

# Journal of Visualized Experiments

## Use of autometallography to localize and semi-quantify silver in cetacean tissues

--Manuscript Draft--

<b>Article Type:</b>	Invited Methods Article - JoVE Produced Video
<b>Manuscript Number:</b>	JoVE58232R2
<b>Full Title:</b>	Use of autometallography to localize and semi-quantify silver in cetacean tissues
<b>Keywords:</b>	Autometallography (AMG); cetacean; cetacean histological Ag assay (CHAA); ImageJ; inductively coupled plasma mass spectroscopy (ICP-MS); silver (Ag); quantitative analysis
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<b>Additional Information:</b>	
<b>Question</b>	<b>Response</b>
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US\$2,400)
Please indicate the <b>city, state/province, and country</b> where this article will be <b>filmed</b> . Please do not use abbreviations.	Room 513, Building 3, Department of Veterinary Medicine, National Taiwan University, No. 1, Sec. 4, Roosevelt Rd., Taipei 10617, Taiwan (R.O.C.)

**TITLE:**

Use of Autometallography to Localize and Semi-Quantify Silver in Cetacean Tissues

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**KEYWORDS:**

Autometallography (AMG); Cetacean; Cetacean Histological Ag Assay (CHAA); ImageJ; Inductively Coupled Plasma Mass Spectroscopy (ICP-MS); Silver (Ag); Quantitative Analysis

**SHORT ABSTRACT:**

A protocol is presented to localize Ag in cetacean liver and kidney tissues by autometallography. Furthermore, a new assay, named the cetacean histological Ag assay (CHAA) is developed to estimate the Ag concentrations in those tissues.

**LONG ABSTRACT:**

Silver nanoparticles (AgNPs) have been extensively used in commercial products, including textiles, cosmetics, and health care items, due to their strong antimicrobial effects. They also may be released into the environment and accumulate in the ocean. Therefore, AgNPs are the major source of Ag contamination, and public awareness of the environmental toxicity of Ag is increasing. Previous studies have demonstrated the bioaccumulation (in producers) and magnification (in consumers/predators) of Ag. Cetaceans, as the apex predators of ocean, may have been negatively affected by the Ag/Ag compounds. Although the concentrations of Ag/Ag compounds in cetacean tissues can be measured by inductively coupled plasma mass spectroscopy (ICP-MS), the use of ICP-MS is limited by its high capital cost and the requirement for tissue storage/preparation. Therefore, an autometallography (AMG) method with an image quantitative analysis by using formalin-fixed, paraffin-embedded (FFPE) tissue may be an adjuvant method to localize Ag distribution at the suborgan level and estimate the Ag concentration in cetacean tissues. The AMG positive signals are mainly brown to black granules of various sizes in the cytoplasm of proximal renal tubular epithelium, hepatocytes, and Kupffer cells. Occasionally, some amorphous golden yellow to brown AMG positive signals are noted in the lumen and basement membrane of some proximal renal tubules. The assay for estimating the Ag concentration is named the Cetacean Histological Ag Assay (CHAA), which is a regression model established by the data from image quantitative analysis of the AMG method and ICP-MS. The use of AMG with CHAA to localize and semi-quantify heavy metals provides a convenient methodology for spatio-temporal and cross-species studies.

**INTRODUCTION:**

Silver nanoparticles (AgNPs) have been extensively used in commercial products, including textiles, cosmetics, and health care items, due to their great antimicrobial effects<sup>1,2</sup>. Therefore, the production of AgNPs and the number of AgNP-containing products are increased over time<sup>3,4</sup>. However, AgNPs may be released into the environment and accumulate in the ocean<sup>5,6</sup>. They have become the major source of Ag contamination, and the public awareness of the environmental toxicity of Ag is increasing.

The status of AgNPs and Ag in the marine environment is complicated and constantly changing. Previous studies have indicated that AgNPs can remain as particles, aggregate, dissolve, react with different chemical species, or be regenerated from Ag<sup>+</sup> ions<sup>7,8</sup>. Several types of Ag

compounds, such as AgCl, have been found in marine sediments, where they can be ingested by benthic organisms and enter the food chain<sup>9,10</sup>. According to a previous study conducted in the Chi-ku Lagoon area along the southwestern coast of Taiwan, the Ag concentrations of marine sediments are extremely low and similar to the crustal abundance, and those of fish liver tissue are usually below the detection limit ( $< 0.025 \mu\text{g/g}$  wet/wet)<sup>11</sup>. However, previous studies conducted in different countries have demonstrated relatively high Ag concentrations in the livers of cetaceans<sup>12,13</sup>. The Ag concentration in the livers of cetaceans is age-dependent, suggesting that the source of Ag in their bodies is most likely their prey<sup>12</sup>. These findings further suggest the biomagnification of Ag in animals at higher trophic levels. Cetaceans, as the apex predators in the ocean, may have suffered negative health impacts caused by Ag/Ag compounds<sup>12-14</sup>. Most importantly, like cetaceans, humans are mammals, and the negative health impacts caused by Ag/Ag compounds in cetaceans may also occur in humans. In other words, cetaceans could be sentinel animals for the health of marine environment and humans. Therefore, the health effects, the tissue distribution, and concentration of Ag in cetaceans are of great concern.

Although the concentrations of Ag/Ag compounds in cetacean tissues can be measured by inductively coupled plasma mass spectroscopy (ICP-MS), the use of ICP-MS is limited by its high capital cost (instrument and maintenance) and the requirements for tissue storage/preparation<sup>12,15</sup>. In addition, it is usually difficult to collect comprehensive tissue samples in all investigations of stranded cetacean cases due to logistical difficulties, a shortage of manpower, and a lack of related resources<sup>12</sup>. The frozen tissue samples for ICP-MS analysis are not easily stored because of limited refrigeration space, and frozen tissue samples may be discarded due to broken refrigeration equipment<sup>12</sup>. These aforementioned obstacles hamper investigations of contamination levels in cetacean tissues by ICP-MS analysis using frozen tissue samples. In contrast, formalin fixed tissue samples are relatively easy to collect during the necropsy of dead-stranded cetaceans. Therefore, it is necessary to develop an easy to use and inexpensive method to detect/measure the heavy metals in cetacean tissues by using formalin fixed tissue samples.

