Mechanosensitive channel-based optical membrane tension biosensor

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ABSTRACT: Plasma membrane tension has been recognized as a global physical organizer of cellular activities. Technical limitations of current membrane tension measurement techniques have hampered in-depth investigation of cellular membrane biophysics and the role of plasma membrane tension in regulating cellular processes. Here, we develop an optical membrane tension biosensor by repurposing an E. coli mechanosensitive channel via insertion of circularly permuted GFP (cpGFP), which undergoes a large conformational rearrangement associated with channel activation and thus fluorescence intensity changes under increased membrane tension.

The plasma membrane is the initial medium through which a cell detects and reacts to external physical stimuli. As a global mechanical regulator, plasma membrane tension coordinates a number of cellular behaviors[1–3]. For instance, membrane tension governs the homeostasis between exocytosis and endocytosis, where high membrane tension promotes exocytosis while low membrane tension supports endocytosis by enhancing vesicle trafficking[4, 5]. In addition, actin-based protrusions have also been suggested to be regulated by plasma membrane tension wherein low membrane tension allows polymerization of actin filaments, which generates lamellipodium-like protrusions at the leading edge during cell spreading[6]. Moreover, plasma membrane coordinates cytoskeletal tension for force transmission to control cell polarity and movement[7].

Although membrane tension has been shown to play critical roles in regulating cell behaviors, the molecular mechanisms by which cells actively regulate membrane tension remain largely unknown. Studies have shown that certain membrane proteins can sense extracellular mechanical forces and convert them into intracellular biochemical signals dubbed as mechanotransduction to modulate cell activities. For example, changes in membrane tension can be recognized and be affected by the activity of membrane-to-cortex proteins, which act as a linker between cellular membranes and actin filaments[8]. Mechanosensitive (MS) channels are another membrane tension sensor with their channel pore opening controlled by changes in plasma membrane tension[9]. Found in all bacteria, mechanosensitive channel of large conductance (MscL) is one of the most studied non-selective MS channels for transporting molecules and ions across the membrane in response to physical stimuli[10]. It is also believed to function as a pressure regulator to prevent lysis of bacterial membrane resulting from the sudden increase of membrane tension during osmotic down-shock[11]. Other eukaryotic MS proteins such as transient receptor potential (TRP) channels[12] and Piezo1[13] are found to mediate a variety of sensations including hearing, touch, and thermo- and osmo-sensation.

Despite the above-mentioned proteins identified as natural membrane tension sensors, a lack of suitable membrane tension measurement tools has hindered detailed studies of the effect of membrane tension on cellular processes. Therefore, development of molecular sensors that report membrane tension of live cells is of significant interest. One of the most widely used technologies for membrane tension quantification is micropipette aspiration[14], in which surface tension is deduced by measured radius of pipette and cells subjected to suction pressure applied by the micropipette using the Laplace law. The use of optical tweezers[15] or atomic force microscopy[16] is another option for indirect membrane tension measurement. However, these methods require special technical expertise and instrumentation. In recent years, several new methods have been developed to measure molecular tension. The development of fluorescence resonance energy transfer (FRET)-based biosensors has revolutionized the imaging of molecular signals with high spatiotemporal resolution[17,18]. However, the limitations in sensitivity and specificity have hindered their broader applications. In addition, current FRET-based tension biosensors focus on the tension change in the actin cytoskeleton or focal adhesion proteins rather than plasma membrane tension[19]. To overcome above-

