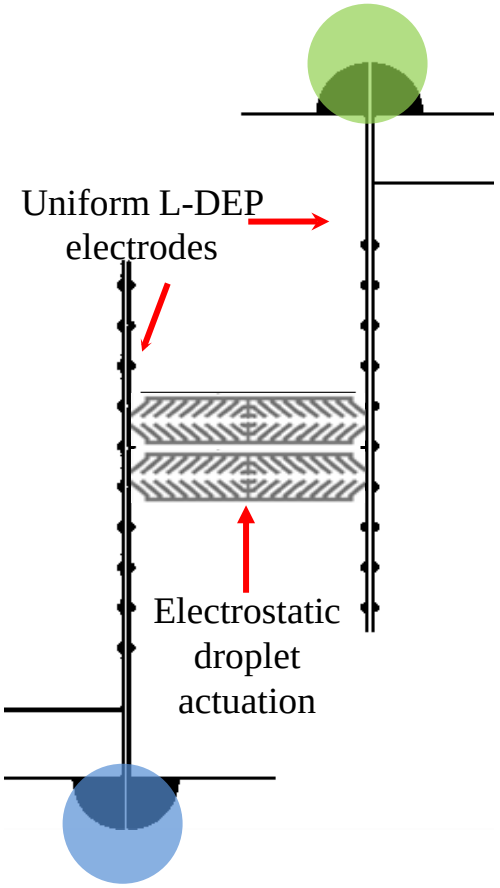


Actuated droplet volume: 1.0 nL

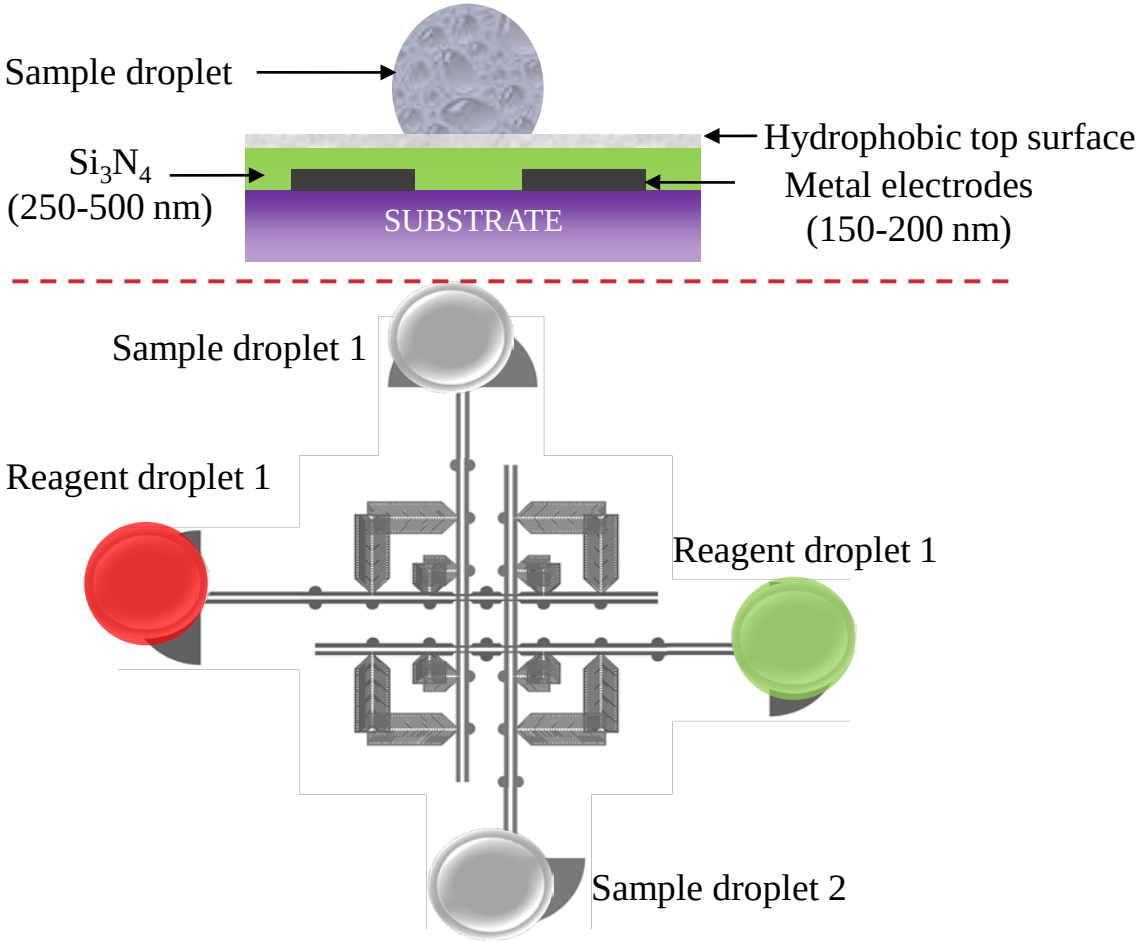
Micro-electrode architectures for chip based qRT-PCR and post amplification detection assays

Integrated DMF electrode structures for post amplification screening[6]

- Integration of the two DEP based droplet actuation methods (L-DEP and D-DEP) results in electrode structures suitable for rapid, multiplexed screening assays



1x1 integrated structure for binary combinatorial assays

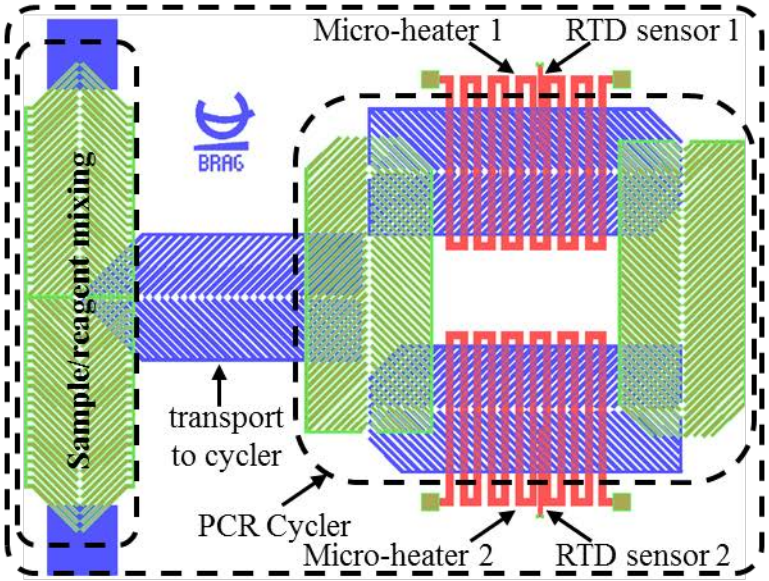


2x2 matrix structure for viral detection assay

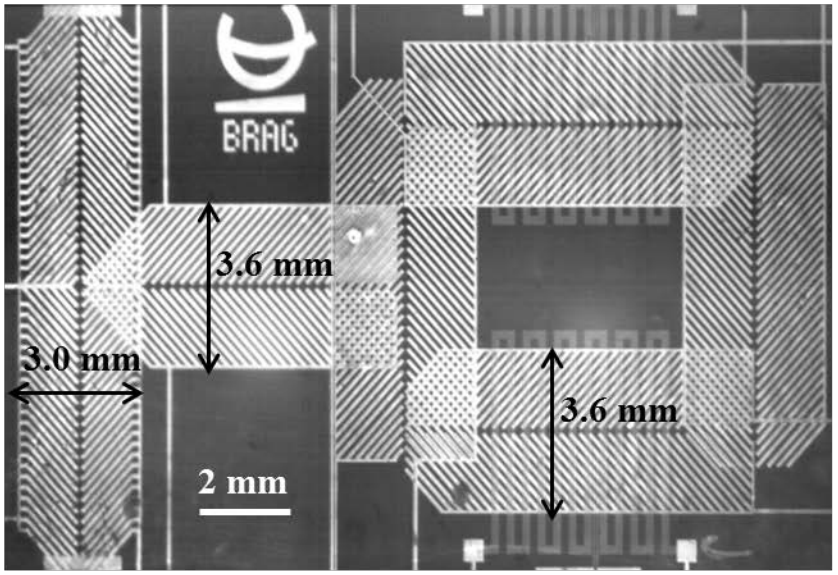
Micro-electrode architectures for the chip based qRT-PCR

qRT-PCR micro-electrode 1

Schematic of the qRT-PCR micro-electrode 1

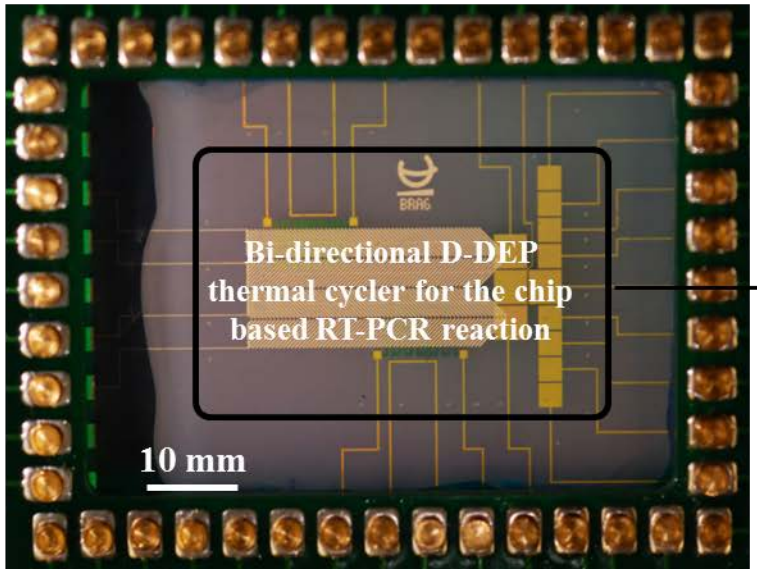


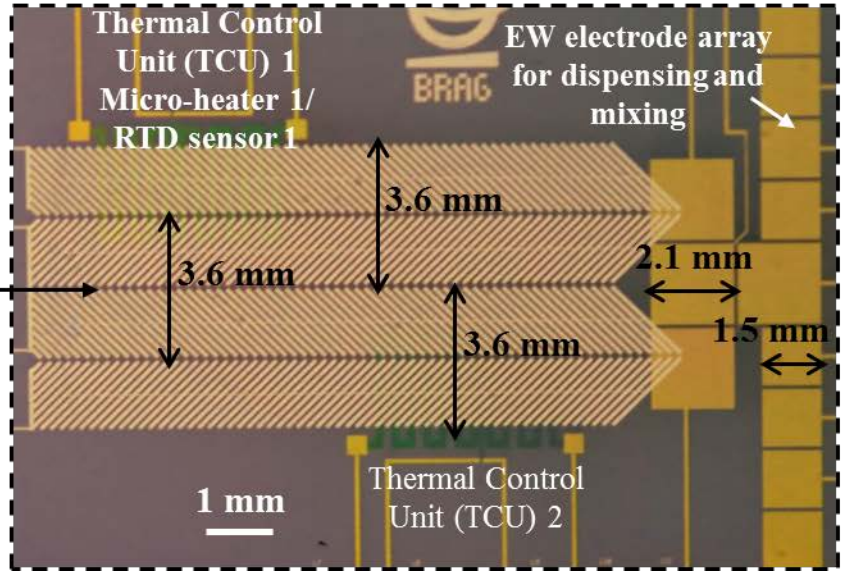
Snapshot of the qRT-PCR micro-electrode 1



qRT-PCR micro-electrode 2

Snapshots showing design attributes of qRT-PCR micro-electrode 2

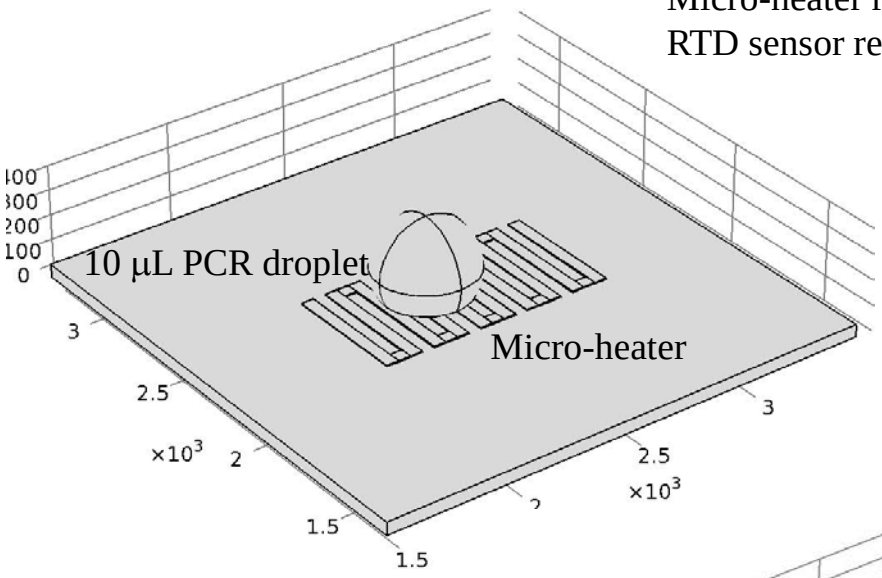




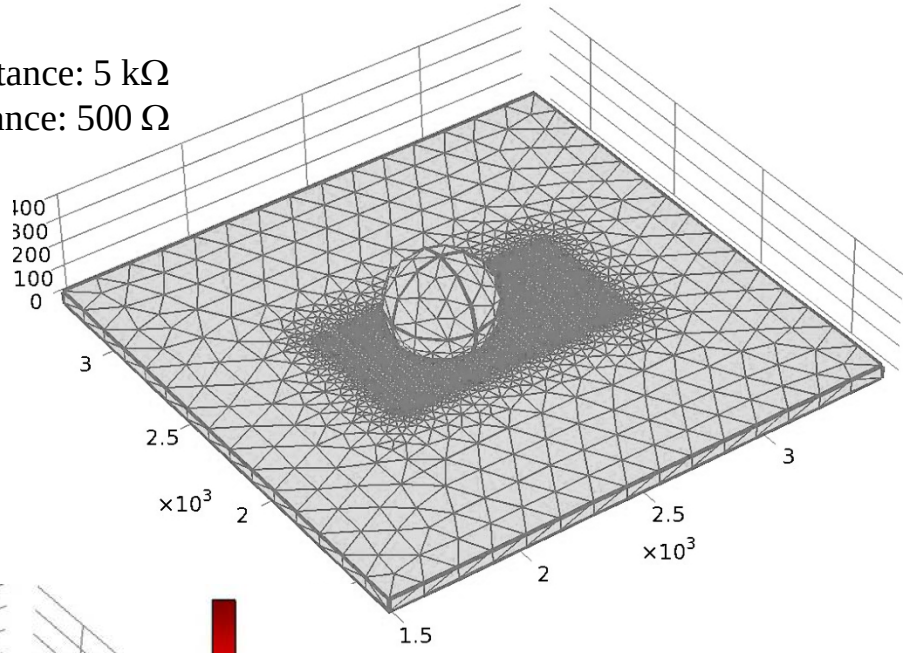
Design and optimization of micro-heater electrode structure for the chip based qRT-PCR assays

