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Detection of microRNA Expression in the Kidneys of Immunoglobulin A Nephropathic Mice

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Dr. Vineeta Bajaj
JoVE (Journal of Visualized Experiments)
June 10, 2020

Dear Dr. Bajaj:

We thank the editor again for the thoughtful comments regarding our manuscript entitled “Detection of microRNA Expression in the Kidneys of Immunoglobulin A Nephropathic Mice” We have revised the manuscript in accordance with these comments. We believe that these revisions have strengthened the manuscript and we hope that it is now suitable for publication in *JoVE*.

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We look forward to hearing from you at your earliest convenience.

Sincerely,

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TITLE:

Detection of microRNA Expression in the Kidneys of Immunoglobulin a Nephropathic Mice

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KEYWORDS:

microRNA, IgA nephropathy, HIGA mouse, qRT-PCR, kidney, expression

SUMMARY:

microRNAs are involved in the pathogenesis of IgA nephropathy. We have developed a reliable method for detecting microRNA expression levels in the kidneys of an IgA nephropathy mouse model (HIGA mice). This new method will facilitate to check for miRNAs involvement in IgA nephropathy.

ABSTRACT:

Immunoglobulin A (IgA) nephropathy is a type of primary glomerulonephritis characterized by the abnormal deposition of IgA, leading to the end-stage renal failure. In recent years, the involvement of microRNAs (miRNAs) has been reported in the pathogenesis of IgA nephropathy. However, there is no established method for profiling miRNAs in IgA nephropathy using small animal models. Therefore, we developed a reliable method for analyzing miRNA in the kidney of an IgA mouse model (HIGA mouse). The goal of this protocol is to detect the altered expression levels of miRNAs in the kidneys of HIGA mice when compared with the levels in kidneys of control

mice. In brief, this method consists of four steps: 1) obtaining kidney samples from HIGA mice; 2) purifying total RNA from kidney samples; 3) synthesizing complementary DNA from total RNA; and 4) quantitative reverse transcription polymerase chain reaction (qRT-PCR) of miRNAs. Using this method, we successfully detected the expression levels of several miRNAs (miR-155-5p, miR-146a-5p, and miR-21-5p) in the kidneys of HIGA mice. This new method can be applied to other studies profiling miRNAs in IgA nephropathy.

INTRODUCTION:

Immunoglobulin A (IgA) nephropathy is a type of primary glomerulonephritis characterized by the abnormal deposition of IgA in the renal glomerular mesangial region[1,2]. It is the most common of the primary glomerulonephritis and leads to the end-stage renal failure in 20%–40% of patients[2]. The cause is still unknown but persistent mucosal infection has been implicated[1,3]. Corticosteroids, immunosuppressants, and renin−angiotensin system inhibitors have been proposed as therapeutic methods[1,3], but have not been completely established[3]. Therefore, further research is required to clarify the etiology and therapeutic methods of treating IgA nephropathy.

microRNAs (miRNAs) are small, non-coding RNAs that play an important role in regulating gene expression[4,5]. miRNAs are reported to be involved in the pathogenesis of various diseases, and some have been identified as disease biomarkers and therapeutic agents[4,5]. In recent years, an association between miRNAs and the pathogenesis of IgA nephropathy has also been reported[2,6,7]. For example, miR-148b was shown to be involved in structural abnormalities of IgA in patients with IgA nephropathy[2,6,7], while miR-148b and let-7b were documented as novel biomarkers for detecting IgA nephropathy[7]. Although understanding the effects of miRNAs on IgA nephropathy may help further elucidate etiology and treatment[2], standard methods for profiling miRNAs in IgA nephropathy using small animal models have not yet been established[2].

We herein developed a simple and reliable method for measuring miRNA expression levels in the kidneys of an IgA nephropathy mouse model (HIGA mice). The HIGA mouse is a characteristic ddY strain showing a particularly high level of serum IgA and the abnormal deposition of IgA in kidney glomeruli[8-11]. Therefore, HIGA mice can be used as an IgA nephropathy mouse model[8-11]. Our method consists of four major steps: first, surgically obtaining kidney samples from HIGA mice; second, homogenizing samples and purifying total RNA using a silica membrane-based spin column; third, synthesizing complementary DNA (cDNA) from total RNA using reverse transcription; and fourth, detecting the expression levels of miRNA by quantitative reverse-transcription polymerase chain reaction (qRT-PCR). The rationale for this method and the reliability of the results are based on previous reports[12,13]. We show that this is a useful technique to accurately measure miRNA expression levels in an IgA nephropathy mouse model, and that it could be used to facilitate future research into miRNAs in IgA nephropathy.