Although the suborgan distributions and concentrations of alkali and alkaline earth metals may be altered during the formalin-fixed, paraffin-embedded (FFPE) process, only lesser effects on transition metals, such as Ag, have been noted<sup>16</sup>. Hence, FFPE tissue has been considered as an ideal sample resource for metal localization and measurements<sup>16,17</sup>. Autometallography (AMG), a histochemical process, can amplify heavy metals as variably sized golden yellow to black AMG positive signals on FFPE tissue sections, and these amplified heavy metals can be visualized under light microscopy<sup>18-21</sup>. Hence, the AMG method provides information on the suborgan distributions of heavy metals. It can provide important additional information for studying the metabolic pathways of heavy metals in biological systems because ICP-MS can only measure the concentration of heavy metals at the organ level<sup>18</sup>. Furthermore, digital image analysis software, such as ImageJ, has been applied to the quantitative analysis of histological tissue sections<sup>22,23</sup>. The variably-sized golden yellow to black AMG positive signals of FFPE tissue sections can be quantified and used to estimate the concentrations of heavy metals. Although the absolute Ag concentration cannot be directly determined by the AMG method with image

quantitative analysis, it can be estimated by a regression model based on the data obtained from the image quantitative analysis and ICP-MS, which is named cetacean histological Ag assay (CHAA). Considering the difficulties in measuring Ag concentrations by ICP-MS analysis in most stranded cetaceans, CHAA is a valuable adjuvant method to estimate Ag concentrations in cetacean tissues, which cannot be determined by ICP-MS analysis due to the lack of frozen tissue samples. This paper describes the protocol of a histochemical technique (AMG method) for localizing Ag at the suborgan level and an assay named CHAA to estimate the Ag concentrations in the liver and kidney tissues of cetaceans.

[Place **Figure 1** here]

## **PROTOCOL:**

The study was performed in accordance with international guidelines, and the use of cetacean tissue samples was permitted by the Council of Agriculture of Taiwan (Research Permit 104-07.1-SB-62).

### **1. Tissue Sample Preparation for ICP-MS Analysis**

Note: The liver and kidney tissues were collected from freshly dead and moderately autolyzed stranded cetaceans<sup>24</sup>, including 6 stranded cetaceans of 4 different species, 1 *Grampus griseus* (Gg), 2 *Kogia* spp. (Ko), 2 *Lagenodelphis hosei* (Lh), 1 *Stenella attenuata* (Sa). Each stranded cetacean had a field number for individual identification. The tissue sample preparation for ICP-MS analysis followed the method established in M.H. Chen's lab, and M.H. Chen's lab conducted the ICP-MS analysis<sup>11,13,25</sup>.

1.1. Collect liver and kidney tissues for ICP-MS analysis from stranded cetaceans and store them at -20 °C until use.

1.2. Collect pair-matched liver and kidney tissues from the same stranded cetaceans for AMG analysis (please see step 2).

1.3. Trim the outer layer of the tissue samples collected for ICP-MS analysis with a stainless-steel scalpel. Cut the inner part of the tissue samples into small cubes (about 1 cm<sup>3</sup>) and place them in zip lock plastic bags. Normally, each bag contains 10 g of the tissues.

1.4. Store the plastic bags containing tissue samples at -20 °C for subsequent procedures.

1.5. Put the 1 cm<sup>3</sup> cubes samples in a freeze dry system (-50 °C, Vacuum pump with a displacement of at least 98 L/min, 0.002 mBar) for at least 72 h till completely dried by weighing to the constant.

1.6. Homogenize the dried cubes into powder with a homogenizer for subsequent tissue digestion.

177  
178 1.7. Weigh 0.3 g of homogenized freeze-dried samples in 30 mL polytetrafluoroethylene  
179 (PTFE) bottles and mix them with 10 mL of 65% w/w nitric acid.

180  
181 1.8. Put closures on the PTFE bottles, but leave the closures untightened.

182  
183 Note: This allows the brown fume to form in the PTFE bottles and reflux inside the bottle for  
184 digestion until the brown fume disappears and turns clear.

185  
186 1.9. Heat the digested samples with a hot plate, from 30 °C to 110/120 °C (according to the  
187 brown fume forming condition) in the PTFE bottles for 2 to 3 weeks until the brownish gas in  
188 the PTFE bottles becomes colorless and the liquid in the PTFE bottles becomes translucent  
189 greenish pale yellow or completely clear.

190  
191 Note: Perform the heating process in chemical fume hood.

192  
193 1.10. Heat the digested samples at 120 °C to evaporate the nitric acid in the PTFE bottles until  
194 only 0.5-1 mL remains.

195  
196 Note: Perform the heating process in a chemical fume hood, and always monitor the  
197 temperature increase to ensure that no brownish gas leaks from the PTFE bottles' closures.

198  
199 1.11. Tighten the closures and cool them at room temperature for about 1 h.

200  
201 1.12. Place the funnels with filter papers on 25 mL volumetric flasks and wash the remaining  
202 liquid with 1 M HNO<sub>3</sub> to a final volume of 25 mL.

203  
204 Note: Wash the bottle for at least three times and the closure twice.

205  
206 1.13. Validate the analytical quality of ICP-MS analysis by using the standard reference  
207 materials, including DOLT-2 (dogfish liver) and DORM-2 (dogfish muscle).

208  
209 1.14. Use duplicates of each analytical sample and triplicates of standard reference materials  
210 for ICP-MS analysis.

211  
212 1.15. Average the Ag concentrations of each analytic samples and present the data as dry  
213 weight basis concentration (µg/g dry weight).

## 214 215 **2. Tissue Sample Preparation for AMG Analysis**

216  
217 2.1. Collect pair-matched liver and kidney tissues for AMG analysis from a stranded cetacean  
218 and fix them in 10% neutral buffered formalin until use.

219

Note: Store the tissue samples in plastic bottles in 10% neutral buffered formalin (NBF, pH 7.0) for 24 to 48 hours. The volume of NBF should be at least 10 times greater than the tissue volume.

2.2. Trim the formalin fixed liver and kidney tissues with stainless steel disposable microtome blades and put the trimmed tissue sections in cassettes with labels.

Note: The size of each tissue sections should be approximately 2 cm x 1 cm and the thickness of each tissue section should not exceed 3 mm. Put the liver and kidney tissues from the same individual in the same cassette.

2.3. Dehydrate the trimmed tissue sections with a tissue processor through a series of graded ethanol (70% for 1 h, 80% for 1 h, 95% for 1 h, 95% for 2 h, 100% for 1 h x 2 staining dishes, and 100% for 2 h), non-xylene (for 1 h and 2 h in different staining dishes), and immerse the dehydrated tissue samples in paraffin (for 1 h and 2 h in different staining dishes).

2.4. Place the dehydrated tissue samples in the bottoms of steel histology molds and embed the dehydrated tissue samples with paraffin.

2.5. Chill the formalin fixed paraffin-embedded (FFPE) tissue blocks at  $-20^{\circ}\text{C}$ . Trim the FFPE blocks with the microtome until the tissue surface is exposed.

2.6. Chill the FFPE blocks at  $-20^{\circ}\text{C}$  again. Section the FFPE blocks at  $5\text{ }\mu\text{m}$  by microtome.