Simulation Results For Optimized Micro-heater Design

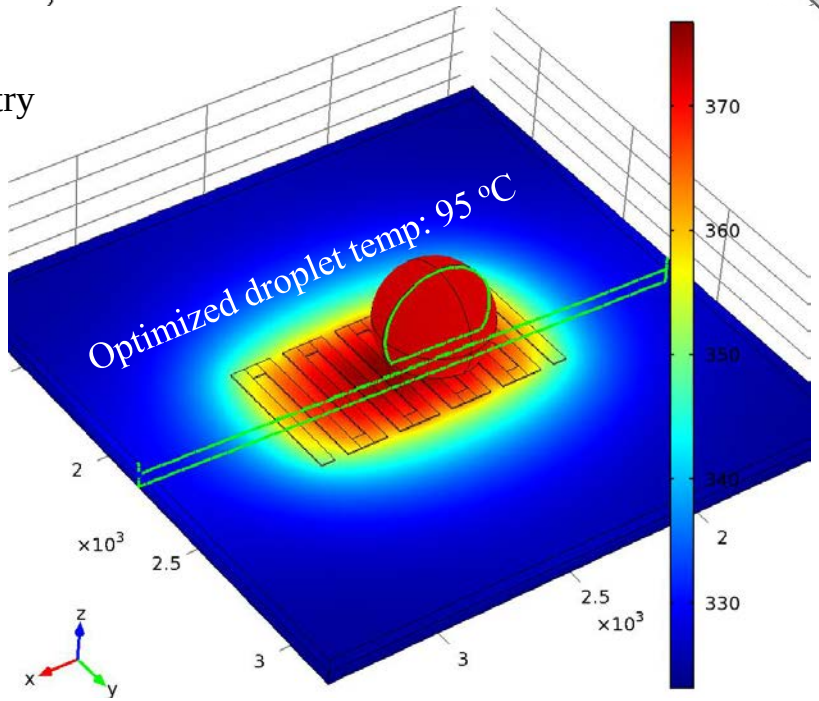
Micro-heater resistance: 5 kΩ
RTD sensor resistance: 500 Ω



Model geometry



Tetrahedral meshing



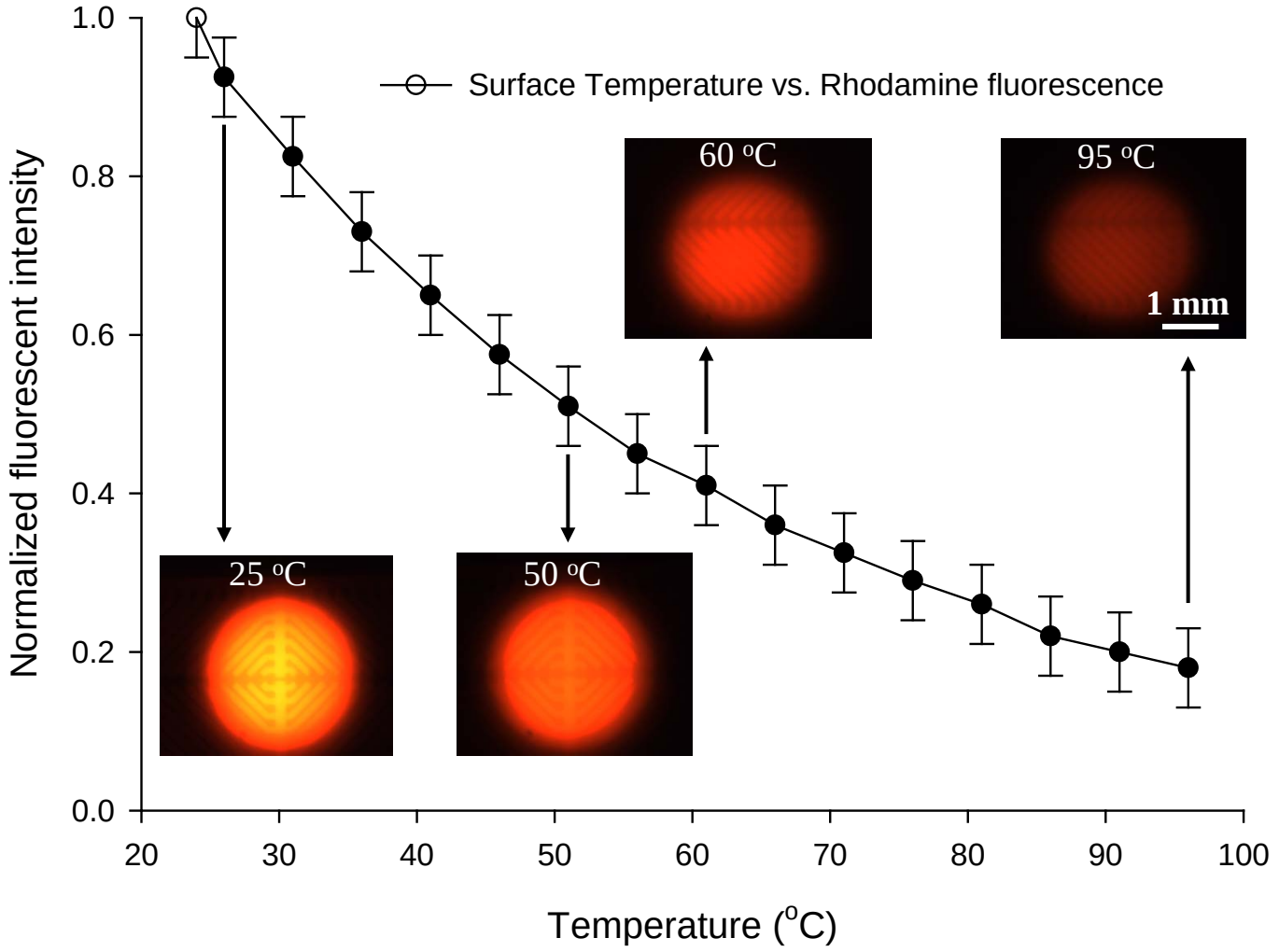
Heat transfer model:

- Localized heat source in the micro-heater electrode **(Power: 1.5 W)**
- Temperature boundary condition (Isothermal platform under the microfluidic chip **(T_{set} : 323 K or, 50 °C)**

Current and power requirement for the micro-heater design was optimized using the heat transfer module of COMSOL 4.2

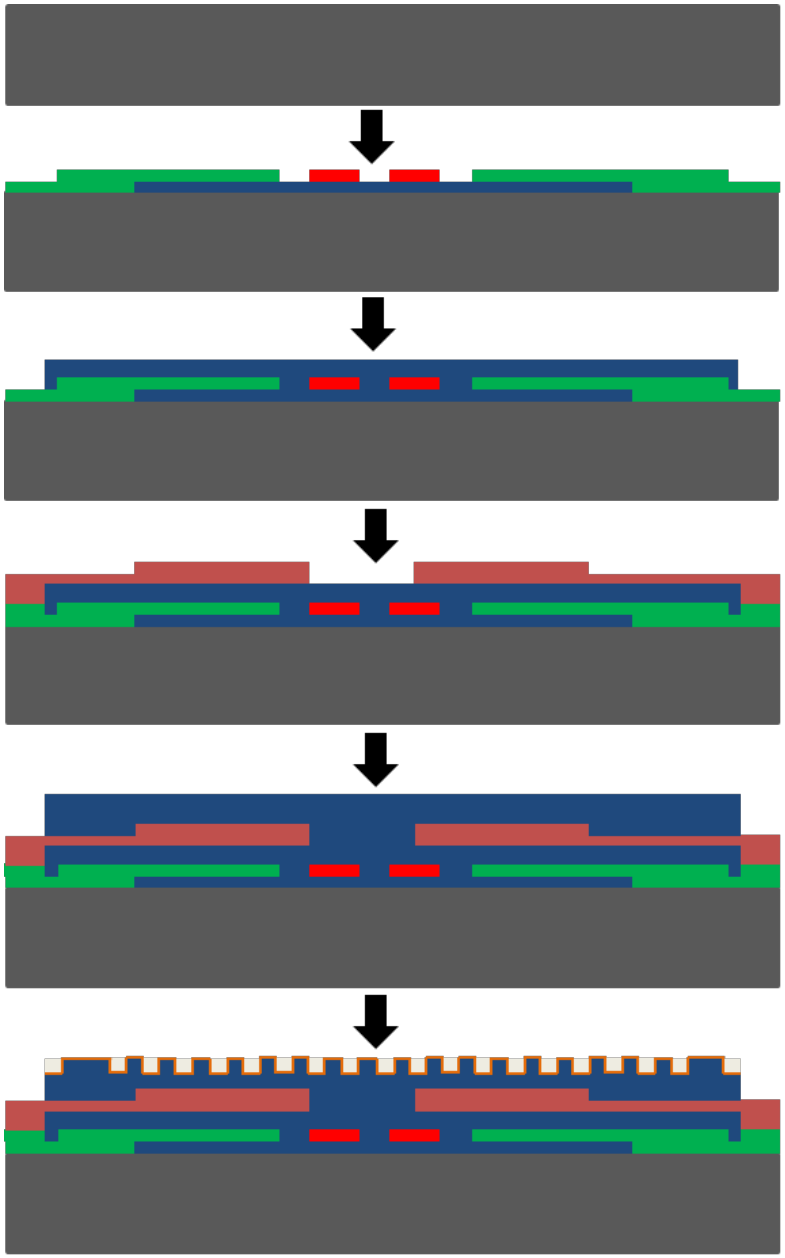
Micro-heater temperature calibration

- Rhodamine dye was used to characterize the thermal zones created by the resistive micro-heaters and monitored using the RTD temperature sensor
- Thermal zones were controlled using the embedded RTD temperature sensor and LabVIEW FPGA interface during the qRT-PCR experiments



qRT-PCR Device Fabrication

Micro/Nano fabrication of qRT-PCR device



Borofloat glass substrate (4" square wafer)

Sputter deposition and lithography patterning of **Au/Cr (200 nm)** layer for contact pads and **Cr (100 nm)** layer for micro-heater/RTD sensor

PECVD deposition and RIE patterning of **Si_3N_4 (600 nm)** layer for dielectric passivation

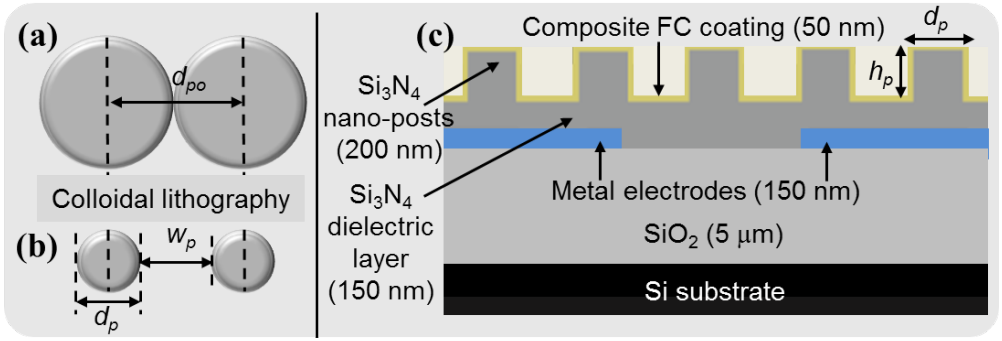
Sputter deposition and lithography patterning of **Al (200 nm)** layer for D-DEP/EW microfluidic electrodes

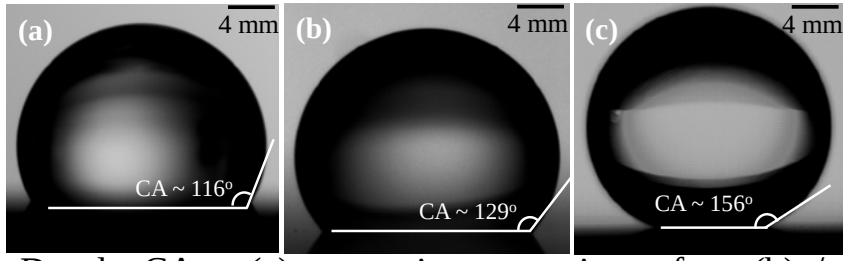
PECVD deposition and RIE patterning of **Si_3N_4 (400 nm)** layer for dielectric passivation

Colloidal lithography and DRIE etching based Nano-texturing of deposited Si_3N_4 layer and composite FC **(60 nm)** coating on top ; Super-hydrophobic top surface

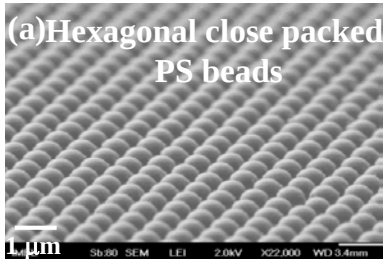
Nano-patterned, Super Hydrophobic Surface for Handling Complex Bio-samples[7]

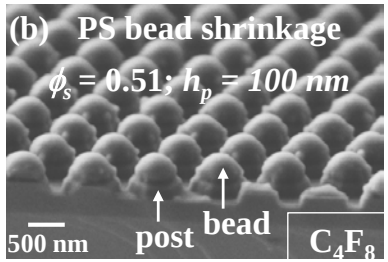
- Nano-patterned superhydrophobic (SH) surfaces with high CA (>140°) are better suited for handling enzymes and other similar bio-markers

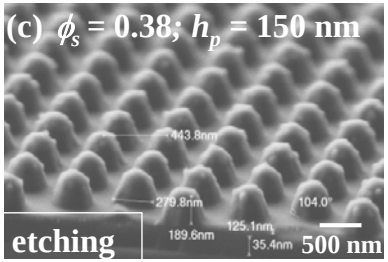




Droplet CA on (a) composite composite surface, (b) ϕ_s = 0.51, h_p =100 nm and (c) ϕ_s = 0.15, h_p =180 nm.