PROTOCOL:

Animal experiments were approved by the Animal Ethics Committee of Jichi Medical University and comply with the Use and Care of Experimental Animals guidelines from the Jichi Medical University Guide for Laboratory Animals.

1. Obtaining kidney samples from HIGA mice

NOTE: HIGA mice show a stable phenotype of IgA nephropathy after 25 weeks of age[8-11]. Balb/c mice should be selected as the control group[8-11]. 25-week-old female HIGA mice (n = 10) and 25-week-old female Balb/c mice (n = 10) were obtained. It is necessary to determine the number of mice required for experiments in advance. This step requires about 7–8 h for a sample size of 10 HIGA mice and 10 Balb/c mice.

1.1. Prepare these items: HIGA mice (25-week-old, female), Balb/c mice (25-week-old, female), inhalation anesthesia apparatus, isoflurane, cork sheet, pin, phosphate-buffered saline (PBS), injection syringes, needles, surgical scissors, and forceps.

1.2. Anesthetize a HIGA mouse using 4%–5% of isoflurane with inhalation anesthesia apparatus and mount it in the dorsal position on the cork sheet using pins. Adjust the concentration of isoflurane to 2%–3% as the maintenance dose after the induction of anesthesia.

NOTE: The depth of anesthesia is maintained at a level at which the pain associated with the invasive procedure is completely eliminated. Hair removal and eye ointment are not essential. It is not necessary to mount the mouse on a heating pad if the procedure can be performed quickly.

1.3. Make a 3–4 cm midline incision of the mouse abdominal wall with surgical scissors and forceps and carefully identify both sides of the kidney.

1.4. Incise the ribs and diaphragm with surgical scissors and forceps to expose the heart. After incising the right atrium, inject PBS into the left ventricle until the kidney color changes to pale yellow (this procedure means that the whole body of the mouse is perfused with PBS.)

1.5. Cut the renal artery, renal vein, and ureter, and remove the kidney. Divide the kidney into 30 mg pieces for use in the next step.

NOTE: The pathology of IgA nephropathy mainly involves the glomerulus in the renal cortex (outer part of the kidney). Although it is difficult to macroscopically and completely distinguish the cortex and the medulla, it is desirable to mainly collect the outer part of the kidney. These samples can be stored at –80 °C for several months.

2. Purifying total RNA from kidney samples

NOTE: In this step, commercially available miRNA isolation kit is used for the extraction of total RNA. In addition, biopolymer-shredding spin column is used. See **Table of Materials** for additional details. miRNA isolation kit contains a silica membrane-based spin column, phenol/guanidine-based lysis reagents, guanidine/ethanol wash buffer (wash buffer 1), ethanol

wash buffer (wash buffer 2), and nuclease-free water. This step requires about 3 h for a sample size of 10 HIGA mice and 10 control mice.

2.1. Prepare the following items: 1.5 or 2.0 mL collection tubes, 100% ethanol, chloroform, silicon homogenizer, micropipettes, pipette tips, centrifuge, biopolymer-shredding spin column, silica membrane-based spin column, phenol/guanidine-based lysis reagents, guanidine/ethanol wash buffer (wash buffer 1), ethanol wash buffer (wash buffer 2), and nuclease-free water.

2.2. Homogenize 30 mg kidney samples using a silicon homogenizer and 700 µL of the phenol/guanidine-based lysis reagent at room temperature.

NOTE: The miRNAs in the sample are vulnerable until they have been exposed to phenol/guanidine-based lysis reagent so these steps should be performed promptly.

2.3. Transfer the lysate to the biopolymer-shredding spin column and centrifuge it at 15,000 *x g* for 2 min at room temperature.

2.4. Add 140 µL of chloroform to the filtrate and mix vigorously for 15 s. Leave for 2–3 min, then centrifuge at 12,000 *x g* at 4 °C for 15 min.

2.5. Gently transfer the clear supernatant without touching the middle layer to a new collection tube and add the determined volume (see below) of 100% ethanol. Vortex for 5 s.