2.7. Fill a water bath with double-distilled water at  $45^{\circ}\text{C}$ . Lift the ribbons of tissue sections and make them float on the surface of the warm water by using tweezers and brushes.

2.8. Separate the ribbons of tissue sections with tweezers. Place a section onto a microscope slide.

2.9. Place the microscope slides on a slide warmer and allow sections to dry overnight at  $37^{\circ}\text{C}$ .

2.10. Put the microscope slides in slide racks and deparaffinize them by soaking them in 3 different staining dishes of pure non-xylene (approximately 200 to 250 mL) for 8, 5, and 3 min.

2.11. Hydrate the tissue sections in slide racks by soaking them in different staining dishes of graded ethanol solutions (100% ethanol twice, 90% ethanol once and 80% ethanol once [1 min each]), and rinse them in double-distilled water.

Note: These solutions are approximately 200 to 250 mL in different staining dishes.

2.12. Rinse the tissue sections in phosphate-buffered saline (PBS) with 0.5% Triton X-100, wash them with PBS for several times, and then rinse them in double-distilled water.

Note: These solutions are approximately 200 to 250 mL in different staining dishes.

2.13. Prepare equal amounts of the three components (initiator, moderator, and activator) provided by silver enhancement kit in dark and mix them thoroughly.

Note: The solutions of moderator and activator are sticky, so please use pipette with wide tip openings (or cut the tips to create wider openings). For each slide, 300  $\mu$ L of the mixed solution (depending on the size of the tissue section) is usually enough. Therefore, if 10 slides are used, the amount of each component (initiator, moderator, and activator) is 1000  $\mu$ L (the mixed solution is 3000  $\mu$ L for 10 slides).

2.14. Incubate the tissue sections in the mixed solution for 15 min in the dark at room temperature. Fully cover the tissue sections on the slides with the mixed solution. A longer incubation time may lead to false-positive AMG signals.

2.15. Wash the slides with double-distilled water and stain them in hematoxylin for 10 s as a counterstain.

2.16. Wash the slides with running tap water, dry them, and mount them with mounting medium.

2.17. Examine the slides under a light microscope.

2.18. Randomly capture ten histological images with a 40X objective lens from each tissue section by using a connected digital camera with computer imaging software.

### 3. Semi-Quantitative Analysis for AMG Positive Values of Histological Images

Note: AMG positive value means the percentage of the area with AMG positive signals.

3.1. Use image analysis software (ImageJ) to analyze the histological images.

3.2. Open the histological image by pressing **File | Open**.

3.3. Split the chosen picture into three color channels (red, blue, and green) by pressing **Image | Type | RGB Stack**.

3.4. Quantify the AMG positive signals by using the blue channel. Nuclear false positive signals are usually decreased under the blue channel when hematoxylin stain is applied for nuclear counterstain (**Figure 2**).

3.5. Measure the percentage of the area with AMG positive signals in each histological image with the threshold tool (**Image | Adjust | Threshold**).



3.6. Manually adjust the cut-off value of the threshold for each histological image (from 90 to 110) based on the presences of false positive areas in nuclei and/or red blood cells.

Note: In default setting, the AMG positive signals should be highlighted in red.

3.7. Press **Analyze | Set Measurements**, and check the box of **Area Fraction** to specify that the area fraction is recorded.

3.8. Press **Analyze | Measure**. The positive percent area of each histological image is displayed in the column of **%Area** of the **Result** window.

3.9. Average the positive percent areas of 10 histological images from each tissue section and define the result as the AMG positive value for each tissue section.

[Place **Figure 2** here]

#### **4. Establishment of the Cetacean Histological Ag Assay (CHAA) by Regression Model**

Note: The following analysis is performed in Prism 6.01 for Windows.

4.1. Evaluate the correlation between the results of ICP-MS and AMG positive values.

4.2. Open the software, create a new project file, and choose **XY** and **Correlation**.

4.3. Input data including the results of ICP-MS and AMG positive values.

4.4. Press **Analysis** and choose **Correlation** under the category **XY Analysis** to analyze the strength of association between the results of the ICP-MS and AMG positive values by Pearson correlation analysis.

Note: The results of the ICP-MS and AMG positive values have to be positively correlated with each other; otherwise, the subsequent regression model should not be developed.

4.5. Statistically compare the regression models, including linear regression, quadratic regression, cubic regression, and linear regression through origin, through statistics software<sup>12,26,27</sup>.

Note: If the regression model generates an unrealistic Ag concentration, the regression model should be abandoned<sup>12</sup>.

4.6. Go back to the Data Table (left panel) and press **Analysis | Nonlinear regression (curve fit)** under the category **XY Analysis | OK**.

4.7. In the window **Parameters: Nonlinear regression**, choose different regression model in the page **Fit** and then compare different regression models in the page **Compare**.

4.8. In the page **Compare**, choose the comparison methods, including the extra sum-of-squares F test and Akaike's information criterion (AIC). According to the results of the comparison methods, use a relatively appropriate regression model in the CHAA.

4.9. Estimate Ag concentrations of the cetacean liver and kidney tissues with unknown Ag concentrations by using the CHAA.

4.10. Evaluate the accuracy and precision of the CHAA for liver and kidney tissues. The difference between precision and accuracy is illustrated in **Figure 3**.

4.11. Accuracy: Calculate the mean standard deviation (SD) from differences between known and estimated Ag concentrations.

4.12. Precision: Perform repeated measurement (at least triplicate) of AMG positive values of serial sections from the same FFPE tissues. Calculate the mean SD of measurements from liver or kidney tissues from differences between known and estimated Ag concentrations

Note: The methods of evaluating the accuracy and precision are depicted in **Figure 4**.

[Place Figures 3 and 4 here]

## **5. Estimation of Ag Concentrations by CHAA.**

5.1. Collect the liver and kidney tissues from stranded cetaceans and fix them in 10% neutral buffered formalin.

5.2. Process the formalin-fixed tissues routinely (please see step 2).

5.3. Estimate the Ag concentrations of the cetacean liver and kidney tissues with unknown Ag concentrations by CHAA (please see steps 3 and 4).

## **REPRESENTATIVE RESULTS:**

Representative images of the AMG positive signals in the cetacean liver and kidney tissues are shown in **Figure 5**. The AMG positive signals include variably-sized brown to black granules of various sizes in the cytoplasm of proximal renal tubular epithelium, hepatocytes, and Kupffer cells. Occasionally, amorphous golden yellow to brown AMG positive signals are noted in the lumen and basement membrane of some proximal renal tubules. There is a positive correlation between the results of ICP-MS and AMG positivity values in liver and kidney tissues, and linear regression through origin is preferred according to the extra sum-of-squares F test and AIC<sup>12,26,27</sup>. In the accuracy test, the mean SDs of the CHAA for liver and kidney are 3.24 and 0.16, respectively. In the precision test, the mean SDs of the CHAA for liver and kidney are 2.8 and

0.35, respectively. The raw data of the accuracy and precision tests are summarized in **Table 1**. The AMG positive values, Ag concentrations estimated by CHAA, and Ag concentrations measured by ICP-MS from the liver and kidney tissues of these six stranded cetaceans are summarized in **Table 2**.