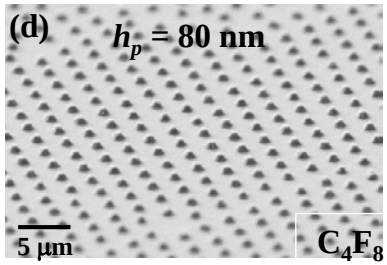


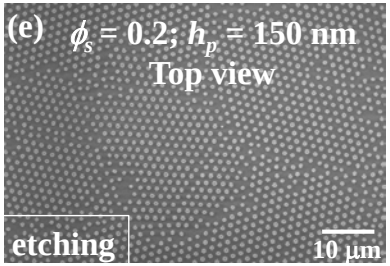
$$\phi_s \text{ (roughness parameter)} = \frac{d_p^{\,2}}{\left(d_p + w_p\right)^2} = \frac{d_p^{\,2}}{d_{po}^{\,2}}$$

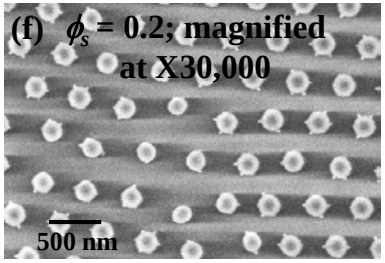
Cassie-Baxter relationship

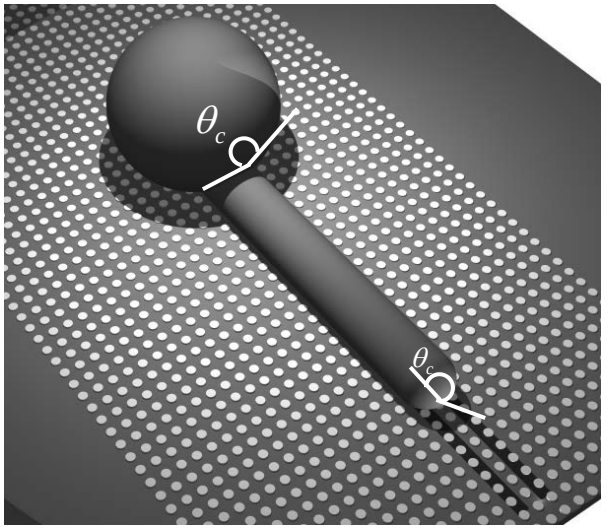
$$\cos\theta_c = \phi_s(\cos\theta_e) + (1-\phi_s)\cos\theta_x;$$

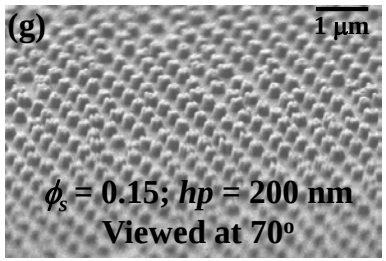
For $\theta_x = \pi$, $\cos\theta_c = -1 + \phi_s(1+\cos\theta_e)$

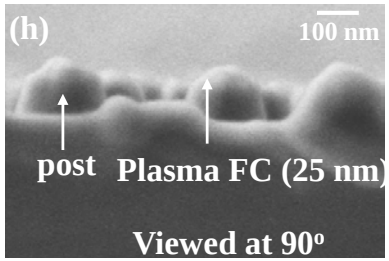


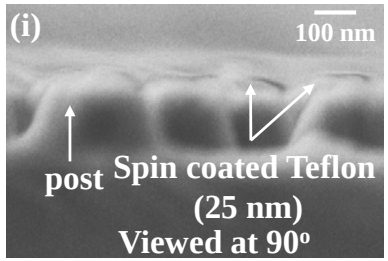








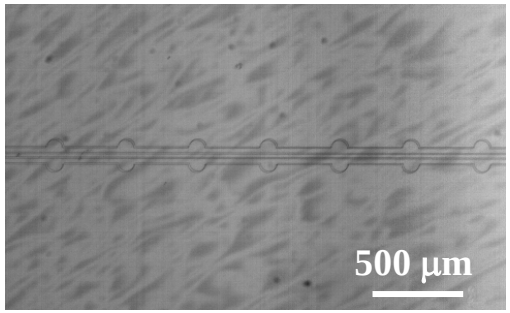




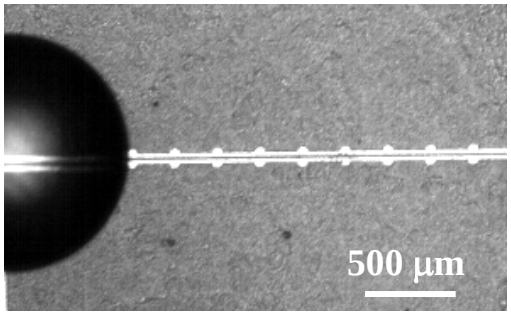
Performance of L-DEP DMF device for TAQ DNA Polymerase actuation over Nano-textured SH Surface[7]

Key advantages of SH surface over composite fluorocarbon coated hydrophobic surface:

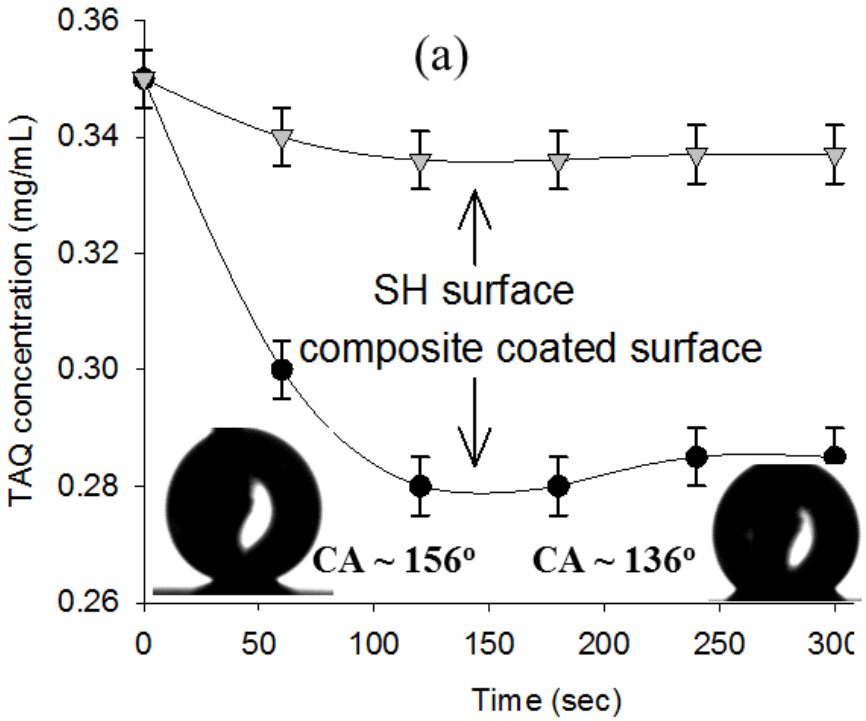
- Loss of CA reduced up to 11.5 %
- TAQ adsorption reduced by up to 40%
- No droplet collapsing