NOTE: 100% ethanol at 1.5x the volume of the obtained supernatant is required.

2.6. Transfer the sample (upper limit 700 µL) to a silica membrane-based spin column and centrifuge the sample at room temperature for 15 s at 8,000 *x g*. Discard the filtrate after centrifugation.

2.7. Add 700 µL of wash buffer 1 to the silica membrane-based spin column and centrifuge the column at room temperature for 15 s at 8,000 *x g*. Discard the filtrate after centrifugation.

2.8. Add 500 µL of wash buffer 2 to the silica membrane-based spin column and centrifuge it at room temperature for 15 s at 8,000 *x g*. Discard the filtrate after centrifugation.

2.9. Add 500 µL of wash buffer 2 to the silica membrane-based spin column and centrifuge it at room temperature for 15 s at 8,000 *x g*. Discard the filtrate after centrifugation.

2.10. Centrifuge the silica membrane-based spin column again without adding anything to remove any additional ethanol, at 15,000 *x g* for 1 min at room temperature. Throw away the filtrate after centrifugation.

2.11. Change the tube attached to the spin column to a new collection tube.

2.12. Add 30 µL of nuclease-free water to the silica membrane-based spin column and centrifuge it at 8,000 *x g* for 1 min at room temperature.

NOTE: The resulting 30 µL solution contains a high concentration of total RNA. This sample can be stored at –80 °C for several months.

3. Synthesis of cDNA from total RNA

NOTE: In this step, a commercially available reverse transcription kit is used. See **Table of Materials** for additional details. This kit contains nucleic acid mix, reverse transcriptase mix, and buffer. This procedure must be performed on ice to prevent progress of the reaction. This step requires about 3 h for a sample size of 10 HIGA mice and 10 control mice.

3.1. Prepare the following items: 1.5 mL collection tubes, eight-well strip tubes, micropipettes, pipette tips, spectrophotometer, nuclease-free water, ice, nucleic acid mix, reverse transcriptase mix, buffer, and thermal cycler.

3.2. Measure the concentration of total RNA using a spectrophotometer. Then, calculate the required volume of nuclease-free water so that 12 µL contains 1 µg of total RNA.

3.3. Prepare a master mix with the following contents: 2.0 µL of nucleic acid mix, 2.0 µL of reverse transcriptase mix, and 4.0 µL of buffer to a total of 8.0 µL per sample.

3.4. Add 8 µL of the master mix to each well of eight-well strip tubes.

3.5. Dilute total RNA in the volume of nuclease-free water calculated in 3.2. Then, add 12 µL of the total RNA solution (containing 1 µg of total RNA) to each well of eight-well strip tubes.

3.6. Incubate the sample in 8 well strip tubes at 37 °C for 60 min using the thermal cycler. Then, incubate at 95 °C for 5 min using the thermal cycler.

NOTE: The solution after incubation contains a high concentration of cDNA.

3.7. Transfer this solution to a 1.5 mL tube and add 200 µL of nuclease-free water.

NOTE: The resulting 200 µL of solution can be used for qRT-PCR as a template cDNA. This sample can be stored at –80 °C for about 1 year.

4. qRT-PCR of miRNA

NOTE: In this step, a commercially available PCR kit is used. See **Table of Materials** for additional details. This kit contains PCR mix, universal primer, and nuclease-free water. Samples should be prepared in duplicate, and the accuracy of the results should be considered in each

case. Expression levels of miRNA are quantified by the ΔΔCT method. This step requires about 4 h for a sample size of 10 HIGA mice and 10 control mice.

4.1. Prepare the following items: 1.5 mL collection tube, 96-well reaction plate, adhesive film for the 96-well reaction plate, micropipettes, pipette tips, PCR mix, universal primer, nuclease-free water, miRNA-specific primers, and a real-time PCR instrument.

4.2. Prepare the master mix with the following contents: 12.5 µL of PCR mix, 2.5 µL of universal primer, 1.25 µL of 5 µM miRNA-specific primer, and 6.25 µL of nuclease-free water for each well.

NOTE: In this assay, primers specific to miRNAs [RNA, U6 Small Nuclear 2 (RNU6-2), miR-155-5p, miR-146a-5p, and miR-21-5p] were used. RNU6-2 was used as an endogenous control[14].