#### FIGURE AND TABLE LEGENDS:

**Figure 1: Flowchart depicting the establishment and application of cetacean histological Ag assay (CHAA) for estimating Ag concentrations.** CHAA = cetacean histological Ag assay, FFPE = Formalin-fixed, paraffin-embedded, ICP-MS = inductively coupled plasma mass spectroscopy.

**Figure 2: The presence of nuclear false positive signals under different color channels (counterstain: hematoxylin stain).** Representative nuclear false positive signals are indicated by yellow arrows. PPA = positive percentage of areas.

**Figure 3: The difference between accuracy and precision.** Accuracy means how close the measurement is to the true value (*i.e.*, Ag concentration determined by ICP-MS); precision means the repeatability of the measurement (*i.e.*, the consistency among the repeated measurements of AMG positive values from the triplicate tissue sections).

**Figure 4: The scheme depicting the methods of evaluating the accuracy and precision.** CHAA = cetacean histological Ag assay; FFPE = Formalin-fixed, paraffin-embedded; ICP-MS = inductively coupled plasma mass spectroscopy; Ai = Each of the Ag concentrations determined by ICP-MS of each pair-matched tissue sample; Bi = Each of the Ag concentrations estimated by CHAA of each pair-matched tissue sample; Ci, Di, and Ei = Each of The Ag concentrations estimated by CHAA of triplicate samples from each pair-matched tissue sample; i = 1 to n. Please see raw data of the accuracy and precision tests in the section of representative results.

**Figure 5: Representative histological images of the AMG positive signals in the liver and kidney tissues of cetaceans (counterstain: hematoxylin stain).** (A) The AMG positive signals in cetacean liver tissue are evenly distributed (*Grampus griseus* (Gg); field code: TP20111116; Ag concentration measured by inductively coupled plasma mass spectroscopy (ICP-MS): 21.82 µg/g dry weight). (B) The AMG positive signals are brown to black granules of various sizes in the cytoplasm of hepatocytes (red arrows) and Kupffer cells (red arrow heads) (Gg; field code: TP20111116). (C) A few AMG positive signals of brown to black granules are shown in the cytoplasm of hepatocytes (red arrows) (*Kogia* spp. (Ko); field code: TC20110722; Ag concentration measured by ICP-MS: 3.86 µg/g dry weight). (D) The AMG positive signals in cetacean kidney tissue are mainly located in the renal cortex (Gg; field code: TP20111116; Ag concentration measured by ICP-MS: 0.42 µg/g dry weight). The black dashed line is placed on the junction between the renal cortex and medulla. (E) Higher magnification of **Figure 5D** (red dashed rectangle). The AMG positive signals in the renal cortex are brown to black granules of various sizes in the cytoplasm of the proximal renal tubular epithelium (red arrows). Amorphous golden yellow to brown AMG positive signals are shown in the lumens (red arrow head) and basement membrane (yellow arrow head) of some proximal renal tubules. No to

minimal AMG positive signals are shown in the glomeruli (green arrow) and distal renal tubules (green arrow head)(Gg; field code: TP20111116). (F) Scattered brown granules of various sizes are shown in the cytoplasm of the proximal renal tubular epithelium (red arrows) (Ko; field code: TC20110722; Ag concentration measured by ICP-MS: 0.05 µg/g dry weight).

**Table 1: The representative results of the accuracy and precision tests for cetacean histological Ag assay (CHAA).** CHAA = cetacean histological Ag assay, ICP-MS = inductively coupled plasma mass spectroscopy, SD = standard deviation.

**Table 2: The AMG positive values, Ag concentrations (µg/g, dry weight) estimated by cetacean histological Ag assay (CHAA), and Ag concentrations (µg/g, dry weight) measured by ICP-MS from the liver and kidney tissues of six stranded cetaceans.** Gg = *Grampus griseus*, Ko = *Kogia* spp., Lh = *Lagenodelphis hosei*, Sa = *Stenella attenuata*.

## DISCUSSION:

The purpose of the article study is to establish an adjuvant method to evaluate the Ag distribution at suborgan levels and to estimate Ag concentrations in cetacean tissues. The current protocols include 1) Determination of Ag concentrations in cetacean tissues by ICP-MS, 2) AMG analysis of pair-matched tissue samples with known Ag concentrations, 3) Establishment of the regression model (CHAA) for estimating the Ag concentrations by AMG positive values, 4) Evaluation of the accuracy and precision of CHAA, and 5) Estimation of Ag concentrations by CHAA.

In this study, the data of ICP-MS were significantly and positively correlated with those of AMG positive values, suggesting that the Ag concentration in cetacean tissues can be estimated by the AMG positive value. Therefore, the CHAA, which is based on the AMG positive value and regression model, has been developed for estimating the Ag concentrations in the liver and kidney tissues of cetaceans. Generally, a regression model with more parameters (*i.e.*, a more complex regression model) fits well into the data, but it is undetermined that the more complex one is actually better than the simpler one. Therefore, the best regression model must be chosen by statistical analysis<sup>26,27</sup>. The results of the statistical analysis indicate that the linear regression model is sufficient to estimate the Ag concentration based on the AMG positive value<sup>12</sup>.

In CHAA for kidney tissue, the mean SD (0.35) of the precision test was larger than that of the accuracy test (0.16). Conversely, in CHAA for liver tissue, the mean SD (2.8) of the precision test was smaller than that of the accuracy test (3.24). Based on this result, it is suggested that the uneven distribution of the AMG positive signals and the relatively low Ag concentrations in cetacean kidney tissue interfere negatively with the precision of CHAA for kidney tissue. Therefore, the CHAA for kidney tissue may be accurate but imprecise. However, the even distribution of the AMG positive signals and the relatively high Ag concentrations in cetacean liver tissues suggest that the CHAA for liver tissue is a reliable method to estimate the Ag concentrations in cetacean liver tissues. Furthermore, if more tissues with known Ag

concentrations determined by ICP-MS are available, a more accurate and precise regression model can be developed to estimate the Ag concentration.