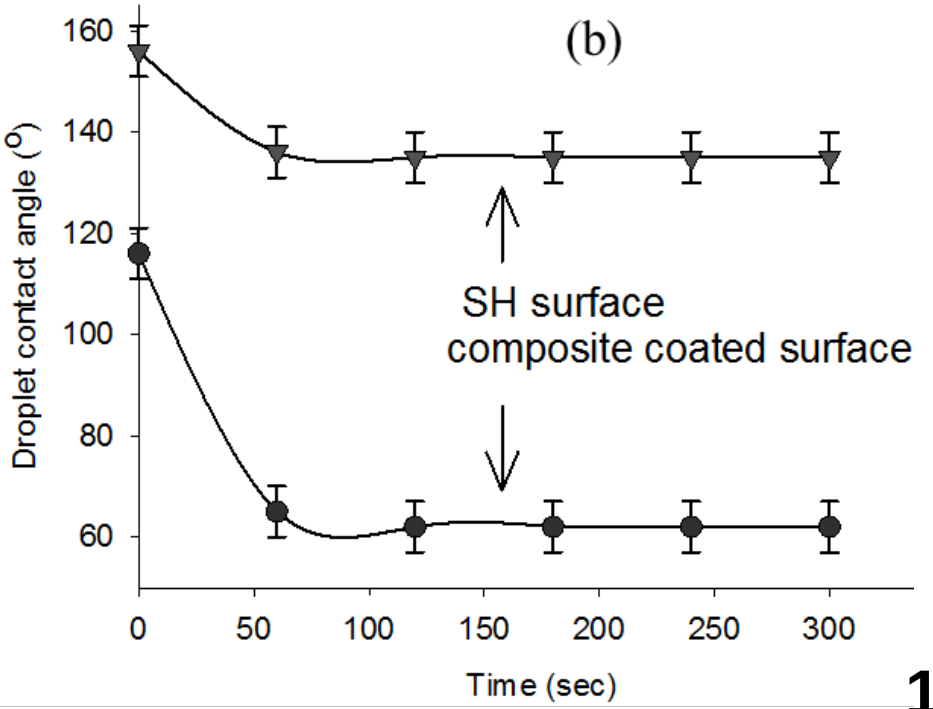


L-DEP actuation of TAQ on hydrophobic surface

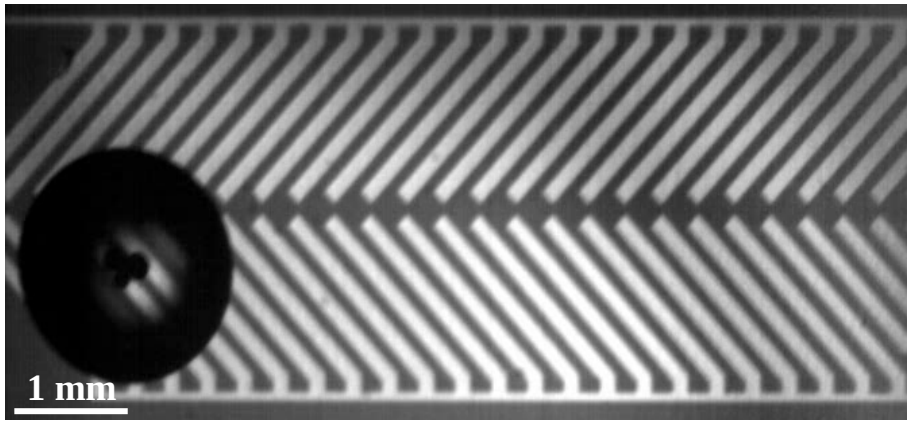


L-DEP actuation of TAQ on SH surface

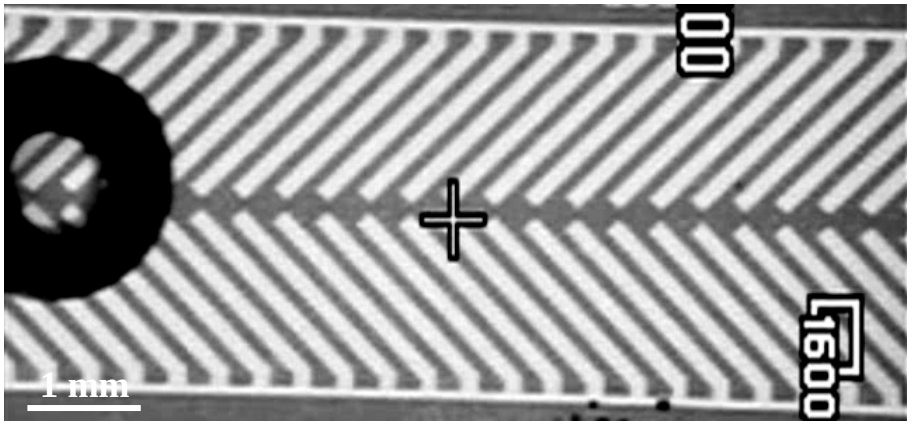




Performance of D-DEP DMF device for TAQ DNA Polymerase actuation over SH Surface

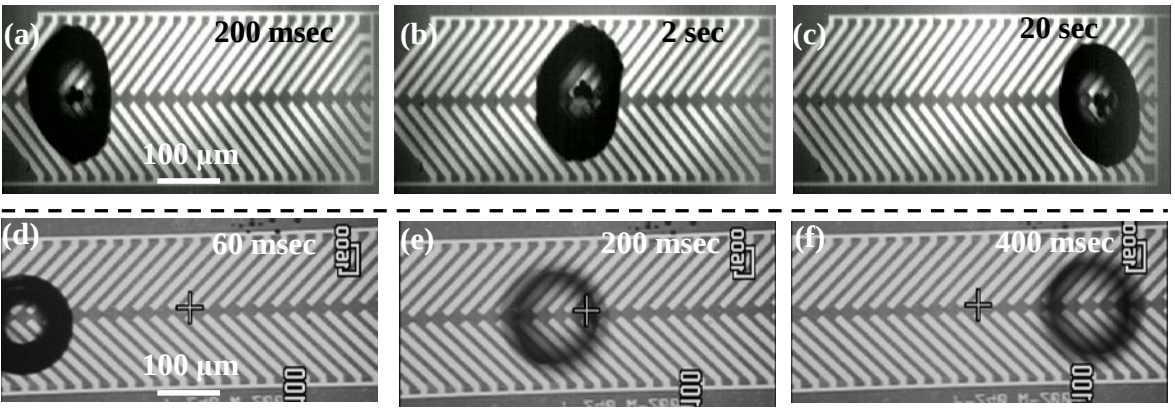


D-DEP actuation of TAQ on hydrophobic surface

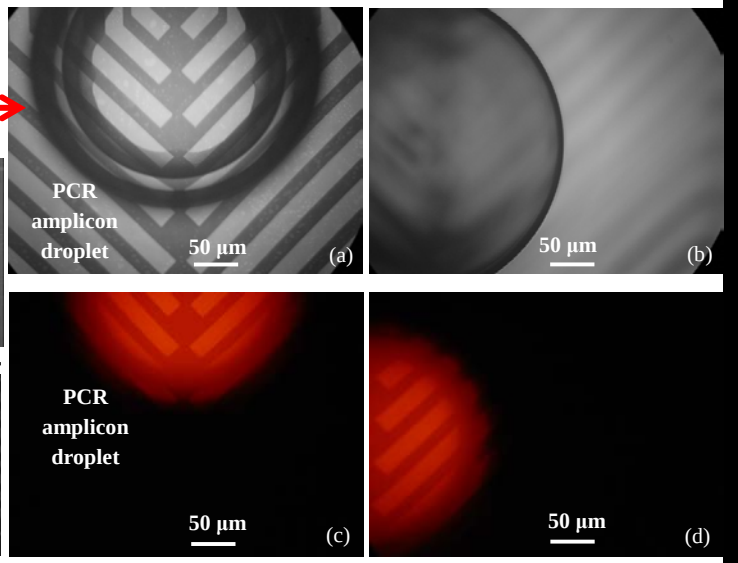


D-DEP actuation of TAQ on SH surface

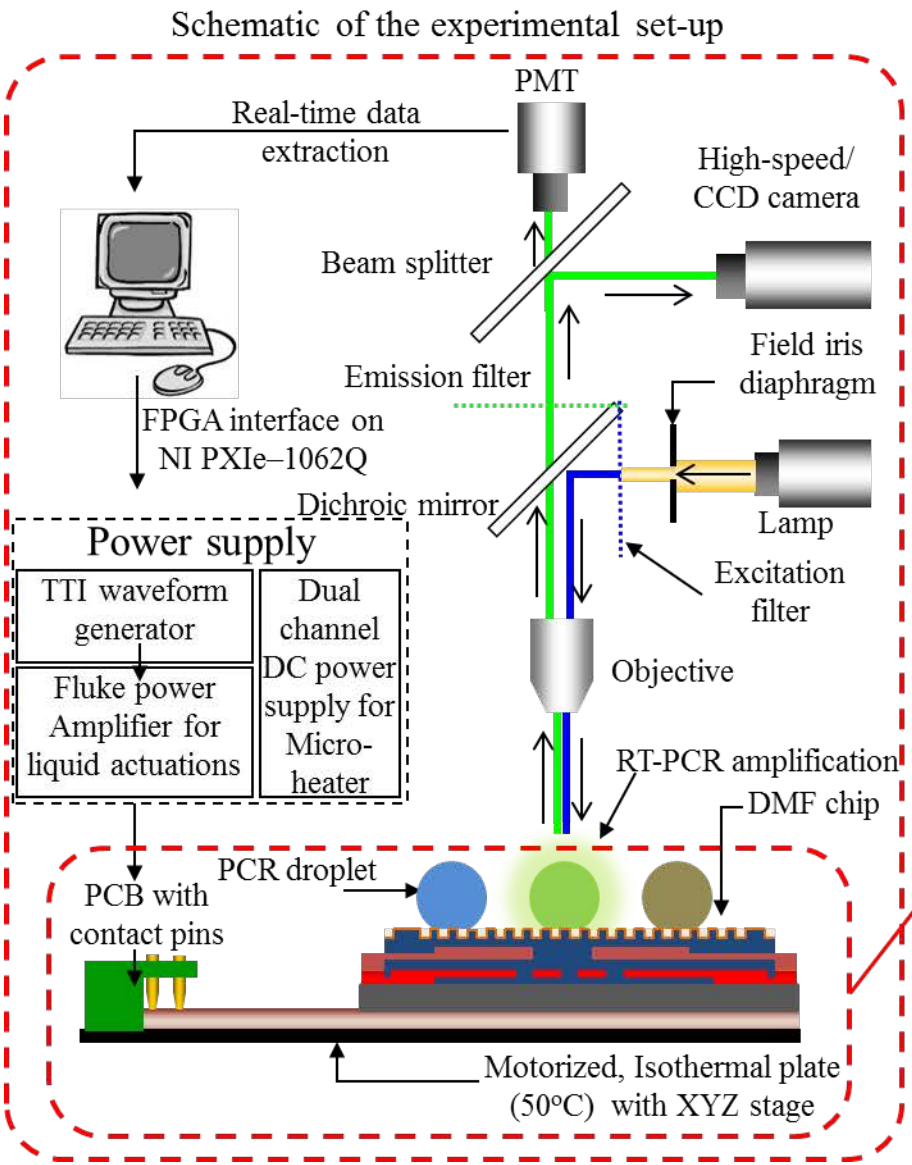
Comparison of TAQ droplet actuation over hydrophobic and SH surface

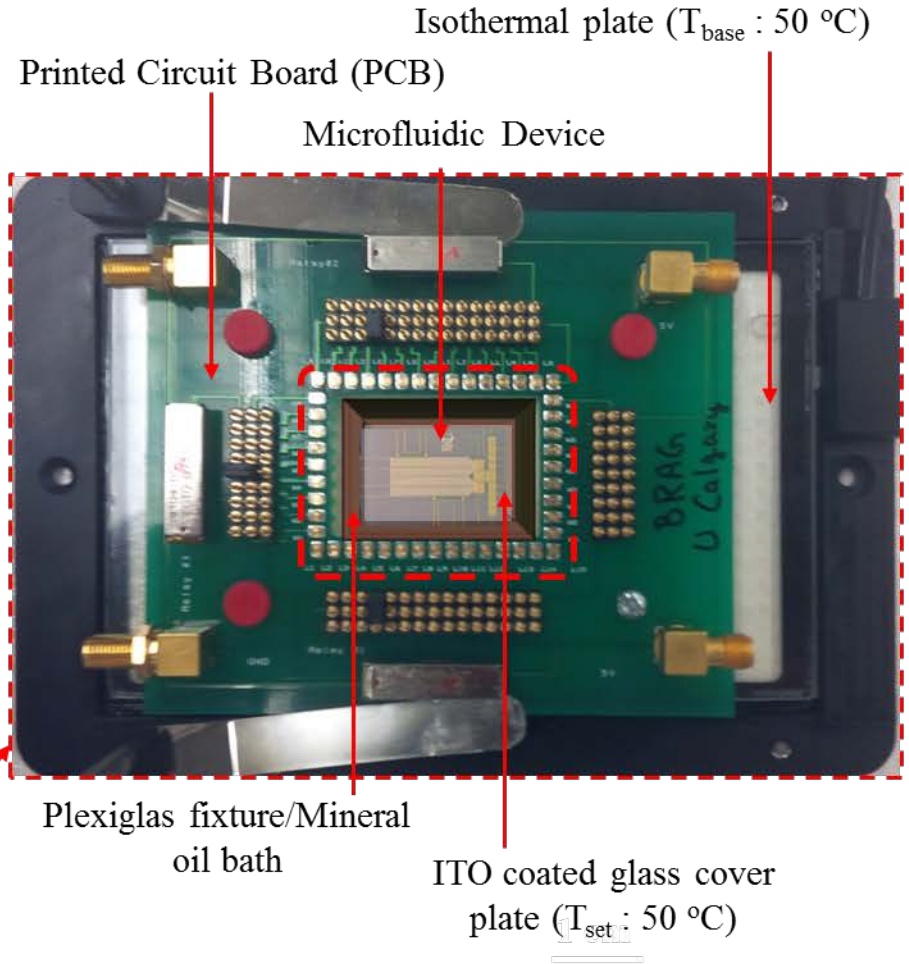


PCR amplicon sample during D-DEP actuation over SH surface



Experimental Set-up for Chip Based qRT-PCR





emSYSCAN and CMC Microsystems contribution towards qRT-PCR device fabrication and experimentation

- Two MNT financial assistance grants were utilized to develop the superhydrophobic top coating and the two integrated qRT-PCR micro-electrode architectures.
- The NI Pxie-1062Q equipment was used to control the electro-actuation of PCR sample/reagent droplets (PXI-5422 waveform generator); the resistive micro-heaters (PXI-4130) and to feedback RTD temperature sensors (PXI-7854-R) using NI LabVIEW FPGA interface.

- RS-232 interface and USB interface used to control photomultiplier tube and CCD imager for fluorescent imaging

Proposed additions:

1) High voltage module with two or more inputs (up to 500 V_{pp} and frequency bandwidth ~ 10 MHz)

2) A more suitable optical platform which can incorporate multiple optical components.

Materials and Methods

Bio-samples and Reagents for On-chip qRT-PCR and Post Amplification Detection Assays

Sample/reagents for on-chip qRT-PCR detection of Influenza A and C viruses[8]

Reagent	Working Conc.	Sample volume (μl)	Final Conc.
Taqman Fast Virus One-Step RT-PCR MMix	4x	2.5	1x
INFC-M-Forward primer	20 μM	0.4	0.8 μM
INFC-M-Reverse primer	20 μM	0.4	0.8 μM
INFC-M-Probe (FAM)	10 μM	0.2	0.2 μM
PCR Water	///	1.5	///
Master Mix Volume	///	**5.0**	///
Influenza viral RNA*	///	5.0	///

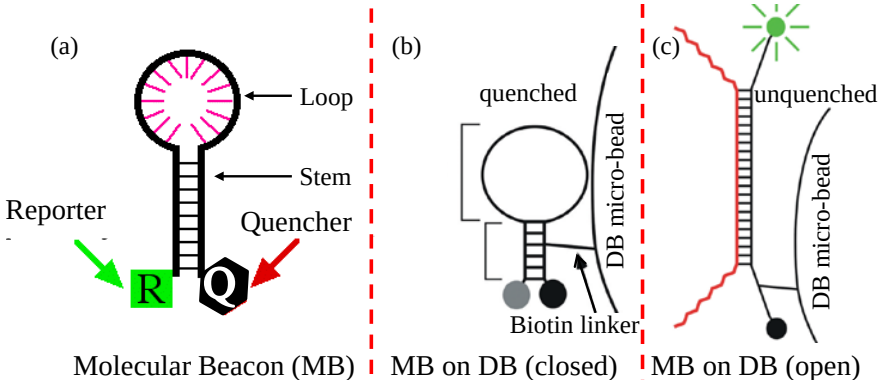
* Influenza virus RNA concentration was varied between 5 to 5E+6 copies per PCR volume

Structure of molecular reagents used in the RT-PCR[8]

RT-PCR reagents	Sequence
INFC-M-For	TGGGAGAGATGGTGTGGAGATA
INFC-M-Rev	TCTTTTTCCATCGAGTCAATTTCA
INFC-M-Probe	FAM-AAAGACCACAATTATGC

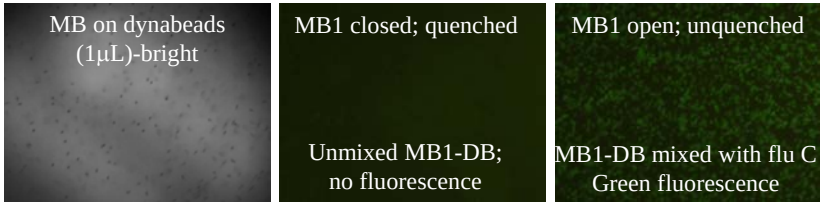
Sample/reagents for bead based post amplification Influenza C detection assay[9]

- Biotinylated molecular beacon (MB1) (**6-FAM** and **BHQ**)
- Biotinylated molecular beacon (MB2) (**HEX** and **BHQ**)
- Blank samples and non-Influenza C amplicons (negative control)
- Streptavidin coated Dynabead (DB; diameter: 2.8 μm)



Molecular Beacon (MB) | MB on DB (closed) | MB on DB (open)

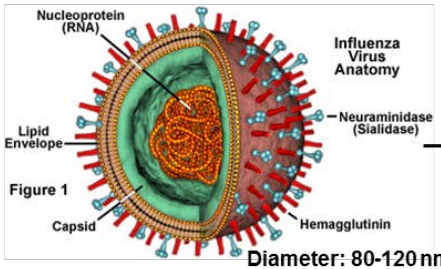
Structure and design of micro-bead anchored molecular beacon probe



Large (1 μL) droplets of MB1-DB; both unmixed and mixed

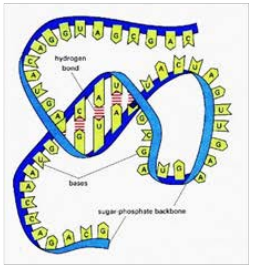
Process flow diagram of the chip based qRT-PCR detection of influenza viruses

Chip based qRT-PCR



Structure of Influenza virus (Microbiology 2009)

RNA extraction



Structure of extracted RNA

Off-chip sample preparation

Off-chip preparation of sample and RT-PCR reagents

Manual sample/reagent loading

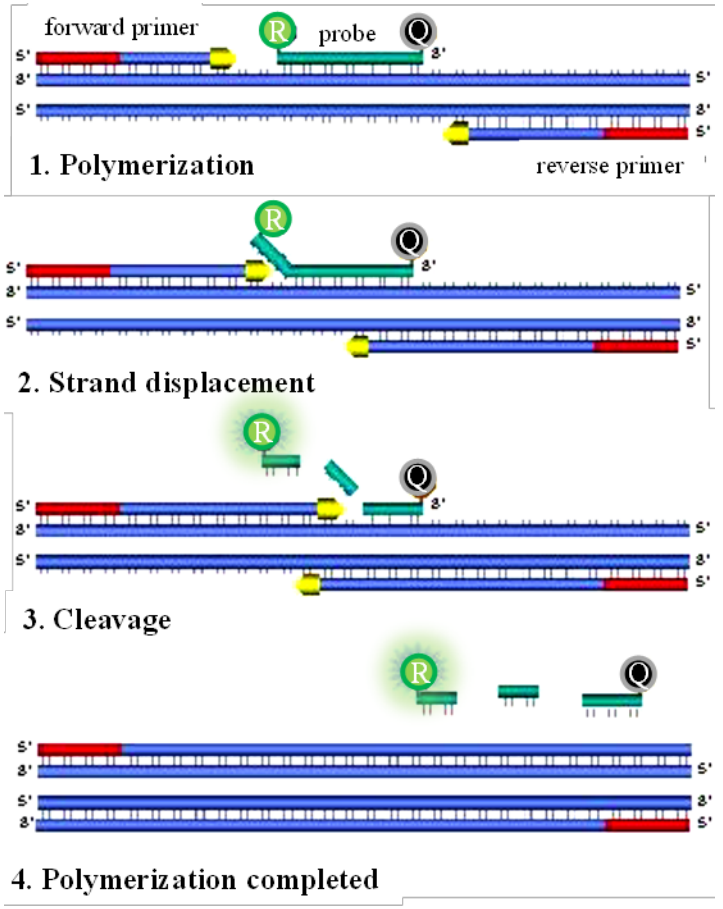
Sample and reagent droplet mixing/transport

Thermal cycling and real-time RT-PCR detection

On-chip qRT-PCR

- RNA to cDNA (RT reaction; 50°C for 5 minutes)
- Enzyme activation (95°C for 20 sec)
- RT-PCR Amplification cycles
  - Denaturation (95°C for 3 sec)
  - Annealing and read-out (60°C for 30 sec)

Schematic of TaqMan RT-PCR reaction



Results of Chip based Nucleic Acid Detection Assays

1. Multiplexed, post amplification detection of Influenza C virus sample amplified using conventional PCR system (ABI 7500)

- ❖ DB-MB based detection (Assay 1) of Influenza C virus over a 2x2 integrated matrix electrode structure
- ❖ Comparison of Assay 1 results with a control experiment over the 2x2 matrix electrode

2. Video illustrations of chip based qRT-PCR reaction (from dispensing to thermal cycling)
3. Chip based quantitative RT-PCR (qRT-PCR) amplification and detection of Influenza C virus
4. Chip based qRT-PCR amplification and detection of blind panel samples
5. Chip based qRT-PCR amplification and detection of Influenza A virus
6. Quantitative analysis of the chip based qRT-PCR reactions
7. qRT-PCR reaction using various reaction volumes (range 1 – 10 µL)