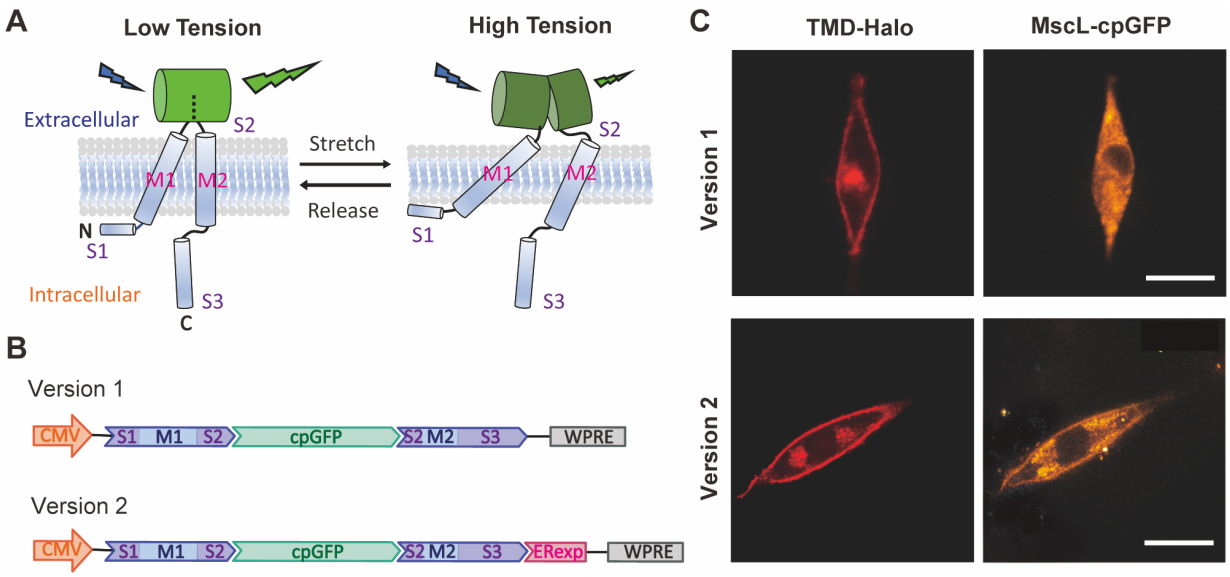

Figure 1. Schematics of the structure of the MscL membrane tension sensor and its expression in living cells. (A) The schematic of engineering MscL as a membrane tension sensor using cpGFP. The substantial conformational changes of the extracellular domain S2 caused by the tilting of the transmembrane domain M1 and M2 under high tension leads to a decrease in fluorescence while cpGFP stays fluorescent under low tension. For simplicity, only a monomer is illustrated with S1, M1, S2, M2, and S3 domains shown. (B) The DNA constructs of the version 1 (top) and version 2 (bottom) MscL membrane tension biosensors. For both constructs, cpGFP was inserted to the sensory domain of MscL G22S after amino acid 61 as an OFF sensor. Version 2 is optimized with the addition of Kir2.1 ER export signal (ERexp) for enhancement of membrane localization. (C) Confocal fluorescence images showing the expression of version 1 (top) and version 2 (bottom) constructs in NIH3T3 cells. Scale bar: 5 μm.

mentioned technical barriers, herein we report the development of an optical membrane tension biosensor using genetically modified MscL.

Specifically, our approach involves the development of a membrane tension OFF senor, by inserting circularly permuted green fluorescence protein (cpGFP), whose optical property depends on its structure, into the extracellular domain of MscL G22S. As a gain-of-function mutant of MscL, MscL G22S has a ~50% lower tension activation threshold (5-6 mN/m) compared to wild type (WT) MscL[20]. MscL G22S (hereafter referred to as MscL) is used instead of WT MscL given the increased sensor sensitivity to mechanical stimuli. An increase in membrane tension causes the tilting of MscL's transmembrane helices and triggers channel opening[21]. We hypothesize that structural rearrangement of MscL caused by membrane tension would be transferred to the connected cpGFP, leading to a conformational change that gives rise to a change in its fluorescence (Figure 1A). A similar concept has been recently utilized for the successful development of a fluorescent voltage sensor[22].

To generate the tension biosensor DNA construct, we first encoded MscL G22S with cpGFP fused to its extracellular loop domain after amino acid 61 into a lentivirus vector, pLVX-Puro vector, under the control of CMV promoter as version 1 (Figure 1B). However, transfection of NIH3T3 cells leads to expression of the sensor version 1 and their notable intracellular accumulation but poor membrane localization (Figure 1C). We speculate a lack of a mammalian-specific export signal, which inhibits heterologous protein retention in the endoplasmic reticulum (ER), is responsible for MscL cytoplasmic accumulation. To overcome this, we next appended Kir2.1 ER export signal (FCYENEV), which is known to mediate protein export from ER to Golgi complex to plasma membrane[23], to the C terminus of MscL as version 2 (Figure 1B). This strategy has previously been shown to promote MscL membrane expression in neurons[24]. To examine localization of the MscL tension biosensors we co-transfected cells with either biosensor design along with a membrane-targeted HaloTag construct (TMD-Halo), where HaloTag was linked to the transmembrane domain of transferrin receptor (TfR) for membrane localization.