4.3. Add 22.5 µL of the master mix to each well of the 96 well reaction plate.

4.4. Add 2.5 µL of cDNA prepared in step 3.7 to individual well of the 96 well plate.

4.5. Cover the 96 well reaction plate with adhesion film, then centrifuge at 1,000 *x g* for 30 s.

4.6. Run the real-time PCR instrument. Program the PCR instrument as follows: initial activation at 95 °C for 15 min, then 40 cycles of denaturation at 94 °C for 15 s, annealing at 55 °C for 30 s, and extension at 70 °C for 30 s.

4.7. Quantify gene expression using the ΔΔCT method. Relative expression levels are determined as $2^{-\Delta\Delta CT}$.

NOTE: Abnormal PCR amplification curves should be considered for exclusion from the results.

REPRESENTATIVE RESULTS:

We investigated the expression levels of miRNAs in the kidneys of HIGA mice (n=10). This result was obtained completely based on the described protocol. The kidneys of Balb/c mice were selected as the control (n=10). In both groups, aged 25 weeks were selected. Only female HIGA mice were available from the supplier. The expression levels of three miRNAs (miR-155-5p, miR-146a-5p, and miR-21-5p; **Figure 1**) were detected, which were previously reported to be associated with IgA nephropathy[15,16]. Relative miRNA expression levels of the two groups were compared using a t-test, with $P < 0.05$ being statistically significant. miR-155-5p was expressed at 3.3-fold higher levels in the HIGA group compared with the control group ($P < 0.001$), while miR-21-5p was expressed at 1.58-fold higher levels in the HIGA group ($P = 0.007$). miR-146a-5p expression did not differ significantly between the two groups.

FIGURE AND TABLE LEGENDS:

Figure 1: Expression levels of miRNAs in the kidneys of HIGA mice. qRT-PCR determined the

relative expression levels of miR-155-5p, miR-146a-5p, and miR-21-5p in HIGA mice (n = 10) and Balb/c mice (n = 10). Relative expression levels are shown as mean values and standard errors. (**A**) miR-155-5p expression was 3.3-fold higher in the HIGA group (P < 0.001). (**B**) miR-146a-5p expression did not differ significantly between the two groups. (**C**) miR-21-5p expression was 1.58-fold higher in the HIGA group (P = 0.007). quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR); microRNAs (miRNAs); not significant (NS); *, p <0.05.

DISCUSSION:

We were able to measure the expression levels of miRNAs in the kidneys of an IgA nephropathy mouse model (HIGA mice) using this new method. IgA nephropathy is an unexplained disease that needs further research to clarify its etiology and therapeutic targets[1,3]. However, obtaining human kidney samples is highly invasive. This new technique is advantageous in that it allows the study of IgA nephropathy using small animals and should facilitate future research into IgA nephropathy and miRNAs. In a future study, this method could be applied to the study of novel miRNAs for the treatment of IgA nephropathy. Artificial modulation of miRNAs in HIGA mice may influence the phenotype of IgA nephropathy. In that case, this method may be useful for verifying the changes of miRNAs. This method is not intended for analyzing miRNAs in serum, but it may be possible to use it for this by modifying the protocol.

The rationale of this method is based on previous reports. We used biopolymer-shredding spin columns to disrupt biopolymers in the sample, which has previously been shown to be an effective technique[12]. Past reports have also demonstrated the accuracy of synthesizing cDNA from total RNA using reverse transcription and performing PCR using the prepared cDNA as a template[13]. As a critical step in this method, evaluation of the PCR results is important. It is necessary to exclude abnormal amplification curves from the results. In addition, changes in endogenous controls should be considered if stable results are not obtained[14].

A major problem that can be encountered with this method is the abnormally low expression of miRNAs. miRNAs are highly degradable compounds so the method should be performed quickly. Additionally, the effects of ribonuclease (RNase) in the experimental environment need to be eliminated. Researchers should always be aware of the presence of RNase in skin and hair[17]. It is necessary to wear disposable gloves, masks, and hair caps for experiments. In addition, laboratory equipment such as micropipettes, pipette tips, and collection tubes must be cleaned to ensure the absence of RNase[17]. The deactivation of RNase using an autoclave should be performed as necessary[17]. Otherwise, researchers should use RNase-free-certified items[17]. All samples should be properly stored under the recommended conditions to reduce the risk of RNase contamination.