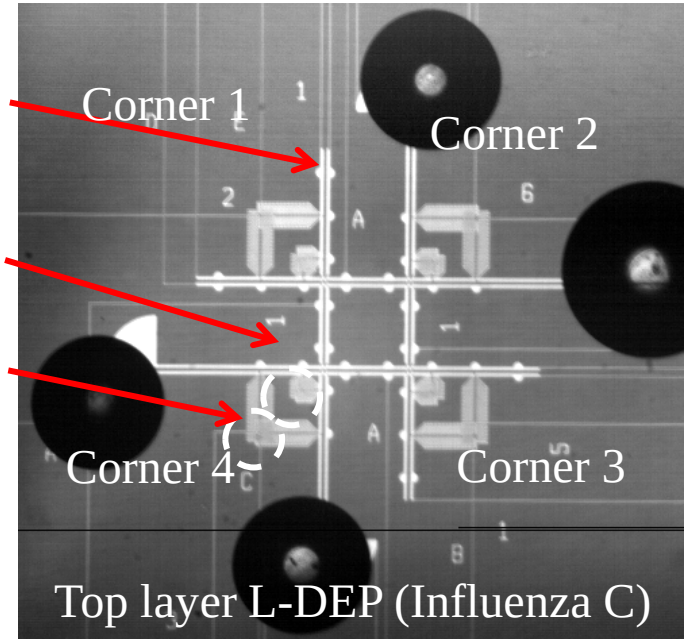
Multiplexed, Post Amplification Detection of Influenza C

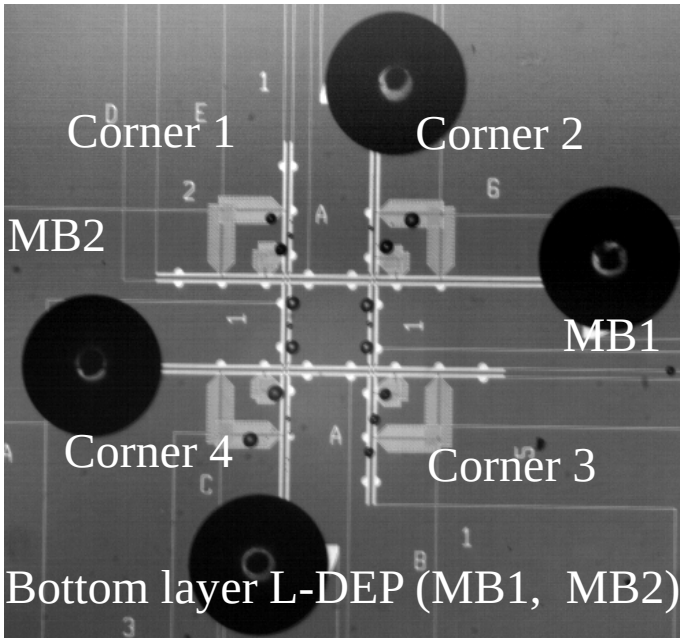
Top layer electrodes

Bottom layer electrodes

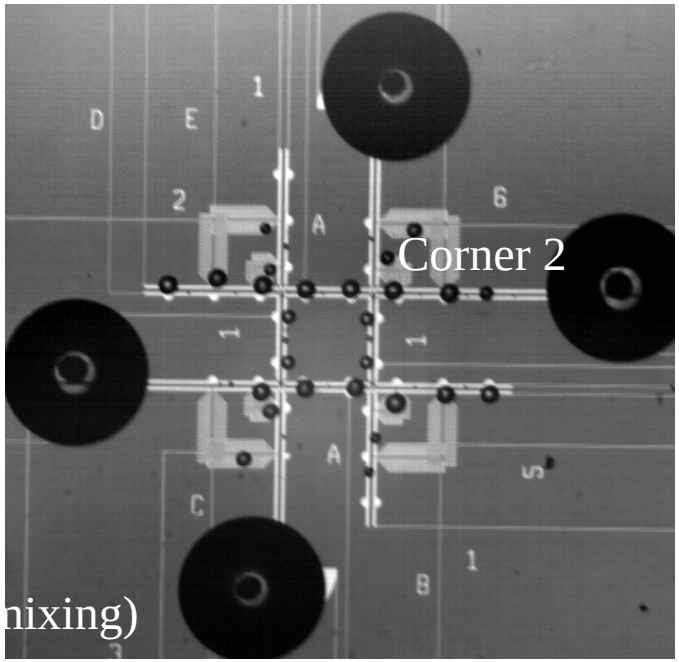
8 binary mixing sites per 2x2 matrix

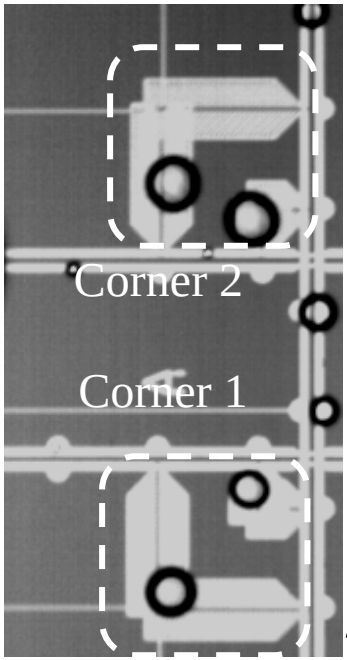
L-DEP actuation over the four L-DEP electrode pairs





D-DEP based transport and E-field assisted mixing of Influenza C samples and DB-MB probes

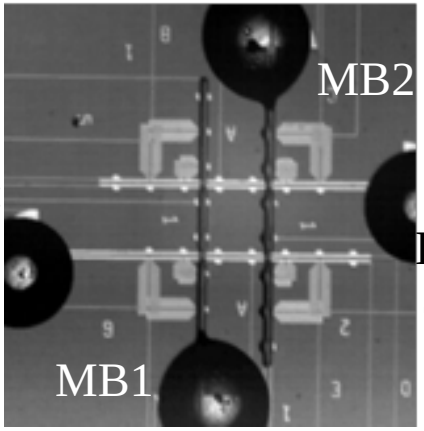




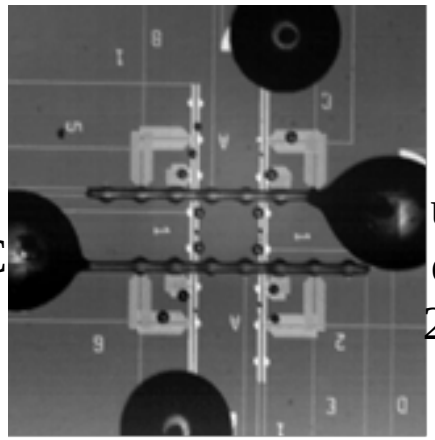
2x2 matrix assays (Assay 1) conducted over superhydrophobic L-DEP devices

Assay 1: MB1, MB2 and Influenza C Amplicon[9]

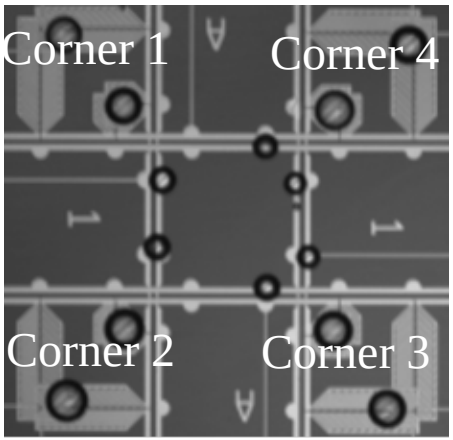
L-DEP actuation: MB1, MB2

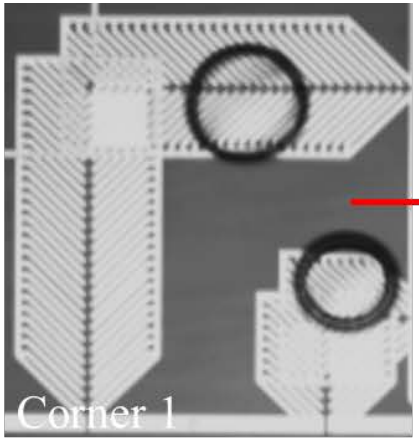


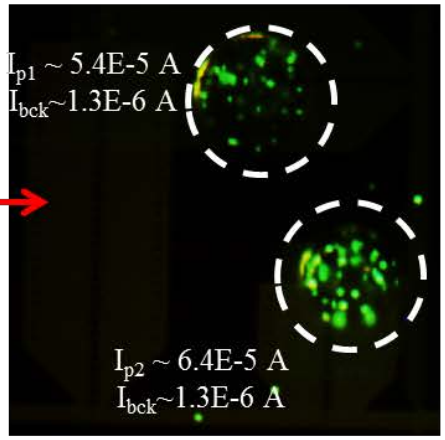
L-DEP actuation: Influenza C (25 ng/μL)

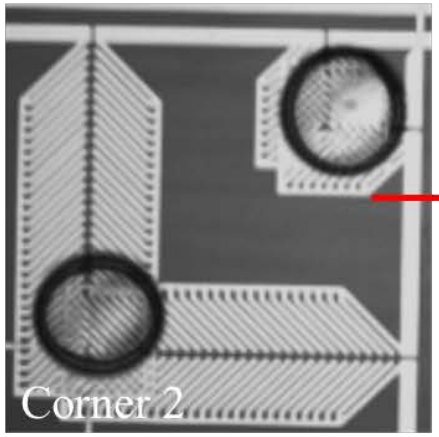


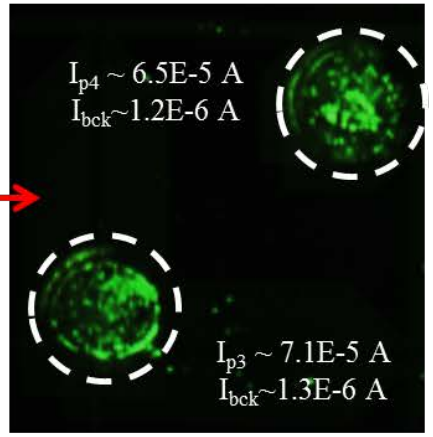
Mixed, unmixed droplets, 2x2 array

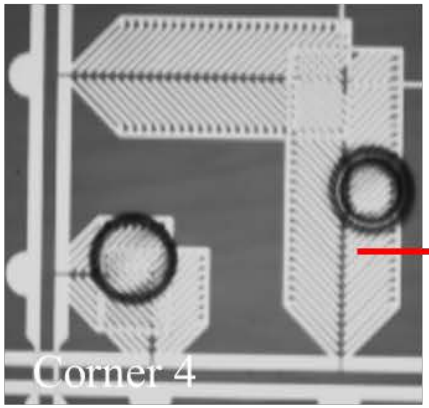


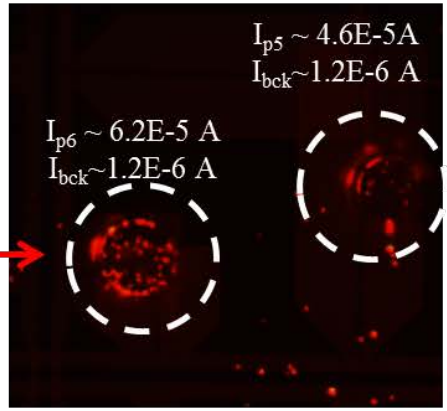


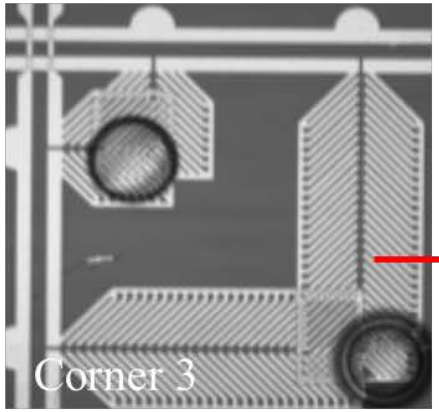


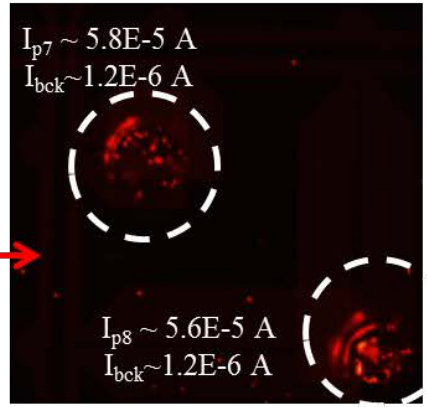






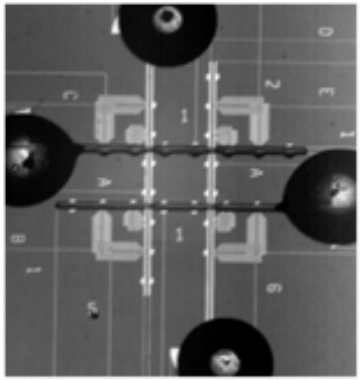




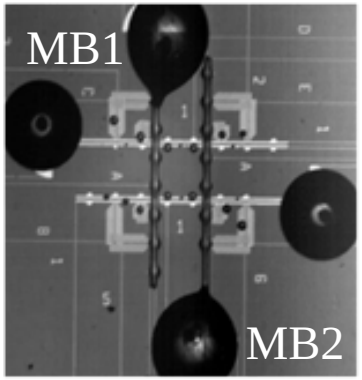


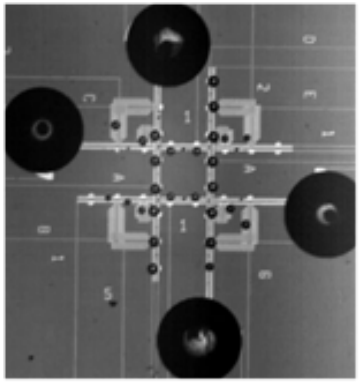
Control: MB1, MB2 and TRIS-MES Buffer

L-DEP actuation: Control (TRIS-MES buffer)