As shown in Figure 1C, reduction of cytoplasmic aggregation and enhancement of membrane expression of version 2 biosensor was confirmed by stronger co-localization of its signal with that of TMD-Halo as compared to that of version 1 biosensor. Version 2 biosensor is referred to as MscL tension sensor and used for the remainder experiments.

To test MscL tension sensor sensitivity, we applied an osmotic pressure test, with increasing osmotic shock leading to increased membrane tension, to NIH3T3 cells transfected with the MscL tension sensor. Previously, we have shown influx of small molecules into mammalian cells expressing MscL in an osmotic pressure dependent

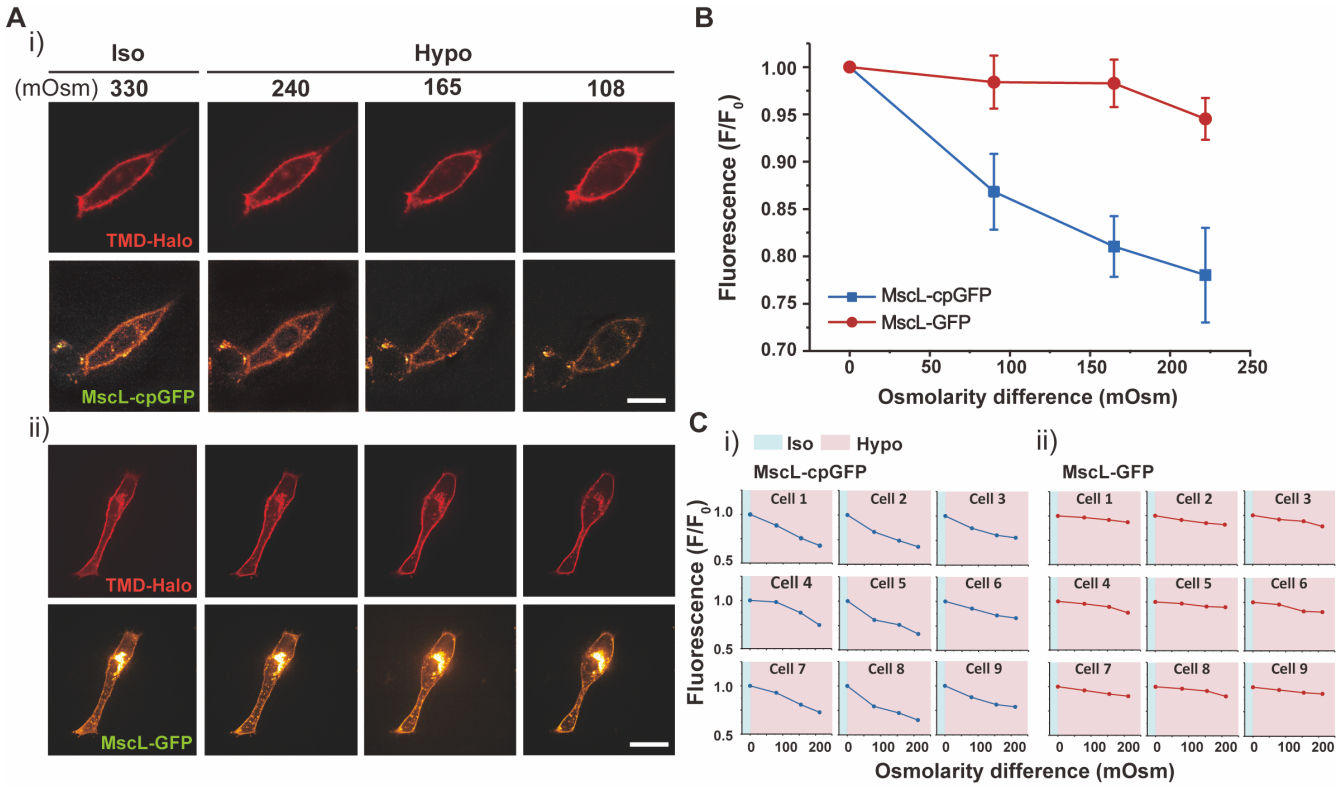

Figure 2. NIH3T3 cells transfected with the MscL tension sensor in response to osmotic pressure. (A) (i) Confocal images showing the membrane localization of the MscL-cpGFP in NIH3T3 cells in response to different osmotic shocks. Transfected cells were cultured in iso-osmotic condition for 2 days and then DI water was sequentially added to the cell culture media to create increasing hypo-osmotic environments. Each image was taken four minutes after the addition of DI water. (ii) Confocal images showing the NIH3T3 cells transfected with the MscL-GFP construct as a control in response to different osmotic shocks. The experiment followed the same method as mentioned in (i). (B) Normalized fluorescence intensities (MscL-cpGFP or MscL-GFP) of the cell membranes of NIH3T3 cells transfected with the MscL tension sensor and MscL-GFP under different osmotic conditions corresponding to the experiment mentioned in (A). (C) Normalized fluorescence intensities of the MscL tension sensor (i) and MscL-GFP control (ii) localizing on cell membranes of nine different NIH3T3 cells, from three separate experiments, at increasing osmotic pressures. Scale bars: 5 μm. The error bars denote standard deviation.

