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Amplitude-Modulated Electrodeformation to Evaluate Mechanical Fatigue of Biological Cells

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SUMMARY:
Presented here is a set of protocols for the biomechanical and mechanical fatigue testing of human red blood cells, using electrodeformation technique. This approach can be used as a general mechanical fatigue platform for biological cells in both health and diseases.

ABSTRACT:
Erythrocytes are known for their remarkable deformability, as they undergo considerable deformation repeatedly when passing through the microcirculation. Reduced deformability is seen in older erythrocytes. Multiple techniques exist to measure cell deformability but are not easily applicable to measure fatigue, the gradual degradation in cell deformability from the cyclic loading. We present a protocol to evaluate mechanical degradation in erythrocytes from cyclic shear stresses, implemented using amplitude shift keying (ASK) modulated electrodeformation in a microfluidic channel. Briefly, the interdigitated electrodes in microfluidic channel are excited with low voltage alternating current at radio frequencies using a signal generator. Erythrocytes in suspension respond to the electric field and exhibit positive Dielectrophoresis (DEP), which moves cells to the electrode edges. Almost promptly, cells are stretched due to the electrical forces exerted on the two cell halves, resulting in uniaxial stretching, known as electrodeformation. The level of shear stress and the resultant deformation can be easily adjusted by changing the amplitude of the excitation wave. This enables quantifications of nonlinear deformability of erythrocytes in response to small and large deformations, at high throughput. Modifying the excitation wave with ASK strategy induces cyclic electrodeformation with programmable loading rate and frequencies, providing a convenient way for characterization of erythrocyte fatigue. Our ASK-modulated electrodeformation approach enabled for the first time a direct measurement of erythrocyte fatigue from cyclic loading. It can be used as a tool for general biomechanical testing for analyses of cell deformability and fatigue in other cell types, diseased conditions, and can also be combined with strategies to control the microenvironment of cells, such as oxygen tension, biological and chemical cues.

INTRODUCTION:

Red blood cells (RBCs) are the most deformable cells in the human body[1]. Their deformability is directly related to their oxygen carrying functionality. Reduced deformability in RBCs has been found to correlate with the pathogenesis of several RBC disorders[2]. Deformability measurements have led us a better understanding of RBC related diseases[3]. The normal lifespan of RBCs can vary from 70 to 140 days[4]. Therefore, it is important to measure how their deformability decreases along with the aging process, e.g., their fatigue behavior due to cyclic shear stresses.[3]

Measuring RBC deformability at high throughput is challenging because of the piconewton

scale forces (~10^{-12} N) that are applied on the individual cells. Over the past decade, many technologies have been developed to measure cell deformability [5]. Deformation measurements of RBCs at the single-cell level can be performed by pipette aspiration and optical tweezers while bulk analyses are done by Osmotic gradient ektacytometry. Ektacytometry analyses provide an abundance of data which provides an opportunity to diagnose blood disorders [6, 7]. Deformability of RBCs can also be analyzed using the viscoelastic theory by colloid probe atomic force microscopy. In this method, a computational analysis is applied to estimate the elastic modulus of RBCs considering both time-dependent and steady state. Deformability of individual RBCs can be measured by using single cell microchamber array method. This method analyzes each cell through membrane and cytosolic fluorescent markers to provide information for RBC deformability and the distribution of cellular characteristics in complex RBC populations to detect hematologic disorders. [8]

Cellular fatigue analysis has long been hampered by the lack of a quick, readily applicable, and quantitative method for implementation of cyclic deformation in cell membranes. This is possible with the utilization of electrical signal modulation and electrodeformation techniques implemented in a microfluidic setting. The Amplitude Shift Keying (ASK) technique as a digital modulation is applied through On-Off Keying (OOK) modulation in this article. The concept of keying refers to transmission of digital signals over the channel which requires a sine wave carrier signal to function[9]. The on and off times can be set equal. Under On-keying, RBCs enter a deformed state while exposed to an external electrodeformation force (F_{dep})[10] created by the nonuniform electric field. Under Off keying, RBCs are in their relaxed state. We observe the fatigue of RBCs, namely a progressive degradation in their ability to stretch with increasing fatigue cycles.

PROTOCOL:

1 Microfluidic Device Preparation

1.1 Tape down the SU-8 master silicon wafer for the microfluidic channel design inside of a plastic 14 cm petri dish and clean with N_2 gas.

1.2 Weigh 60 g of polydimethylsiloxane (PDMS) base and 6 g of PDMS curing agent in a paper cup. Mix the two parts using a senior tongue depressor until the mixture is a cloudy white color.

1.3 Pour the PDMS mixture into the plastic petri dish containing the silicon wafer. Place the petri dish into a vacuum desiccator to remove air bubbles from the PDMS.

1.4 Run the vacuum desiccator and reintroduce air in approximately 5 minutes cycles. Repeat until all air bubbles are removed from the features of the channels.