Although the current protocols provide an adjuvant method to investigate Ag in animal tissues, some limitations on the AMG method should be noted. First, false-positive AMG signals may present due to interference from other heavy metals, such as mercury, bismuth and zinc<sup>28</sup>. Therefore, the results of the AMG method have to be interpreted with other specific methods, such as ICP-MS, to monitor the actual composition of heavy metals<sup>28</sup>. Second, it is difficult to detect a homogenously distributed heavy metal because it may generate brighter amorphous AMG positive signals, which may not be identified by visualization under microscopic examination. Furthermore, the amorphous and brighter AMG positive signals are difficult to analyze with image analysis software because the color of the AMG positive signals may be similar to that of the background (*e.g.*, the amorphous AMG positive signals found in the lumen of proximal renal tubules). Therefore, the AMG positive signals cannot be highlighted after the adjustment of the cut-off value of the threshold in the image analysis software. Third, because the AMG positive values are based on the percentage of the area of AMG positive signals, it is possible that the values of highly concentrated heavy metals may be underestimated.

FFPE samples are relatively easy to collect and store, and our previous study has demonstrated that the current AMG method can successfully amplify FFPE samples stored for over 15 years<sup>12</sup>. The mechanism of AMG is not affected by different animal species, for it has been widely used in various animal species<sup>20,29-31</sup>. Although the current article is focused on the cetaceans, the protocols described here may also be used in different animal species. In addition, the cost of the AMG method with ICP-MS is relatively low (as compared to laser ablation-ICP-MS), and thus the current protocols are valuable for researchers or countries lacking sufficient research funding to investigate the distribution and concentration of heavy metals in animal tissues. In conclusion, the use of AMG with quantitative analysis to localize and semi-quantify heavy metals provides a convenient methodology for spatio-temporal and cross-species studies.

#### **ACKNOWLEDGMENTS:**

We thank the Taiwan Cetacean Stranding Network for sample collection and storage, including the Taiwan Cetacean Society, Taipei; the Cetacean Research Laboratory (Prof. Lien-Siang Chou), the Institute of Ecology and Evolutionary Biology, National Taiwan University, Taipei; the National Museum of Natural Science (Dr. Chiou-Ju Yao), Taichung; and the Marine Biology & Cetacean Research Center, National Cheng-Kung University. We also thank the Forestry Bureau, Council of Agriculture, Executive Yuan for their permit.

#### **DISCLOSURES:**

The authors have nothing to disclose.