manner[25, 26]. In the osmotic pressure test, the osmolarity of culture medium was gradually decreased from 330 to 108 mOsm. As shown in Figure 2Ai, MscL tension sensors were colocalized to TMD-Halo in NIH3T3 cells over the range of hypo-osmotic conditions. MscL-cpGFP fluorescence decreased as the osmolarity of culture medium decreased, supporting that the MscL tension sensors function properly in living cells.

To verify that the declining fluorescence intensity was not caused mainly by photobleaching, we fused GFP to the C-terminus of MscL G22S as a control and carried out the same osmotic pressure test. While the membrane localization of the control sensor was similar to that of the MscL tension sensor, no significant change of the green fluorescence intensity along the plasma membrane was detected (Figure 2Aii). We analyzed the fluorescent intensity by normalizing the fluorescence with the initial fluorescence intensity localized to the plasma membranes (using a mask generated from the TMD-Halo image) of cells under iso-osmotic condition (Figure S1). Cells with MscL tension sensors displayed a substantial decrease in fluorescence in response to increasing osmotic pressure. For example, osmotic pressure with an osmolarity difference of 220 mOsm reduced fluorescence intensity by about 23%, whereas control sensors manifested only 5% fluorescence reductions under the same osmolarity difference (Figure 2B). This is also evident from normalized fluorescence of MscL tension sensors in individual cells for both conditions (Figure 2C). As another control, we used a bicistronic construct using self-cleaving peptide P2A to express G22 MscL and GFP and found that GFP fluorescence at the membrane did not reduce with increasing osmolarity difference, suggesting that volume changes of cells due to osmotic downshock did not appreciably change fluorescence at the membrane (Figure S2).

To further verify the sensitivity of fluorescence shift of the MscL tension sensor in response to changes in membrane tension, we performed cyclic osmotic test on NIH3T3 cells co-transfected with MscL tension sensor and TMD-Halo. The osmolarity was repeatedly switched between iso-osmotic (~330 mOsm) and hypo-osmotic conditions (~165 mOsm). As shown in Figure 3A, MscL tension sensor was located on plasma membrane as ex-