1.5 Place the petri dish inside of an oven at 70˚ C for 4 hours. Once time has elapsed, remove the petri dish, allow cool down to room temperature, and place it on a cutting mat.

1.6 Using a scalpel, cut out the portion of the PDMS above the silicon wafer. Place the cut out PDMS in between two sheets of parafilm for identifying locations of the microfluidic channels.

1.7 Using a razor blade, cut out an individual channel from the large PDMS. Punch a 3 mm inlet hole and 1.5 mm outlet hole using biopsy punches respective to the two sizes (**Figure 1A**).

1.8 Place the hole punched channel, with the channel side facing up, on to a clean glass slide. Place a 20 mm x 15 mm ITO substrate containing thin-film interdigitated electrodes on the

same glass slide with electrodes facing up.

1.9 Gently place the glass slide with PDMS and substrate into a plasma cleaner. Close the gas valve, turn on the pump switch and wait 2 minutes to obtain a sensor reading of 600 – 800 mTorr.

1.10 Turn on the power switch and wait 30 seconds. Increment the RF power knob from low to high and wait 1 minute.

1.11 After waiting, reverse the sequence by turning RF to low, power switch off, pump switch off, and open the gas valve.

1.12 Immediately after opening the chamber of the plasma cleaner, lift and rotate the PDMS so that the channel side is facing down (180˚). Place the channel on top of the ITO substrate. The bonding process has begun.

1.13 Using tweezers, gently press down on the corners of the PDMS. Avoid pressing onto the channel itself. The bonding process is now complete.

1.14 Load prime medium into a 1 mL syringe with a 23 -gauge needle. Carefully wet the channel by inserting the needle straight into the inlet well and release medium. Operate slowly. Do not introduce air bubbles. Incubate for 3 or more minutes.

1.15 Remove the prime medium using a 10 µL pipette tip. Wash the channel with DEP medium 3 times by inserting DEP medium into the channel. Keep the channel wet at all times.

2 Test Fixture

2.1 The test fixture is designed using SolidWorks, including a base housing unit and a top unit (**Figure 1B**). Then it is manufactured using a 3-axis CNC milling machine (not shown).

2.2 Pre-solder wires into the solder cup ends of two sets of spring piston connectors.

2.3 Insert the spring piston connectors into the top unit and create a permanent bonding by adding a drop of epoxy glue.

3 Electrodeformation Working Buffer Preparation

3.1 To prepare DEP medium, weigh 12.75 g of sucrose and 0.45 g of dextrose using a scale.

3.2 Dissolve both powders into a single container with 150 mL of deionized (DI) water and 3.5 mL phosphate buffered saline (PBS).

3.3 Using a low range conductivity tester, measure the conductivity to be $0.04\ S/m$. (**Figure 2)**

3.4 Using an osmometer, confirm the osmolarity within the normal range of blood plasma, 275 to 295 mOsm/kg of water (**Figure 3**). Store at 4˚ C. The DEP medium is now prepared.

3.5 In a 15 mL tube, dissolve 0.5 g of Bovine Serum Albumin (BSA) into 10 mL of the DEP medium. Mix thoroughly. The Device's prime medium is now prepared.

4 Cell Suspension Preparation

4.1 Wash 20 µL of whole blood by centrifuging the blood with 1 mL of PBS at 268 RCF for 3 minutes. Discard the supernatant.

4.2 Resuspend the RBCs in 1 mL of PBS. Gently pipette to mix.

4.3 Wash the RBCs for 3 minutes at 268 RCF and discard the supernatant.

4.4 Extract 5 µL of RBC pellet and wash with 1 mL of DEP medium by centrifuging at 268 RCF for 3 minutes.

4.5 Discard the supernatant and resuspend the RBCs in 1 mL of DEP medium. Gently pipette to mix.

4.6 Wash the RBCs for 3 min at 268 RCF and discard the supernatant. Pipette 2 µL of RBC pellet into 500 µL of DEP medium. The cell suspension is now prepared.

5 Electrodeformation Setup and Fatigue Testing

5.1 Place the microfluidic device into the bottom part of the test fixture. Align the top part of the fixture to the device and assemble the two parts using two sets of nylon screws and nuts (**Figure 4**).

5.2 Place the test fixture on the microscope stage. Locate one desired set of electrodes under the microscope.

5.3 Connect the corresponding pair of electrode wires that match the located electrode set to the output terminal of the function generator (**Figure 4**).

5.4 Remove 5 µL of DEP medium from the 3 mm inlet of the microfluidic channel. Slowly load 5 µL of cell suspension into the inlet using a 10 µL pipette tip.

5.5 Allow cells to settle down for 1 minute. If necessary, add additional DEP medium to the inlet to push cells into the channel.