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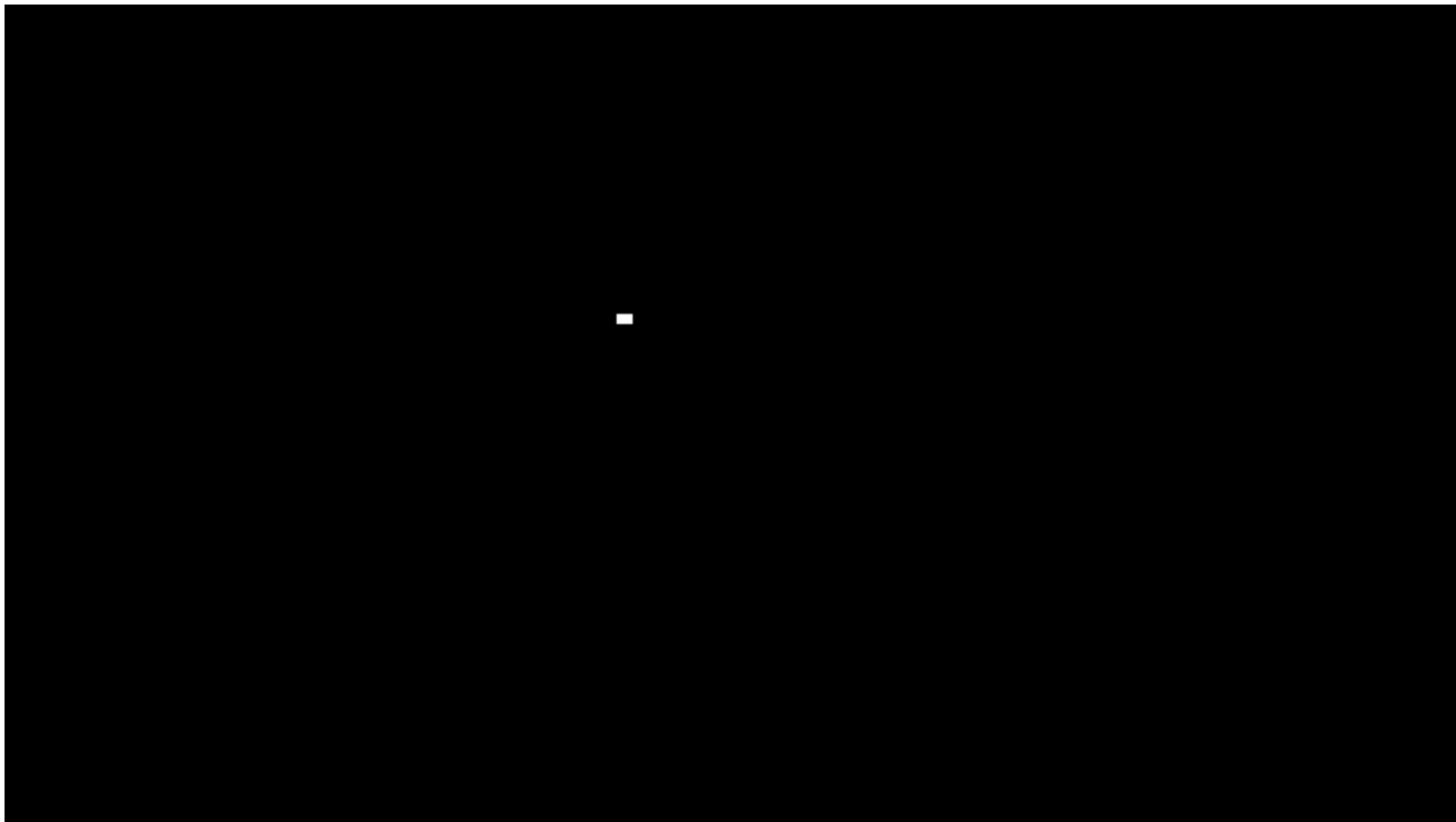
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