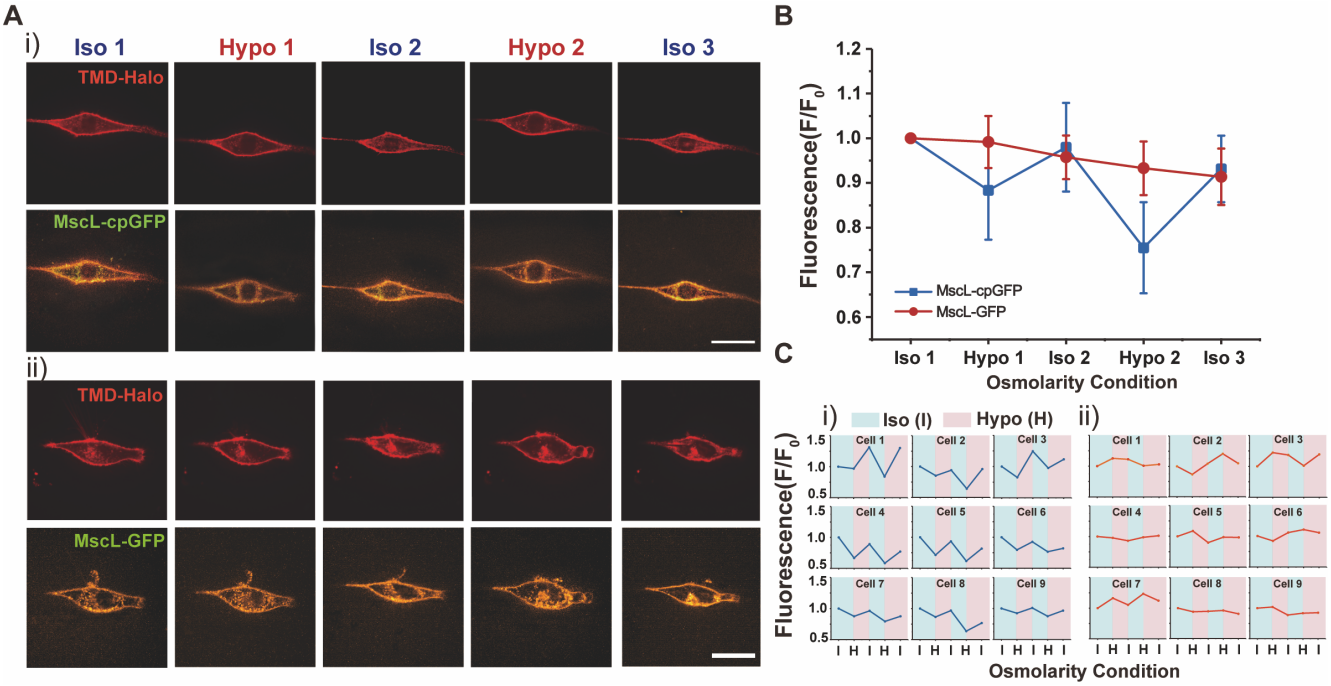

Figure 3. NIH3T3 cells transfected with the MscL tension sensor in response to cyclic pressure (A) (i) Confocal images showing the membrane localization of MscL tension sensor in NIH3T3 cells in response to the cyclic pressure test, which was carried out by alternating between iso-osmotic and hypo-osmotic conditions repeatedly. Transfected cells were cultured in iso-osmotic condition for 2 days. DI water was first added to the cell culture media to create hypo-osmotic environments and then 10X PBS solution was added back to the media to increase the osmolarity for iso-osmotic environments. The osmolarity of the media is ~330 mOsm for iso-osmotic conditions and ~165 mOsm for hypo-osmotic conditions. Each confocal image was taken four minutes after the addition of DI water or PBS solution. (ii) The confocal images showing the NIH3T3 cells transfected with the MscL-GFP construct as a control in response to the same cyclic pressure cycle test as mentioned in (i). (B) Normalized fluorescence intensities of the cell membranes of NIH3T3 cells transfected with the MscL tension sensor and MscL-GFP construct corresponding to the cyclic pressure cycle test. Nine cells were analyzed for each condition from three independent experiments. (C) (i) Fluorescence intensity traces of MscL-cpGFP on cell membranes of nine different NIH3T3 cells during the cyclic osmotic tests (ii) Fluorescence intensity traces of MscL-GFP on cell membranes of nine different NIH3T3 cells as a control. Scale bars: 10 μm. The error bars denote standard deviation.

pected. MscL-cpGFP fluorescent intensity varied depending on membrane tensions resulting from the cyclic iso-osmotic and hypo-osmotic conditions. We observed an alternating pattern of high and low fluorescence in iso-osmotic and hypo-osmotic conditions, respectively. When quantified as before, fluorescence intensities of the MscL tension biosensor displayed a 'W' shape trend (Figure 3B, Ci). A similar cyclical test was also conducted on NIH 3T3 cells transfected with MscL-GFP as a control, in which no obvious trend of MscL fluorescence could be correlated to the osmolarity changes (Figure 3Aii, B, Cii). Our second control where GFP is not attached to MscL also lacked such a trend (Figure S3). Together, these results demonstrate the reversibility of the MscL tension sensor.