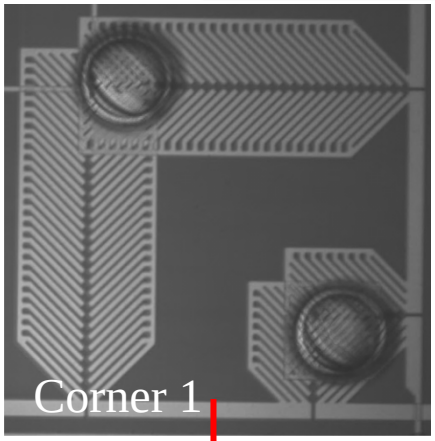


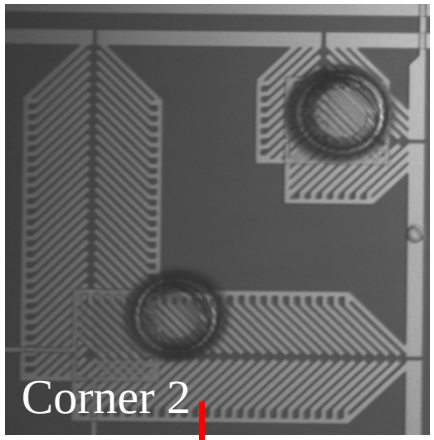
L-DEP actuation: MB1, MB2

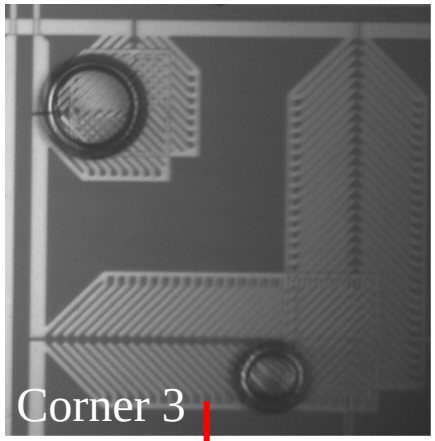


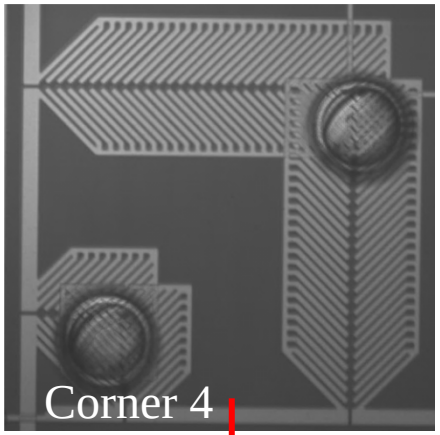


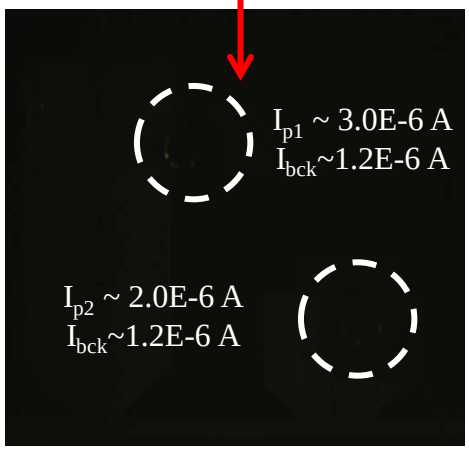
Unmixed droplets, 2x2 array

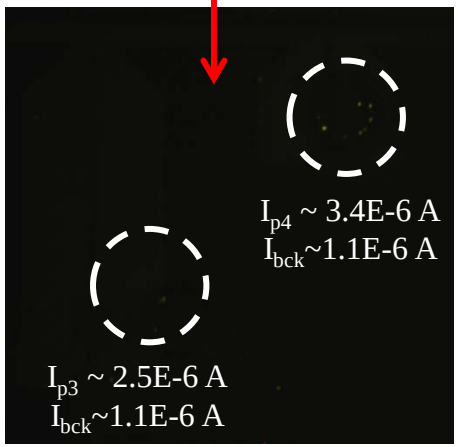


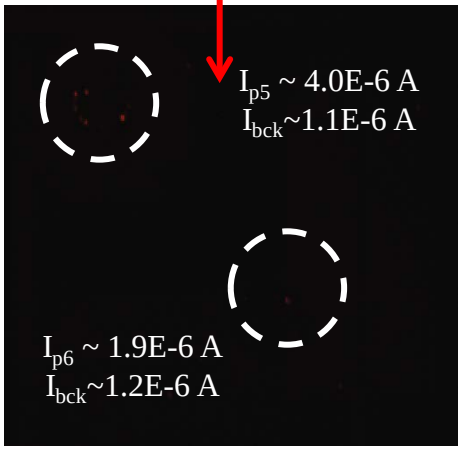












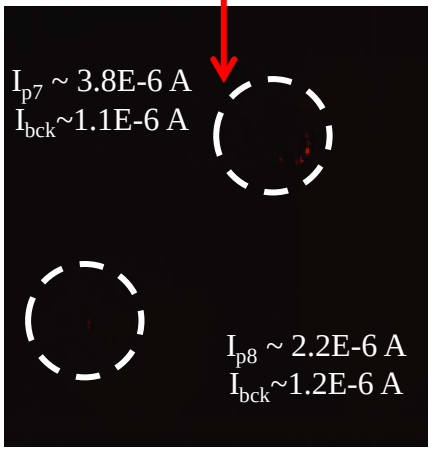
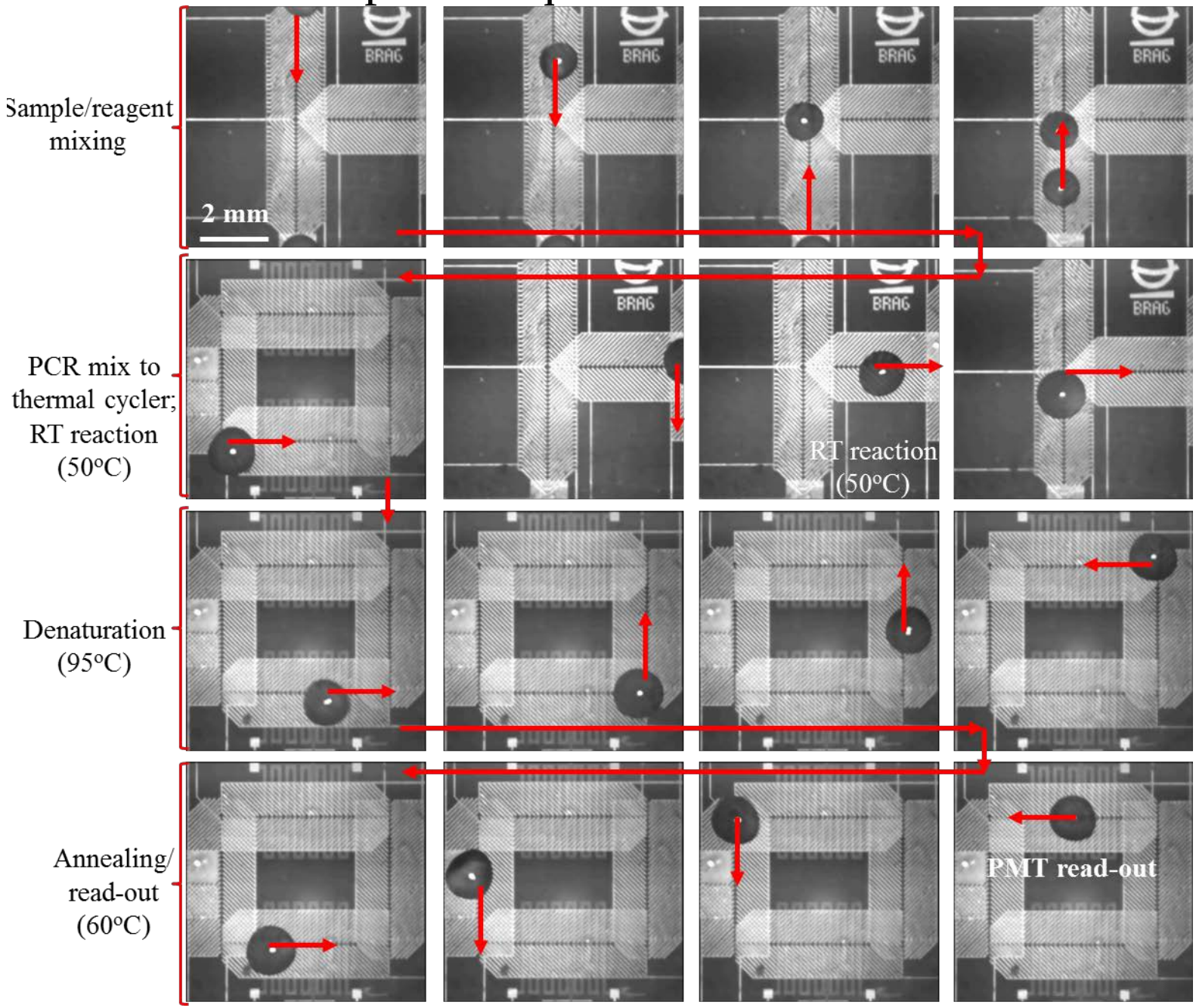
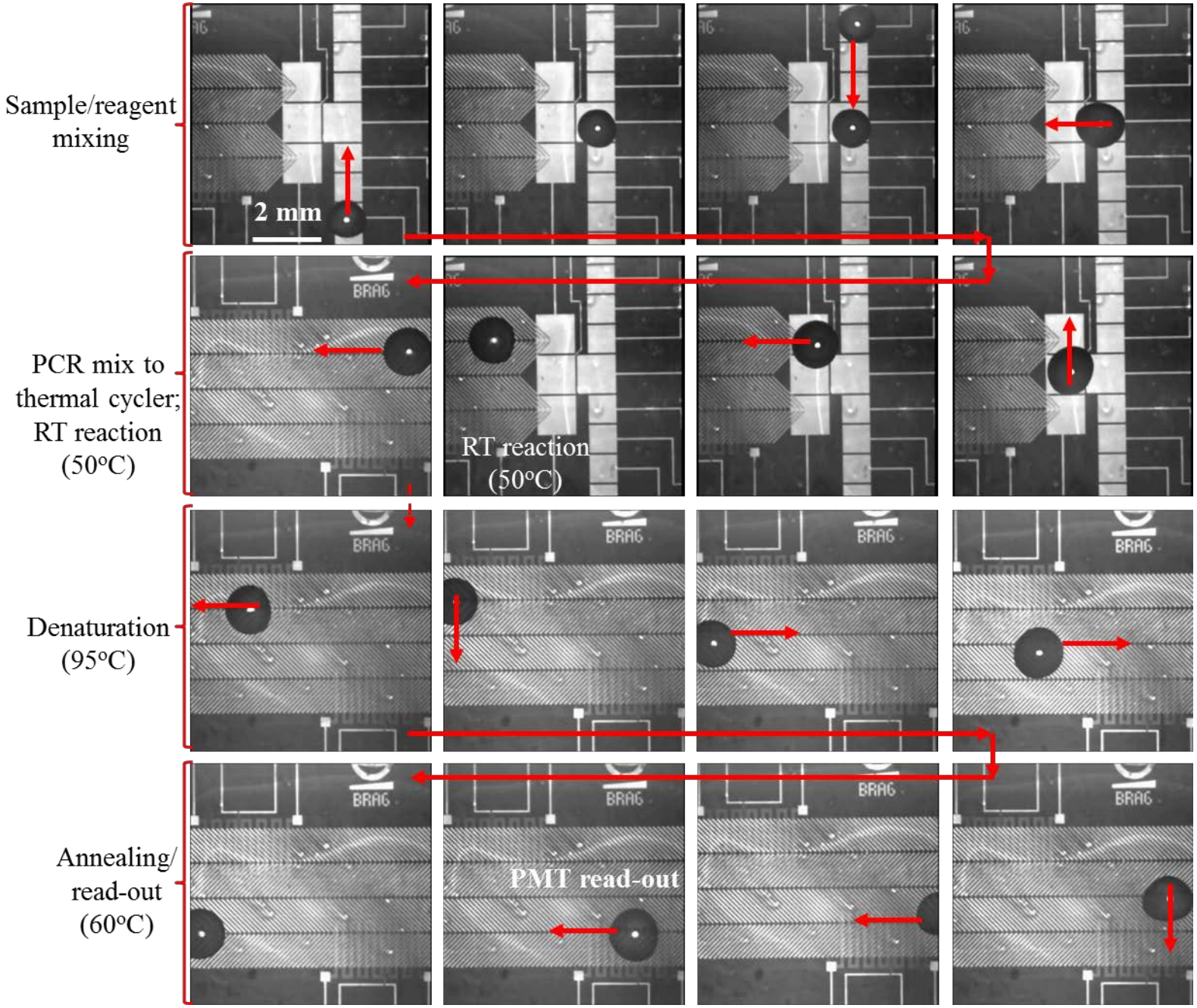


Illustration of chip based qRT-PCR on micro-electrode structure 1



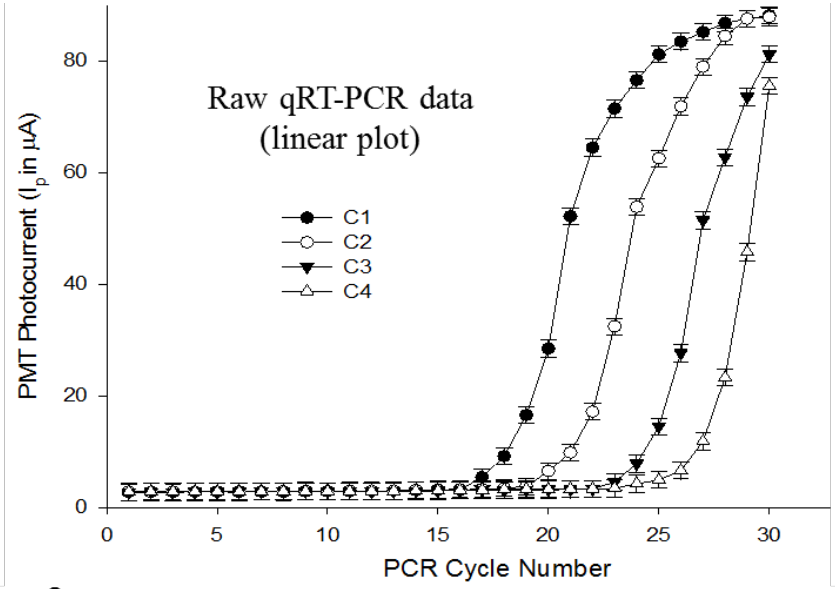
PCR droplet volume: 10 µL

Illustration of chip based qRT-PCR on micro-electrode structure 2



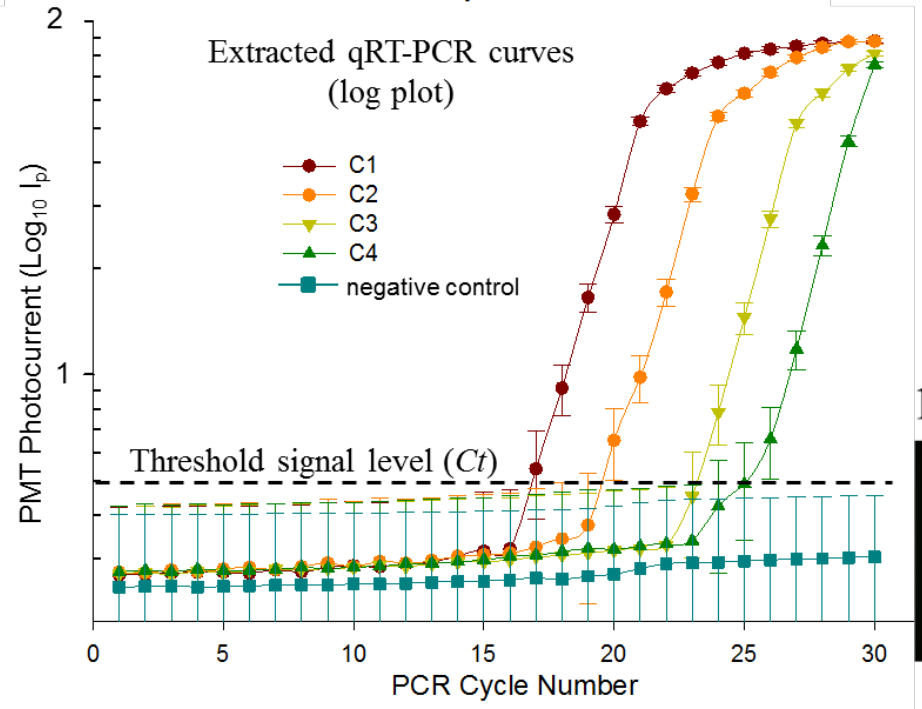
PCR droplet volume: 10 µL

Outcomes of the chip based qRT-PCR of Influenza C Virus



Thermal cycling time for the chip based qRT-PCR: 10 sec for ramp-up (60 °C – 95 °C); 10 sec for ramp-down (95 °C – 60 °C); 25 sec for annealing and data acquisition at 60 °C per RT-PCR cycle