Our method has three important limitations. The first is that we did not demonstrate whether it can be applied to other animals and other organs. This is relevant because miRNAs are known to play an important role in blood cells in IgA nephropathy[2,6,7], but we did not investigate whether our method can be used in blood cells. Second, the sample size of our experiments may be small. Sample size should be determined depending on the study design, statistical analysis, required time, and required costs. Therefore, each researcher must determine the appropriate sample

size before starting this experiment. Third, the severity and phenotype of IgA nephropathy in HIGA mice may vary between individuals[9]. In addition, the bias of sex and age has not been fully investigated. It is recommended to add immunohistochemistry to confirm the severity and phenotype of IgA nephropathy, if necessary. In addition, the methods other than PCR, including microarray, northern blotting, and ribonuclease protection assays, are recommended to perform as necessary.

In conclusion, we developed a reliable method for measuring the expression levels of miRNAs in the kidneys of an IgA mouse model (HIGA mice).

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DISCLOSURES:

The authors declare that they have no conflicts of interest.

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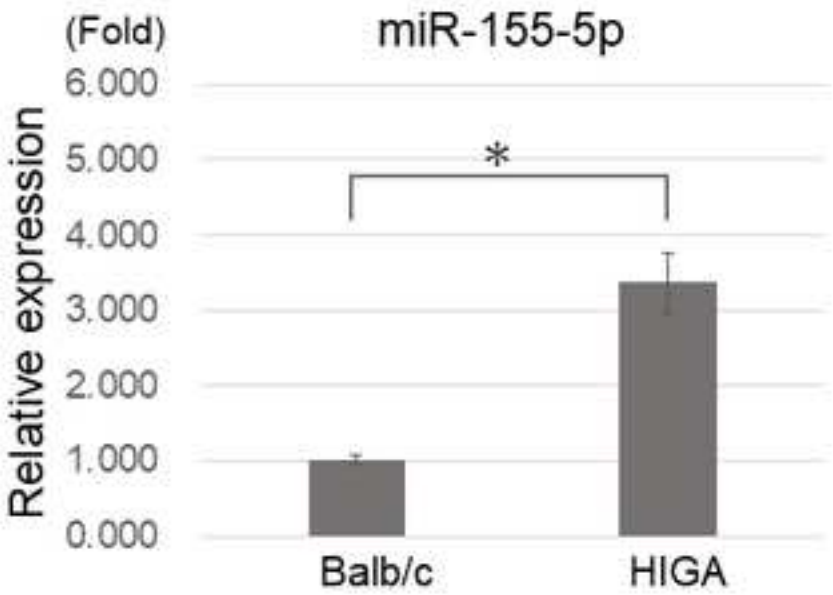
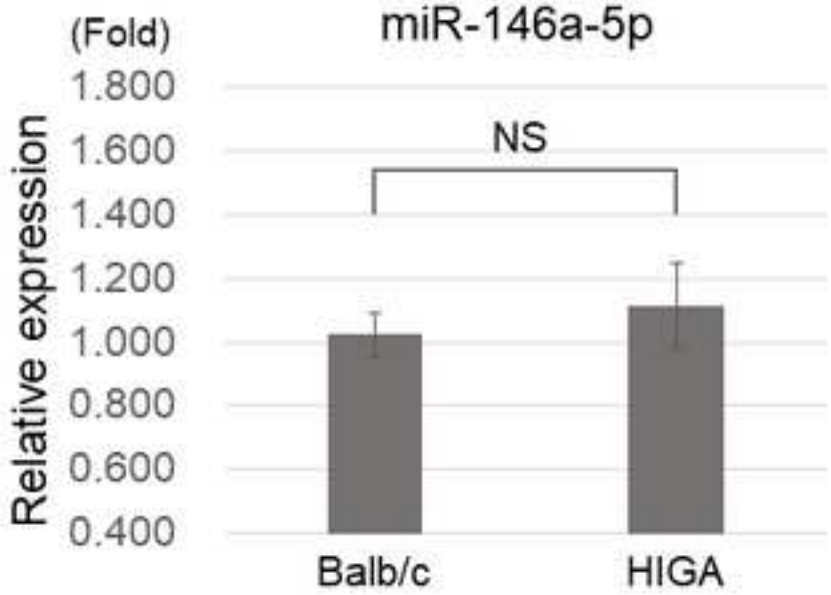
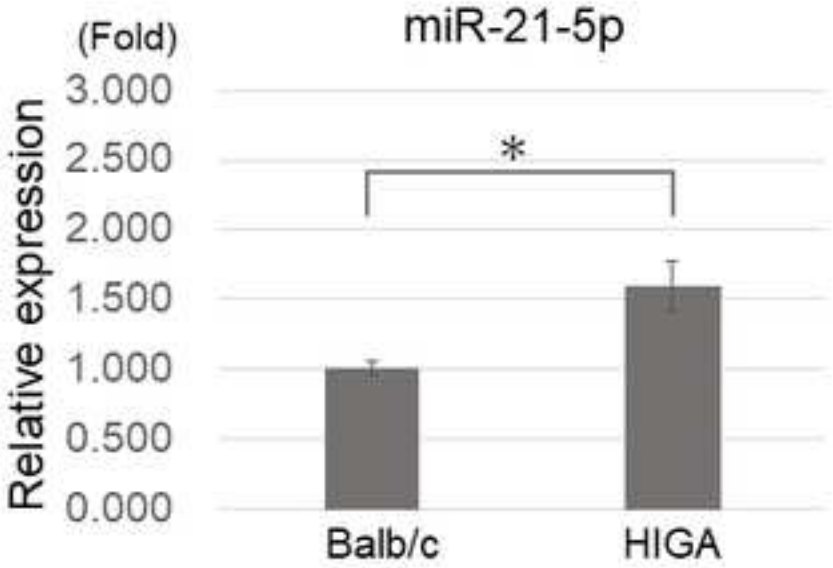
Figure 1