Finally, we used polydimethylsiloxane (PDMS) micropost arrays to further demonstrate the applicability of the MscL membrane tension sensor in cells under different spreading conditions. Micropost array technologies have been widely used to study the mechanosensitive behaviors of cells. Cells attached to fibronectin-coated microposts with different post heights, therefore stiffnesses, experience different degrees of cell spreading[27–29]. Cells attached to shorter microposts have greater membrane tension as they spread more on more rigid substrates. Conversely, longer microposts cause lower plasma membrane tension due to limited cell spreading on softer substrates. For our assay, fibronectin (50 µg/mL) was microcontact printed onto micropost arrays to create an adhesive surface. Human mesenchymal stem cells (hMSCs) co-transfected with the MscL tension sensor and TMD-Halo were seeded at a density of around 1,500 cells/cm2 on micropost arrays with posts of 1.83 µm diameter and 2.3 or 6.4 µm height. We observed successful expression and membrane localization of the MscL tension biosensor colocalizing with TMD-Halo, and cells seeded on short microposts exhibited lower GFP membrane fluorescence in compared to those attached to longer microposts (Figure 4A), consistent with the data from the osmotic pressure experiments in Figure 2Ai. As expected, cells spread more on short microposts compared to long microposts, irrespective of the constructs expressed (Figure 4B). While cells expressing the MscL tension sensor responded to changes in cell sprea-

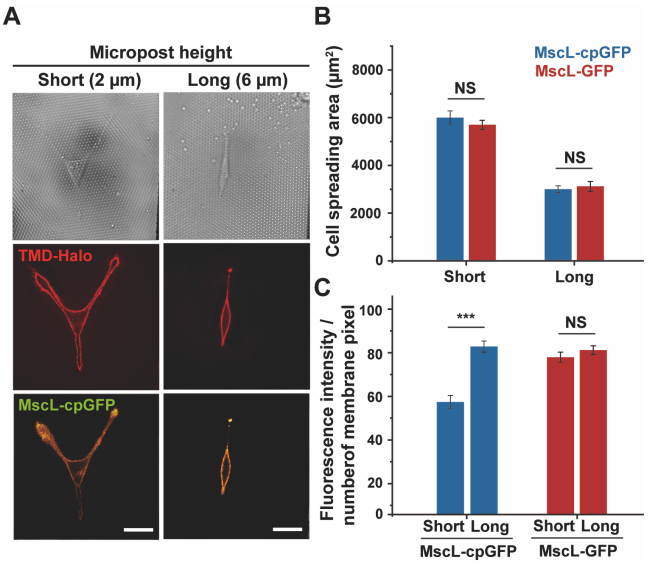

Figure 4. Fluorescence response of the MscL tension biosensor expressed in hMSCs attached to micropost arrays with different rigidity. (A) Confocal images showing the expression of the MscL tension biosensor in hMSCs spreading on short (left) or long (right) microposts. (B) Cell spreading areas of hMSCs on microposts with different heights. (C) The average fluorescence intensity of cpGFP or GFP signals localized to the cell membranes in response to different substrate rigidity. Nine cells were analyzed for each post height from three independent experiments. Scale bars: 50 μm. The error bars denote standard error. NS denotes not significant. *** represent $p < 0.001$.

ding, with ~33% reduction in average of membrane fluorescent intensity from longer to shorter microposts, the control MscL-GFP expressing cells showed no difference in GFP membrane fluorescence under the same conditions (Figure 4C).

In summary, we successfully developed an optical membrane tension biosensor by repurposing MscL through insertion of cpGFP. As an OFF sensor, fluorescence of the MscL tension biosensor is negatively correlated to membrane tension. Our study demonstrates that the MscL tension biosensor can be expressed efficiently on cell membranes and used in different cell types for membrane tension measurement. The MscL membrane tension biosensor is user friendly, thus offering a useful approach for detailed investigations of spatial and temporal dynamics of membrane tension, which will help advance research in cell mechanics, mechanotransduction, and cell signaling.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Experimental details, micropost array fabrication, additional results, including figures and tables, data analysis and image processing (PDF)

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Author Contributions

A.P.L. and J.F. conceived the study. Y.Y.H., A.P.L. designed the experiments. Y.Y.H. performed the experiments. A.M.R. wrote the codes for image processing. Y.Y.H. and A.M.R. carried out image processing. Y.Y.H., A.M.R. and A.P.L. wrote the paper. All authors contributed to the manuscript revision and approved the final version.

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