5.6 Observe the channel under 20x magnification. Use a 414/46 nm bandpass filter to enhance the contrast of the imaging.

5.7 Program a sine wave with a $2\ V_{RMS}$ amplitude and 3 MHz frequency.

5.8 Press the "Mod" button to enable modulation. Change the wave mode into ASK by pressing the "type" option.

5.9 Set the modulation frequency to 250 mHz which corresponds to a 4 second loading-unloading period (**Figure 5A**). Turn on the output of the function generator.

5.10 Record a 1-minute video every 10 minutes at 30 frames per second (fps).

6 Characterization of Erythrocyte Deformation

6.1 Using the VirtualDub application, open .avi files recorded in the previous step by pressing Ctrl+O. Use the timeline to choose a frame of interest and set the selection start and end frames to be identical by pressing the 'Home' key and then the 'End' key on the keyboard.

6.2 Export the image frame. Select the Output format to be 'JPEG' and press 'OK'.

6.3 Open the ImageJ application and load the images saved in the previous step. Begin by setting the needed measurements by pressing Analyze → Set Measurements and ensuring the check boxes for 'Area', 'Perimeter', and 'Fit Ellipse' are checked. Press 'OK'.

6.4 Next, convert the image to grayscale by choosing Image → Type → 8-bit.

6.5 Then convert the image to binary using Image → Adjust → Thresholding. In the 'Threshold' dialog box, adjust the two sliders as needed. Press 'Apply' and then close the 'Threshold' dialog box.

6.6 Choose Analyze → Tools → ROI Manager. In the ROI Manager, press the checkbox labeled 'Show All'. Do not close this box.

6.7 Select the 'Wand (tracing) tool' and select an applicable cell in the image and press 't' on the keyboard. The selected cell will be numbered. A new cell can be selected again. Select all applicable cells to be measured.

6.8 Return to the ROI Manager box and press 'Measure'. This opens the 'Results' box. Columns labeled Major and Minor are the lengths of the fitted ellipse major and minor axes (in pixels) respectively. Choose File → Save As to export the measurements as a CSV formatted file.

6.9 Using any appropriate computational analysis software, calculate the quotient of Major and Minor.

REPRESENTATIVE RESULTS:

Under On-Keying phase, RBCs are stretched due to electrodeformation; under On-Keying phase, RBCs are relaxed (**Figure 5B**). When tracking individual RBCs during the 1-hour fatigue testing, we observed a gradual decrease in cellular deformation (**Figure 5C**). Deformability is quantified by the ratio of the major and minor axes of measured RBCs, using open-source imaging software, e.g., ImageJ (**Figure 6**). Images of interest were opened in the software program. It was not necessary to calibrate pixel size into length scale for the deformability measurement. Numerical data can be further analyzed and plotted in software, such as Excel, Origin, and MATLAB. In this protocol, plot was performed in MATLAB from data collected in 60 minutes of experimentation in time intervals of 10 minutes. Noted deformability loss, cellular fatigue for RBCs during 1 hour of cyclic mechanical loading (250 mHz) was found to be -18% (**Figure 7**).

FIGURE AND TABLE LEGENDS:

Figure 1. (A) Schematic of the microfluidic channel with biopsy punched holes of 1.5 mm and 3 mm for sample outlet and inlet, respectively. (B) Exploding view of the test fixture assembly.

Figure 2. A conductivity meter was used to verify the conductivity of DEP medium to be $0.04\ {}^{S}/_{m}$. The sensing probes at the base of the meter are submerged in the sample to obtain a reading.

Figure 3. An osmometer was used to verify the osmotic concentration of DEP medium. Step 1 - Snap a sample tip into place on the sampler and load a 20 microliter of sample. Step 2 - Rest the sampler within the operating cradle and beneath the cradle top. Step 3 - Push the entire operating cradle down until it reaches a positive stop. Step 4 - The instrument runs the test for approximately one minute and display the result.

Figure 4. Picture of the experimental setup for fatigue testing including the test fixture assembly and a function generator. ITO electrode pads are connected to the function generator by BNC-to-alligator clip cable via the wires pre-soldered into the Pogo pin cups pressed into the top unit of the test fixture. The microfluidic device with two independent parallel channels sits on the bottom unit of the test fixture.

Figure 5. (A) On-Off keying modulated sine wave for 1-hour fatigue testing: sine wave of 2 V_{RMS} amplitude at 3 MHz for electrodeformation action, modulation frequency of 250 mHz resulting in 2 s of stretching and 2 s of relaxation. (B) Under On-Keying phase, RBCs are stretched due to electrodeformation; under Off-Keying phase, RBCs are relaxed. (C) Electrodeformation of a representative cell shows gradual degradation in membrane deformation during 1 hour of cyclic stretching.

Figure 6. Characterization of RBC deformability using ImageJ. Step 1 – Import the image into ImageJ and convert into 8-bit grayscale image. Step 2 - Adjust threshold to convert images to binary. Step 3 - Select cells with the wand (tracing) tool and manage selections with the ROI manager. Step 4 – Select the cells to obtain measurements for major and minor axes.