Name of Material/Equipment	Company	Catalog Number
BALB/cCrSlc (25-week-old, female)	Japan SLC, Inc.	none
HIGA/NscSlc (25-week-old, female)	Japan SLC, Inc.	none
MicroAmp Optical 96 well reaction plate for qRT-PCR	Thermo Fisher Scientific	4316813
MicroAmp Optical Adhesive Film	Thermo Fisher Scientific	4311971
miScript II RT kit	Qiagen	218161
miRNeasy Mini kit	Qiagen	217004
miScript Primer Assay (RNU6-2)	Qiagen	MS00033740
miScript Primer Assay (miR-155-5p)	Qiagen	MS00001701
miScript Primer Assay (miR-146a-5p)	Qiagen	MS00001638
miScript Primer Assay (miR-21-5p)	Qiagen	MS00009079
miScript SYBR Green PCR kit	Qiagen	218073
QIA shredder	Qiagen	79654
QuantStudio 12K Flex Flex Real-Time PCR system	Thermo Fisher Scientific	4472380
QuantStudio 12K Flex Software version 1.2.1.	Thermo Fisher Scientific	4472380
takara biomasher standard	Takara Bio	9790B

Comments/Description
Mouse for control
IgA nephropathy mouse model
96-well reaction plate
adhesive film for 96-well reaction plate
Experimental kit for synthesis of cDNA
Experimental kit fot extraction of total RNA
miRNA-specific primer
miRNA-specific primer
miRNA-specific primer
miRNA-specific primer
Experimental kit for real-time PCR
biopolymer-shredding spin column
real-time PCR instrument
real-time PCR instrument software
silicon homogenizer

Response to Editor:

We thank the editor again for the thoughtful comments and suggestions. As outlined below, we have addressed and/or clarified each of the issues raised in a point-by-point manner. The revised components are highlighted in red. We believe that these changes have greatly improved the quality of the manuscript.

1. The editor has formatted the manuscript to match the journal's style. Please retain and use the attached version for revision.

We have confirmed the formatting by the editor.

2. Please address all the specific comments marked in the manuscript.

We have revised the manuscript in accordance with each of the specific comments.

3. Once done, please ensure that the highlight is no more than 2.75 pages including headings and spacings.

We have confirmed that this part does not exceed 2.75 pages.

4-5. Given the current situation with the Covid-19 pandemic restricting filming in many areas of the world, we are allowing authors to move forward with publishing their text manuscript online before filming the video. We will proceed with filming of the video once institutional and laboratory restrictions are lifted. However, if you film yourself, review/peer review would have to occur on the video as well. So, we will have to reopen the review process for the article.

Thank you for your consideration. We would like the text to be published before the video is taken.

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