Total qRT-PCR reaction time: 35 minutes

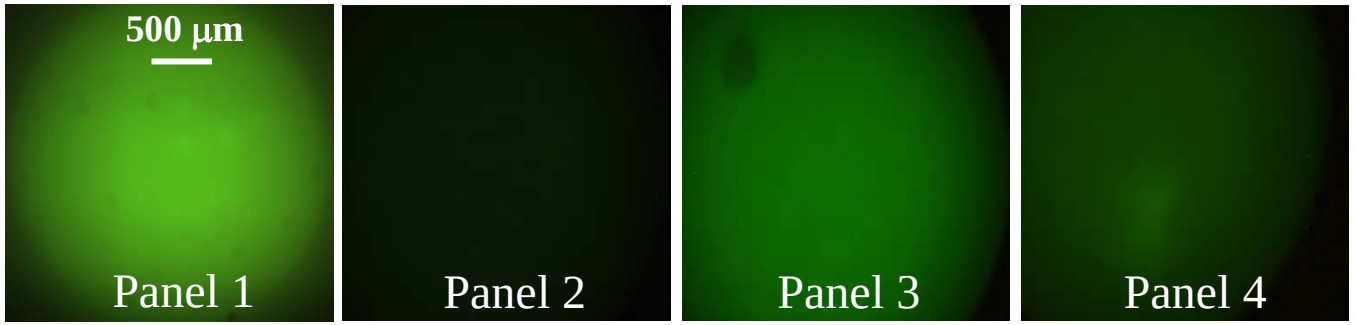


Serial dilution (X10) of stock Influenza C sample:
C1: 4510 copies;
C2: 451 copies;
C3: 45 copies;
C4: ~ 5 copies
negative control sample

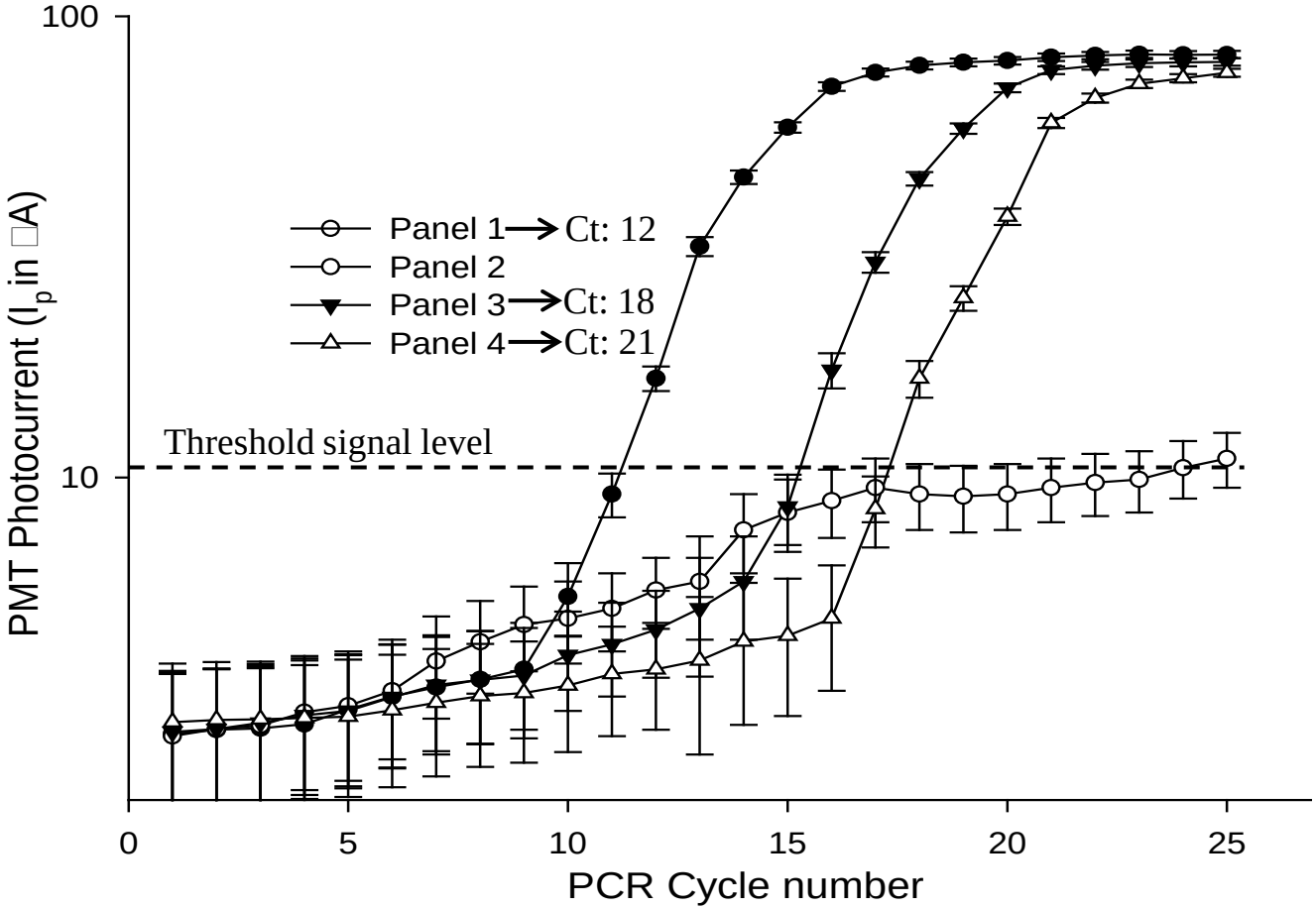
10 μL PCR sample (**C1**) droplet after:

Results of the chip based blind panel qRT-PCR test of Influenza C

CCD images of panel samples after 20 RT-PCR cycles

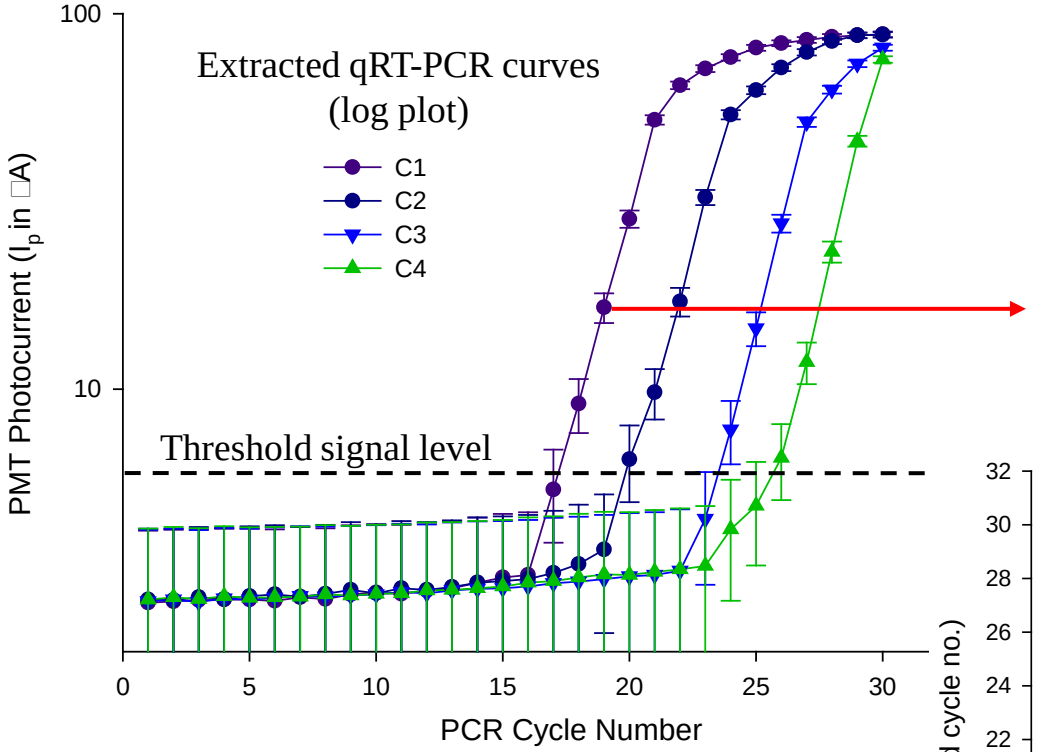


- Blind panel, prepared at the Provincial Health Laboratory Calgary, comprised of four samples of Influenza M-gene RNA with unknown viral concentration/ copy count
- The panel was investigated using chip based qRT-PCR and results validated by the conventional qRT-PCR set-up (ABI 7500) at ProvLab Calgary



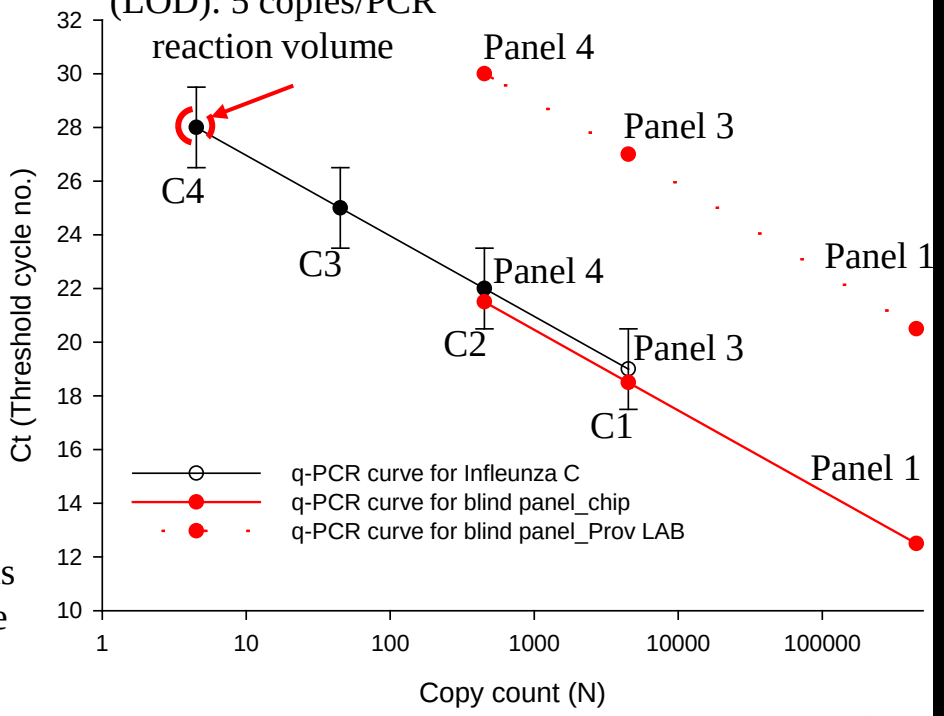
Outcomes of chip based quantitative RT-PCR assay for Influenza C

- Sequential dilution (X10) of stock Influenza C sample C1 (4510 copies/5 µL)



10 µL RT-PCR sample (**C1**) after:

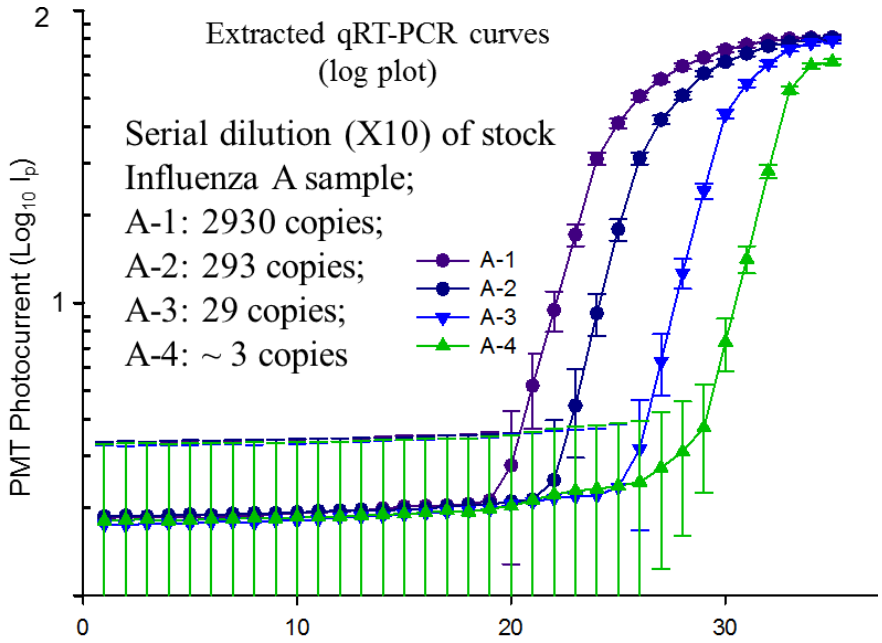
Limit of detection (LOD): 5 copies/PCR reaction volume