Figure 7. Degradation in RBC deformability due to cyclic electrodeformation. Error bar shows standard deviation.

DISCUSSION:

The ASK OOK modulation of a DEP force-inducing sine wave can be used to test the mechanical fatigue of RBCs over a long period of time. In this protocol, we limited the in vitro fatigue testing to 1 hour to prevent the potential adverse metabolic effects on the cell deformability. Comprehensive fatigue testing condition can be programmed using the ASK-modulated electrodeformation technique. Parameters such as loading frequency, amplitude, and loading rate can all be programmed. The loading frequency can be programmed to values different from 250 mHz to determine the dependence of fatigue on loading frequency as well as differences between cyclic loading and static loading[11]

The stretching magnitude can be easily adjusted by using a different voltage level for small or large deformations. However, when using high voltage levels to include large deformation, it is noted that electrodeformation will result in flame-like shaped RBCs (**Figure 6, Step 3**). This can introduce error when fitting cell shapes with ellipse, which cut off the pointed edges of cells. Under this circumstance, especially when using the electrodeformation to extract membrane shear viscoelasticity parameters, two strategies can be used: (i) using a computational model of true cell shapes will provide accurate results than the simple analytical elliptical shape model; (ii) using a smaller voltage level to stretch cells so cell shape can be well fitted with ellipses.

In current protocol, the medium conductivity was 0.04 S/m, which can be adjusted as needed. As electrodeformation-induced cellular stretching is highly related to the real part of the Clausius Mossotti factor, the sine wave frequency can be selected different from 3 MHz, where the key is to use minimum level of voltage to induce electrodeformation with negligible Joule heating effect. An optimum electrical excitation can be determined using DEP theory or using computational tool for dielectric modeling of cells, such as My DEP [12]

It should note that cell immobilization is not required in this protocol as cells undergoing electrodeformation are inherently exhibiting positive DEP, which moves cells to the electrode edges spontaneously. This allows us to perform testing on cell suspension and immobilize all cells interacting with the electrode and simultaneously stretching the cells. Once the testing is done, cells can be easily removed from the device by flushing the channel with medium. The characteristic of the current protocol which makes it work well with suspending cells may limit its application to test adherent cells. However, we can detach adherent cells from substrate using chemicals such as Ethylenediaminetetraacetic acid. Since the testing can be designed to be completed in a relatively short time from minutes to 1 hour, we should have enough time window for mechanical fatigue before they anchor and spread.

In the current protocol, a commercially available ITO chip with 100-µm band interdigitated

electrodes was used. Interdigitated electrode design is advantageous for measuring multiple cells at a time. Throughput of the measurement is also dependent on the field of observation, where cell size and deformability set limitation on the minimum gap of the electrodes. The band width of the electrodes can be further decreased to increase the number of cells of observation for higher throughput. The electrode materials can be other metals, such as titanium or gold; however, the transparency of the electrode materials may be a better choice as part of cell membranes can be blocked by the non-transparent electrodes. The testing can still be performed, if a relevant mathematical shape model of cell can be used during imaging processing. Theoretically, this electrodeformation and ASK-modulated electrodeformation techniques can work on other cell types, given the right condition, e.g., medium conductivity and electrical excitations. A limitation is how much elongation we can observe. RBC is a good cell model for its large deformability and circulating nature. Current protocol has been applied to study human RBCs in both health and disease and readily equipped with gas microenvironment to study hypoxic fatigue [11, 13].

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DISCLOSURES:
The authors have nothing to disclose.