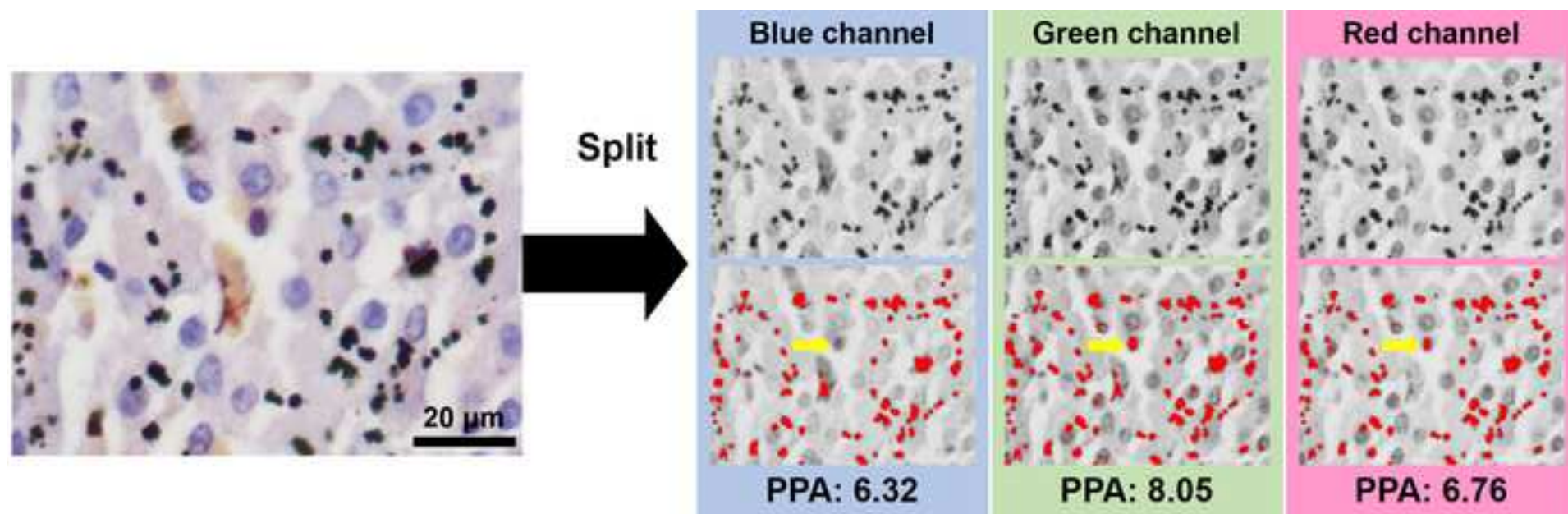
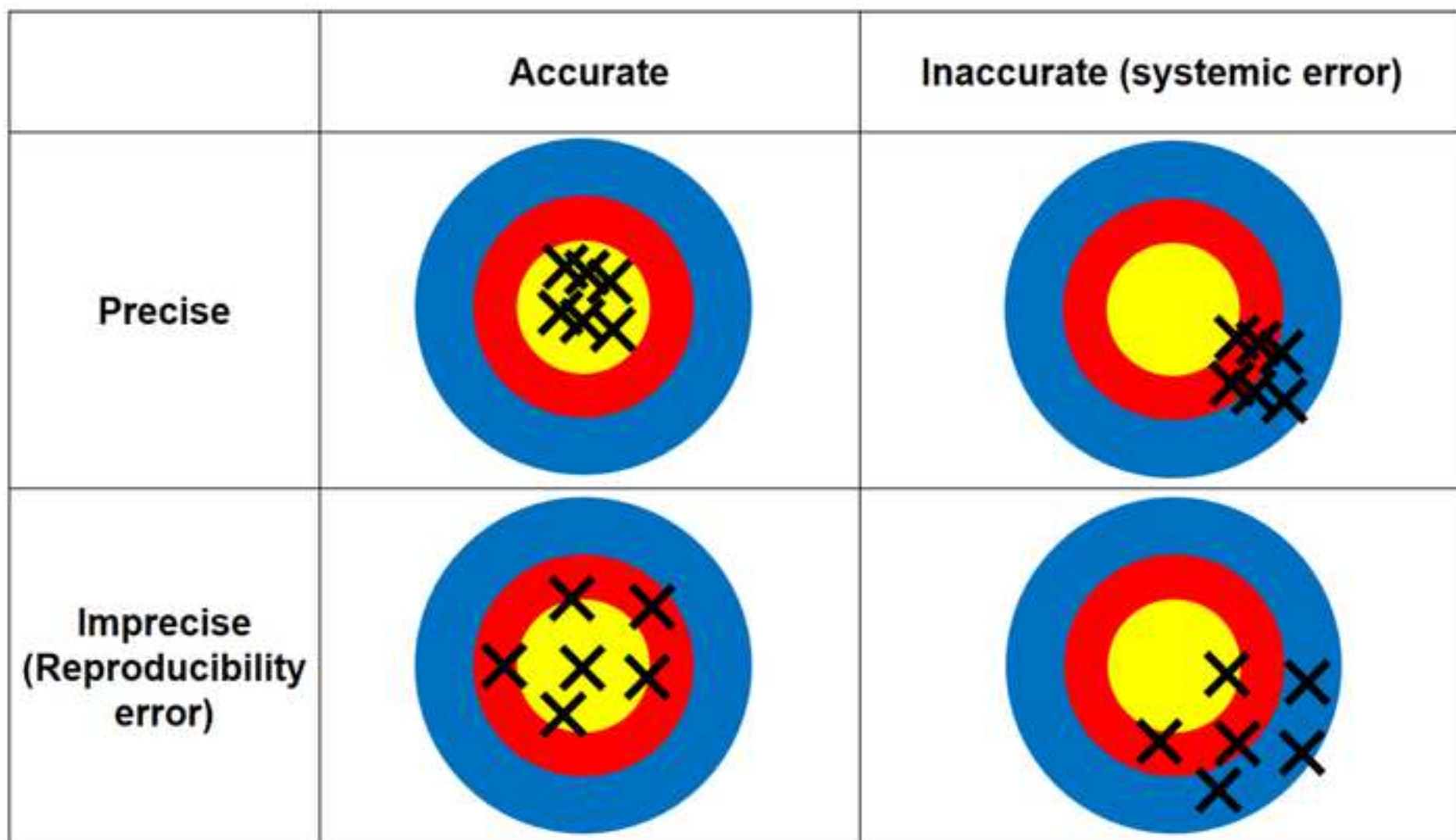
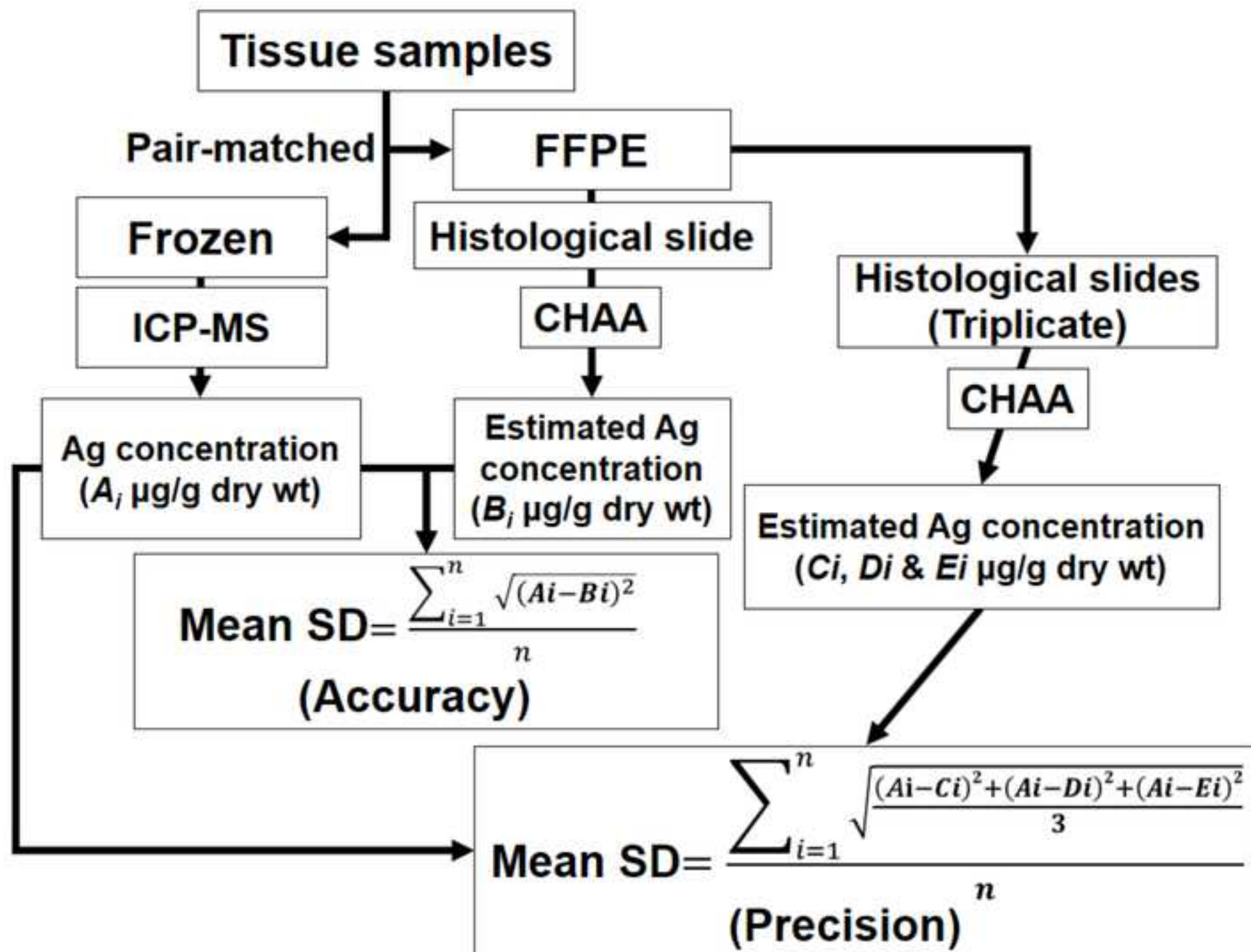
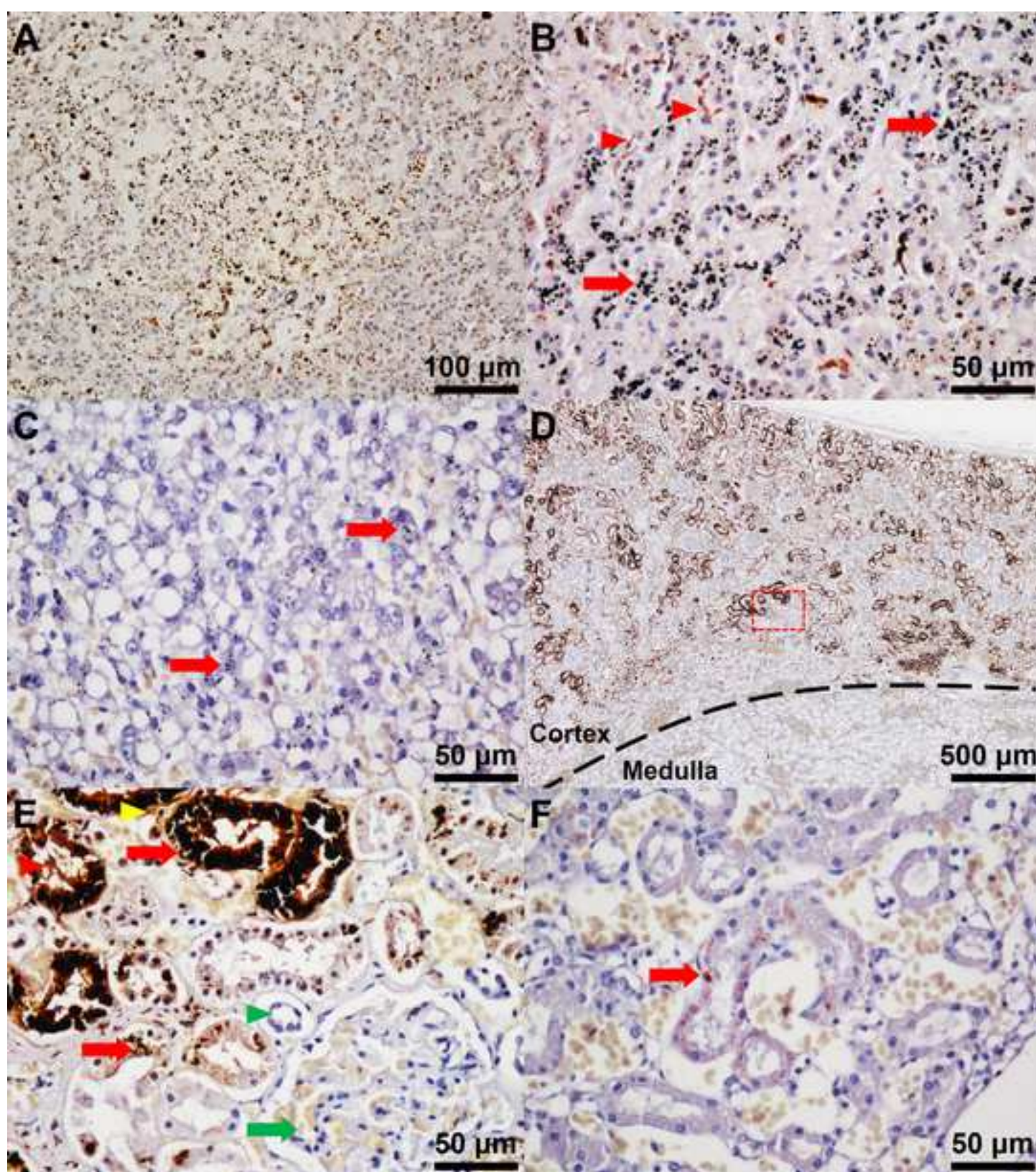