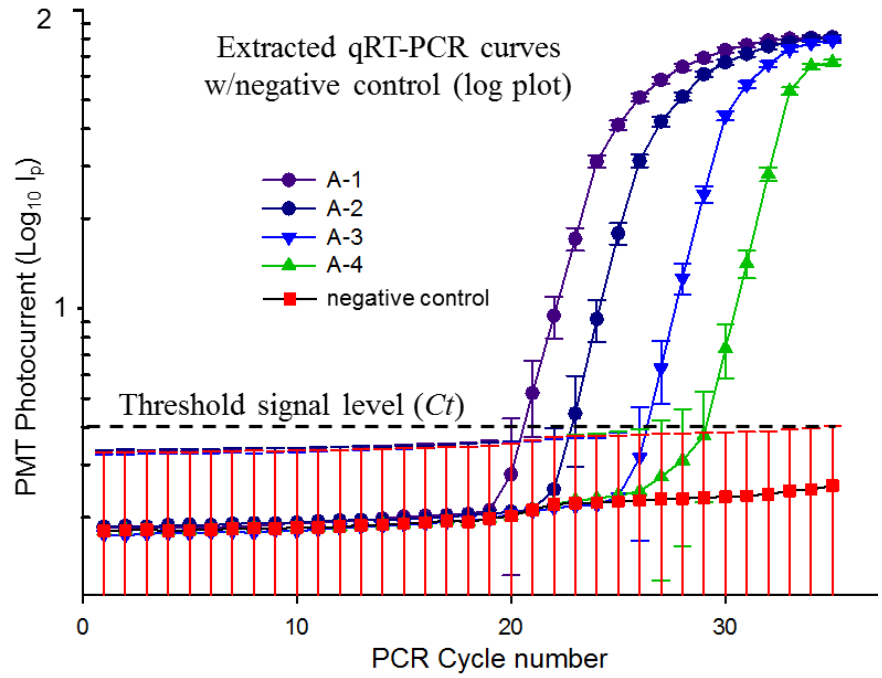


Efficiency of the on-chip qRT-PCR amplification of Influenza C: 96.4 %

Panel 2 identified as negative during the on-chip qRT-PCR reaction

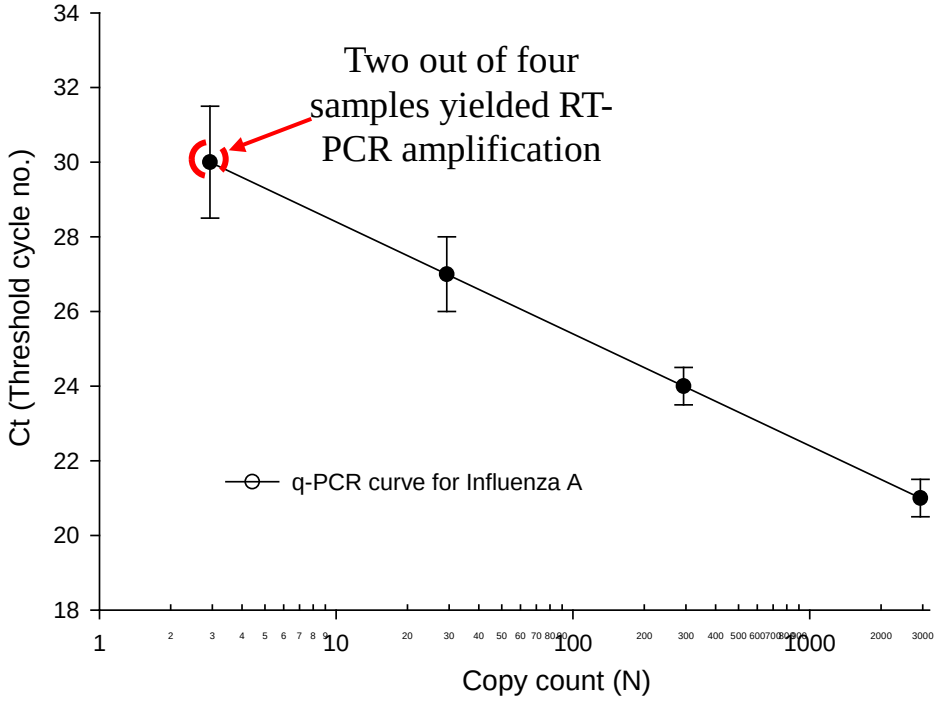
Outcomes of chip based qRT-PCR amplification of Influenza A Virus





- Sequential dilution (X10) of stock Influenza A;
- A-1: 2930 copies;
- A-2: 293 copies;
- A-3: 29 copies;
- A-4: ~ 3 copies

Efficiency of the on-chip qRT-PCR amplification of Influenza A: 94.8 %

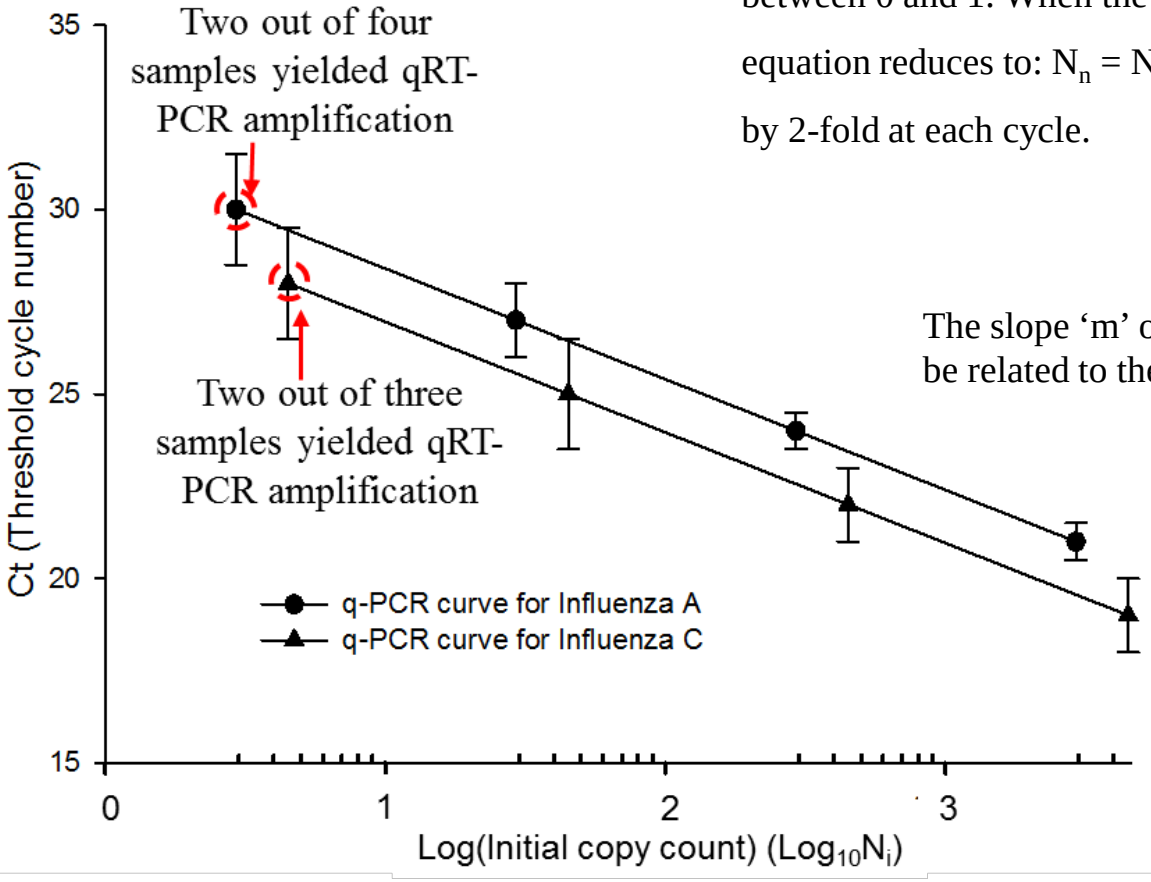


Results of the chip based qRT-PCR detection assays of Influenza A, C

- Standard PCR quantification curves are essential to a quantitative PCR set-up;
- The standard quantification curve is obtained by plotting the extracted *Ct* values vs. the initial DNA/RNA copy number (*Ni*) on a logarithmic scale
- PCR efficiency, related to the exponential amplification region of the PCR curve is given as:

$$N_n = N_i (1 + E)^n$$

N_i = initial copy number; N_n = copy number at cycle n; n = number of cycles and E = efficiency of target amplification, with theoretical values between 0 and 1. When the reaction efficiency is a maximum (E = 1), the equation reduces to: $N_n = N_i (2^n)$ and the target DNA copy count increase by 2-fold at each cycle.

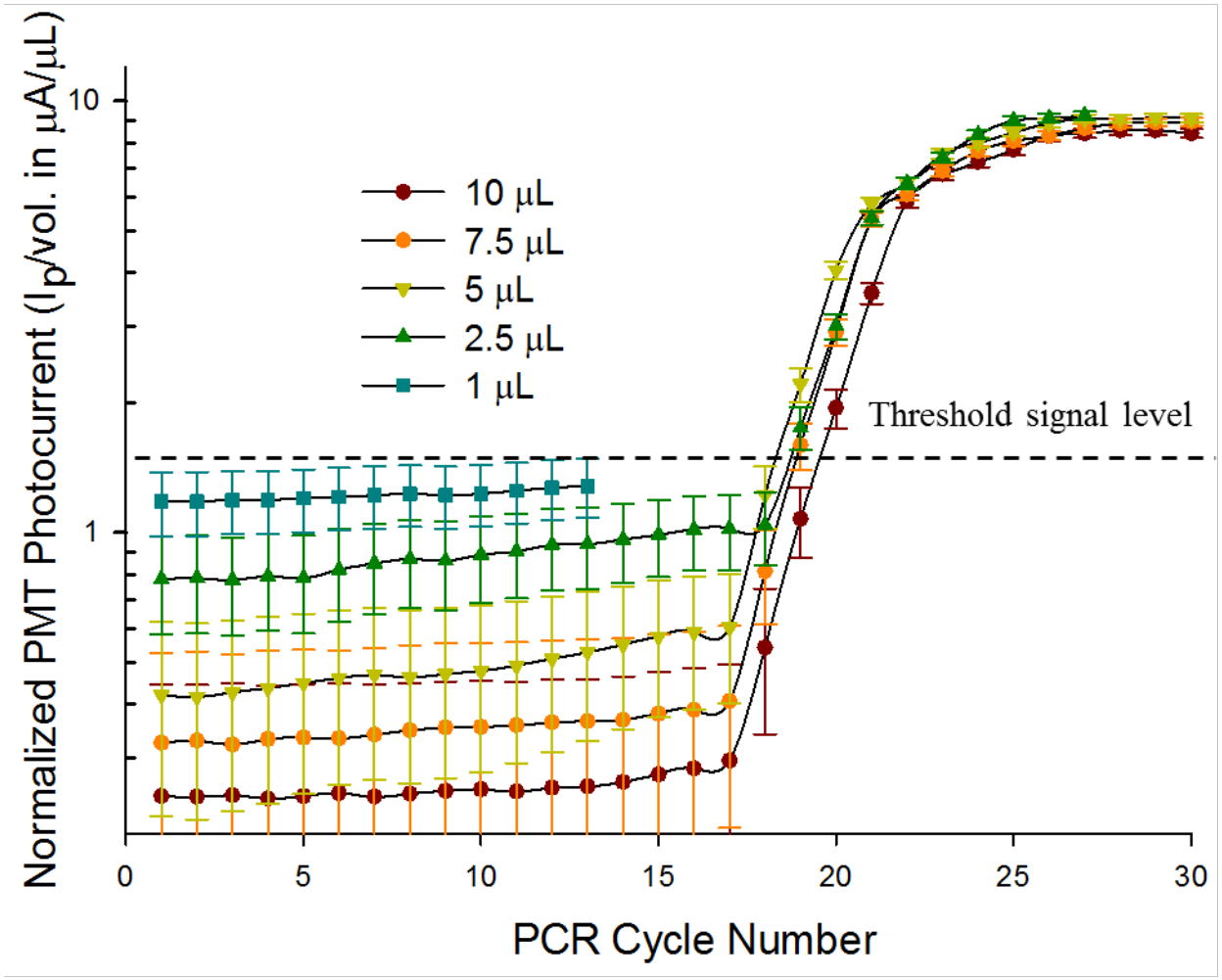


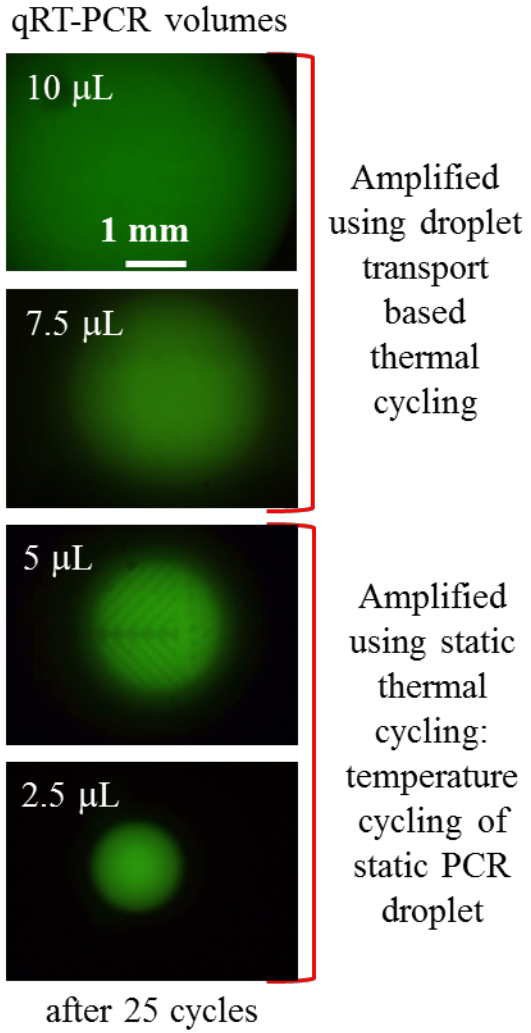
The slope 'm' of the linear, standard quantification curve can be related to the PCR efficiency E as:

$$E = 10^{-1/m} - 1$$

qRT-PCR reaction using various reaction volumes (range 1 – 10 µL)

- Transport based droplet RT-PCR reaction for PCR volumes 5 – 10 µL for Influenza C virus sample (C1)
- Static droplet RT-PCR reaction for PCR volumes ≤ 5 µL by thermal cycling the micro-heater





- Cycle time for static RT-PCR = 2.5x Cycle time for droplet transport based RT-PCR

Conclusions

- Design and micro-fabrication of microfluidic device to handle PCR samples and reagents
- Post amplification detection of amplified PCR products using molecular beacon probe
- On-chip mixing/preparation of RT-PCR reaction mix
- Integration of micro-heater/RTD sensor based thermal control unit with DEP/EW based miniaturized droplet handling technology
- qRT-PCR on a droplet microfluidic device
- On-chip qRT-PCR amplification and real-time detection of Influenza viruses
- Limit of detection (LOD) for the targeted Influenza viruses < 5 **copies** per PCR reaction

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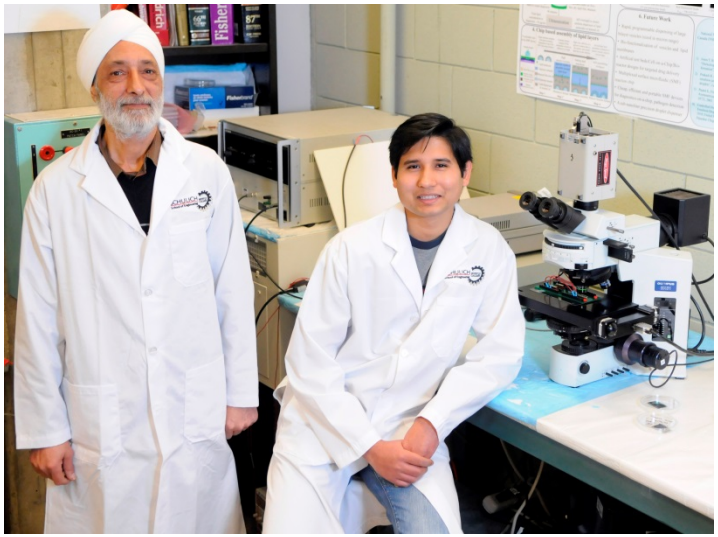
Research team:



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Thank You