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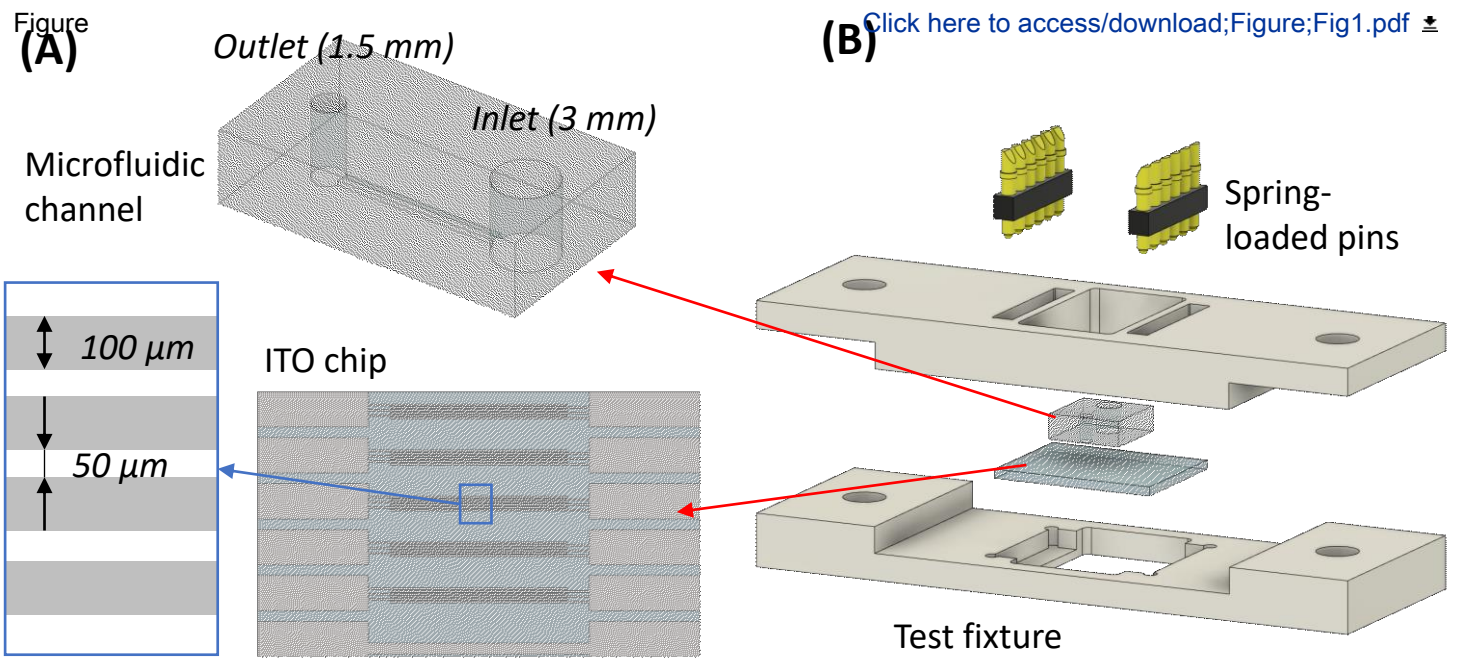
Figure



Figure

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Step 1

Step 2

Step 3

Step 4

Figure

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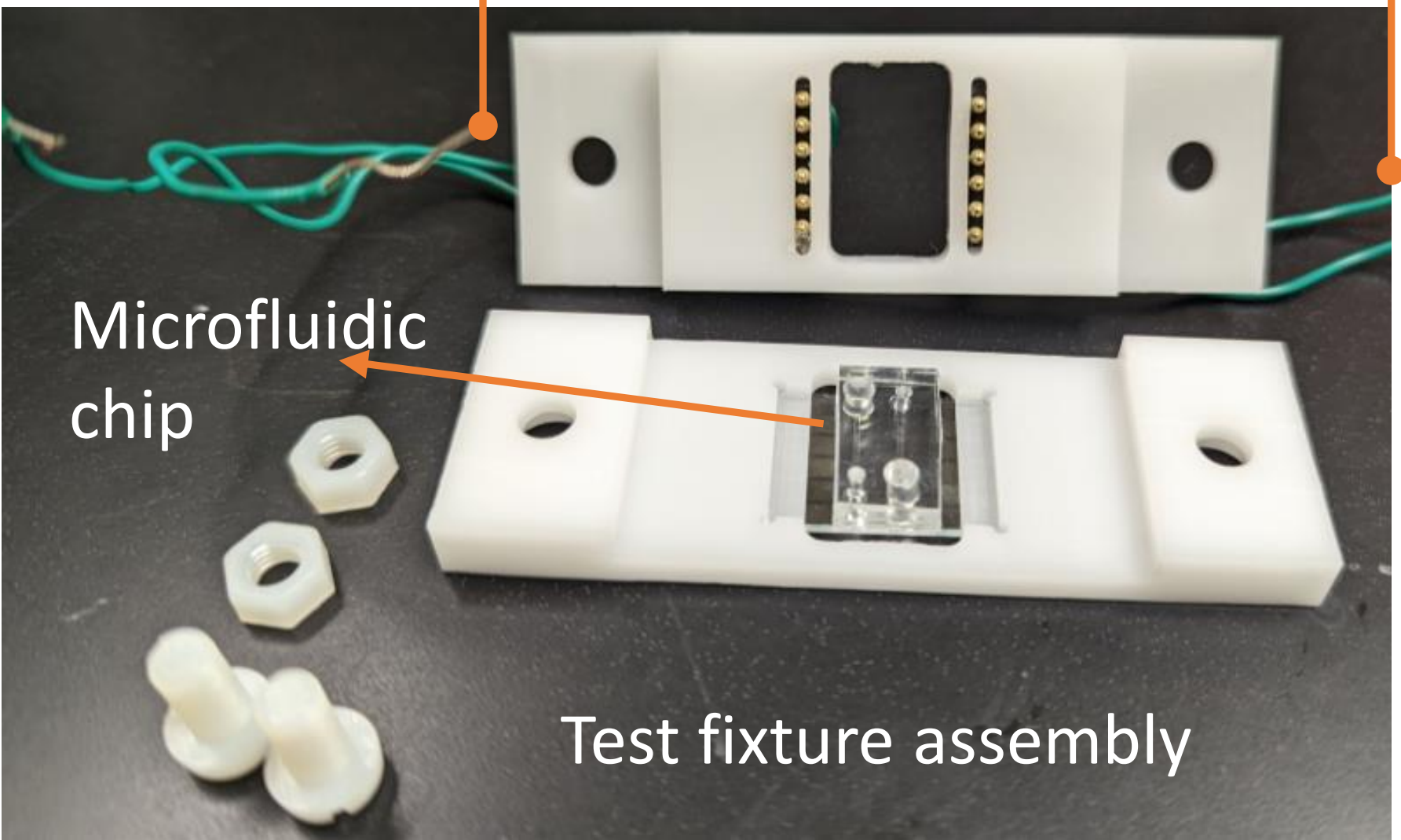

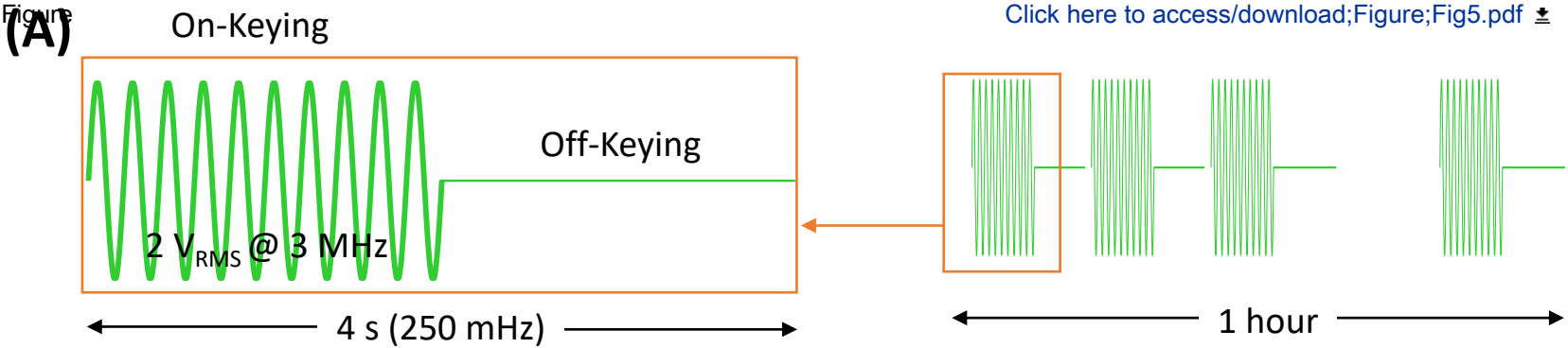

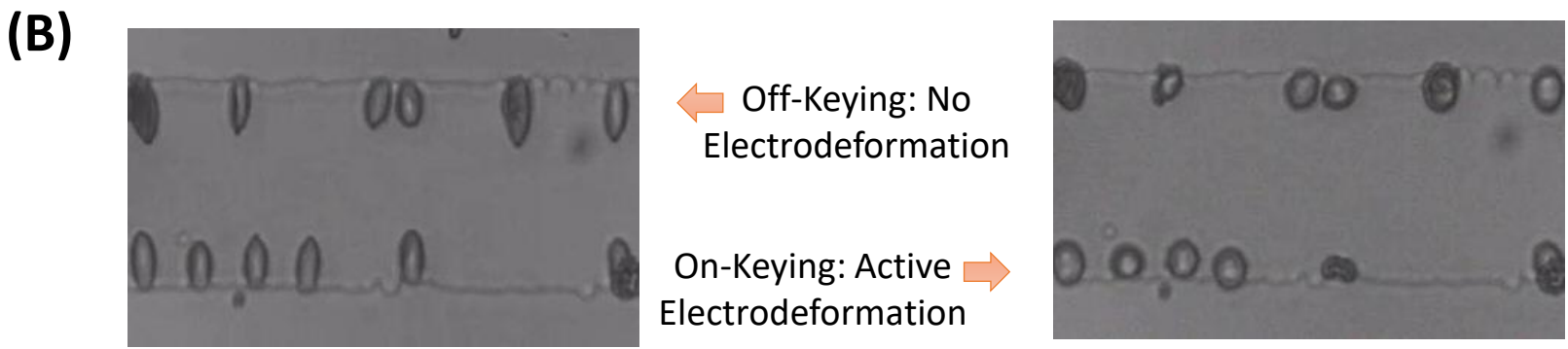

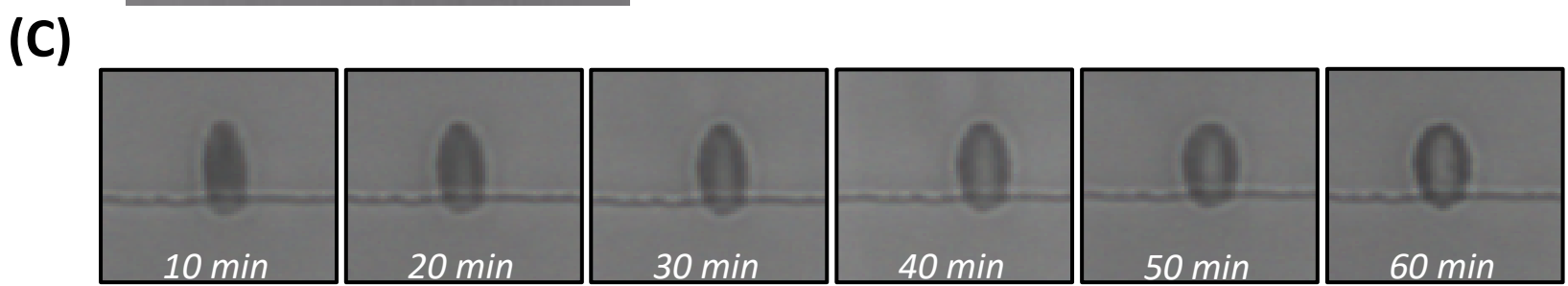

Figure

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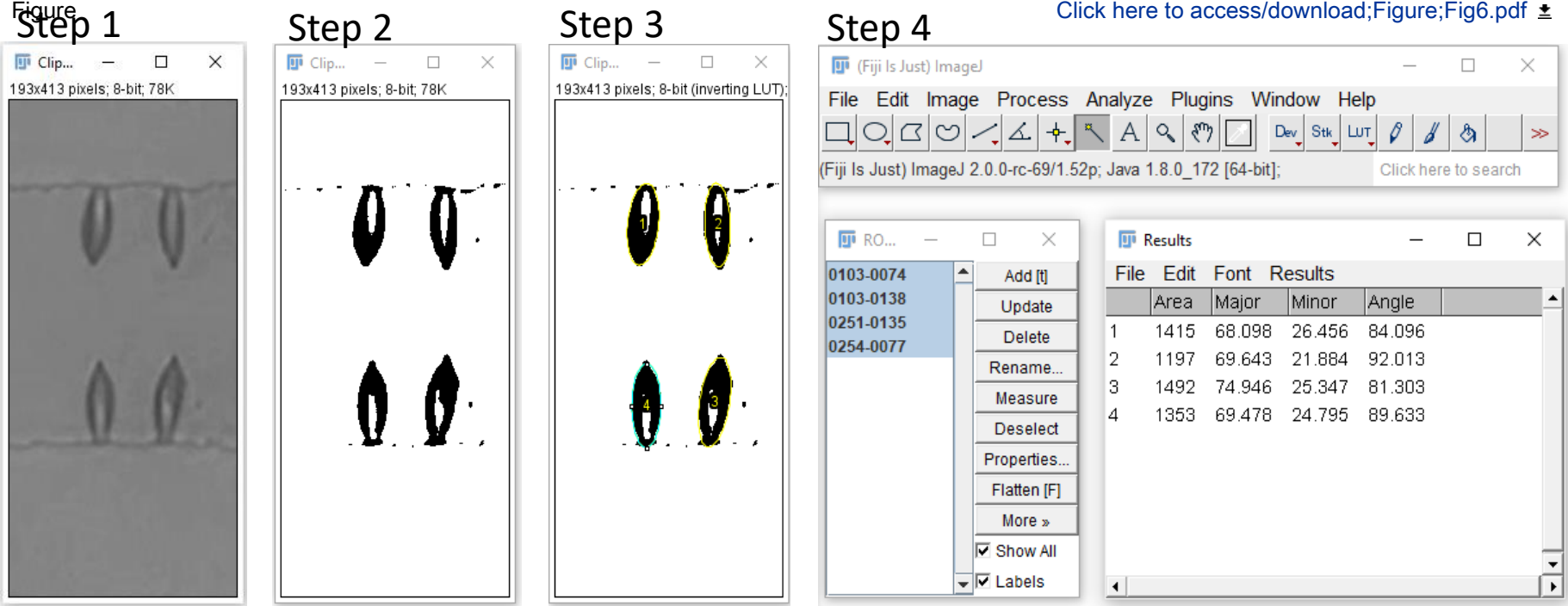

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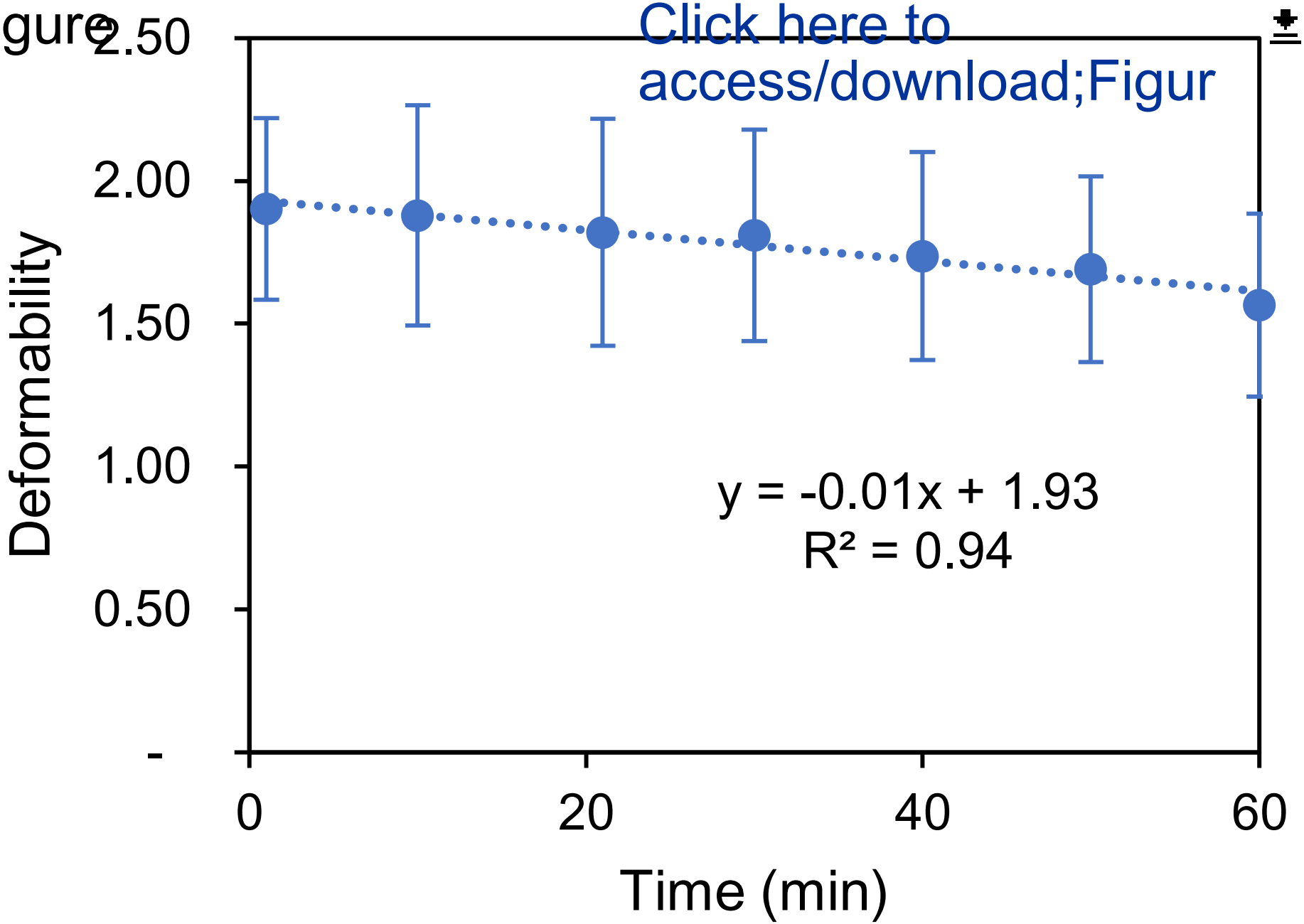

Table of Materials

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Name of Material/ Equipment	Company
Balance Scale	ViBRA
Bandpass filter	BRIGHTLINE
Bottle – 150 mL	CORNING
EC Low Conductivity meter	ecoTestr
Centrifuge	SCILOGEX
Glass/ITO Electrode Substrate	OSSILA
Eppendorf	www.eppendorf.com
23-gauge Needle	BSTEAN
Function Generator	SIGLENT
Biopsy Punch	INTERGRALIFE
Inverted Microscope	OLYMPUS
Micro Osmometer - Model 3300	Advanced Instruments Inc.
Lab Oven	QUINCY LAB (QL)
Parafilm	PARAFILM
Petri dish	FALCON
Plasma Cleaner	Harrick plasma PDCOOL
1 mL Syringe	BD
50 mL Conical Tube	fisherbrand
Vacuum Desiccator	SPBEL-ART
Phosphate Buffered Saline (PBS)	LONZA
Bovin Serum Albumin (BSA)	RMBIO
SUCROSE	SIGMA
DEXTROSE	SIGMA

Catalog Number	Comments/Description
HT-224R	
414/46 BrightLine HC	
	www.corning.com
358/03	
911015119999	
S161	
X001308N97	
SDG830	
	WWW.INTERGRALIFE.COM, 1.5 and 3 mm
IX81 - SN9E07015	
S/N: 03050397P	
MODEL 30GCE	Digital Model
	WWW.PARAFILM.COM
SKU=351006	ICSI/Biopsydish 50*9 mm
NC0301989	
05-539-13	
F42400-2121	
04-479Q	
	CAS number :57-50-1
	CAS number :50-99-7