Figure 3









Accuracy test						
Field number	Liver			Kidney		
	CHAA*	ICP-MS	SD	CHAA*	ICP-MS	SD
TP20111116	16.82	21.82	4.99	0.64	0.42	0.22
TC20110611	10.12	2.77	0.96	0.11	0.05	0.35
TC20110722	2.70	3.86	1.15	0.01	0.05	0.04
TD20110608	0.76	0.06	7.35	0.02	0.05	0.06
TP20110830	13.97	14.93	4.28	0.69	1.04	0.24
IL20110101	6.00	1.73	0.72	0.38	0.14	0.03
	Mean SD		3.24	Mean SD		0.16
Precision test						
Field number	Liver			Kidney		
	CHAA*	ICP-MS	SD	CHAA*	ICP-MS	SD
TP20111116	20.90			0.21		
	16.11	21.82	4.08	0.22	0.42	0.44
	17.75			0.14		
TD20110608	1.52			0.00		
	2.40	0.06	1.71	0.00	0.05	0.02
	1.12			0.00		
TP20110830	13.12			0.45		
	12.50	14.93	2.70	0.26	1.04	0.59
	11.35			0.33		
	Mean SD		2.83	Mean SD		0.35

\*The regression equations of the CHAA for livers and kidneys were respectively  $Y = 2.249 \times X$  (adjusted  $R^2 = 0.74$ ) and  $Y = 0.07288 \times X$  (adjusted  $R^2 = 0.69$ ).

Field number	Species	Liver			
		AMG	CHAA*	ICP-MS	AMG
TP20111116	Gg	7.48	16.82	21.82	8.82
TC20110611	Ko	4.50	10.12	2.77	1.52
TC20110722	Ko	1.20	2.70	3.86	0.11
TD20110608	Lh	0.34	0.76	0.06	0.21
TP20110830	Lh	6.21	13.97	14.93	9.43
IL20110101	Sa	2.67	6.00	1.73	5.26

\*The regression equations of the CHAA for livers and kidneys were respectively  $Y = 0.74X$  and  $Y = 0.07288 \times X$  (adjusted  $R^2 = 0.69$ ).

Kidney

CHAA\*

ICP-MS

0.64

0.42

0.11

0.05

0.01

0.05

0.02

0.05

0.69

1.04

0.38

0.14

= 2.249 × X (adjusted R2

Field number	Species	Liver			Kidney		
		AMG	CHAA*	ICP-MS	AMG	CHAA*	ICP-MS
TP20111116	Gg	7.48	16.82	21.82	8.82	0.64	0.42
TC20110611	Ko	4.50	10.12	2.77	1.52	0.11	0.05
TC20110722	Ko	1.20	2.70	3.86	0.11	0.01	0.05
TD20110608	Lh	0.34	0.76	0.06	0.21	0.02	0.05
TP20110830	Lh	6.21	13.97	14.93	9.43	0.69	1.04
IL20110101	Sa	2.67	6.00	1.73	5.26	0.38	0.14

\*The regression equations of the CHAA for livers and kidneys were respectively  $Y = 2.249 \times X$  (adjusted  $R^2 = 0.74$ ) and  $Y = 0.07288 \times X$  (adjusted  $R^2 = 0.69$ ).



<b>Name of Material/ Equipment</b>	<b>Company</b>	<b>Catalog Number</b>
HQ Silver enhancement kit	Nanoprobes	#2012
Surgipath Paraplast	Leica Biosystems	39601006
100% Ethanol	Muto Pure Chemical Co., Ltd	4026
Non-Xylene	Muto Pure Chemical Co., Ltd	4328
Silane coated slide	Muto Pure Chemical Co., Ltd	511614
Cover glass (25 x 50 mm)	Muto Pure Chemical Co., Ltd	24501
Malinol	Muto Pure Chemical Co., Ltd	20092
GM Haematoxylin Staining	Muto Pure Chemical Co., Ltd	3008-1
10% neutral buffered formalin solution	Chin I Pao Co., Ltd	---
Tip (1000 µL)	MDBio, Inc.	1000
PIPETMAN Classic P1000	Gilson, Inc.	F123602
15 ml Centrifuge Tube	GeneDireX, Inc.	PC115-0500
Dogfish liver	National Research Council of Canada	DOLT-2
Dogfish muscle	National Research Council of Canada	DORM-2
Inductively coupled plasma mass spectrometry (ICP-MS)	PerkinElmer Inc.	PE-SCIEX ELAN 6100 DRC
FreeZone 6 liter freeze dry system	Labconco	7752030
BRAND® SILBERBRAND volumetric flask	Merck	Z326283
30 mL standard vial, flat interior with 33 mm closure	Savillex Corporation	200-030-12
Nitric acid, superpur®, 65.0%	Merck	1.00441
Hot Plate/Stirrers	Corning®	PC-220
High Shear lab mixer	Silverson	SL2T
Sterile polypropylene sample jar (250mL)	Thermo Scientific™	6186L05
Digital camera	Nikon Corporation	DS-Fi2
Light microscope	Nikon Corporation	ECLIPSE Ni-U
Shandon™ Finesse™ 325 manual microtome	Thermo Scientific™	A78100001H
Accu-Cut® SRM™ 200 rotary microtome	Sakura	1429
Microtome blade S35	FEATHER®	207500000
Slide staining dish and cover	Brain Research Laboratories	#3215
Steel staining rack	Brain Research Laboratories	#3003
Shandon embedding center	Thermo Scientific™	S-EC
Shandon Citadel® tissue processor	Thermo Scientific™	69800003

Slide warmer	Lab-Line Instruments	26005
Water bath	Shandon Capshaw	3964
Filter paper	Merck	1541-070
Prism 6.01 for windows	GraphPad Software	
ImageJ	National Institutes of Health	
Stainless steel tissue embedding mould	Shenyang Roundfin Trade Co., Ltd	RD-TBM003

**Comments/Description**

Paraffin

For freeze drying

For diagection

For diagection

For diagection

For homogenization

For homogenization

Statistic software

For paraffin emedding



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Author(s):

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**Dear Dr. Alisha DSouza and reviewers,**

The attached files are revised manuscript with track changes (**58232-R2**) and response letter (**Response letter**). In the revised manuscript, we have made the following changes according to the editorial comments.

### **Responses to editorial comments**

1. *The language in the manuscript it not publication grade. There are missing articles and many awkward sentences throughout the manuscript. Also, there are verb tense issues as well as missing punctuation.*

**Response:** The language used in this manuscript has been edited by Mr. John Ring, who is a native speaker of English and a language teacher on our campus with prolonged experience in editing scientific papers.

2. *Please highlight up to 2.75 pages of the protocol to be featured in the protocol section of the video. Please ensure that the highlighted steps represent a coherent protocol or story.*

**Response:** The protocol section has been highlighted. The procedure of ICP-MS is performed by Dr. Chen's Lab, and thus the protocol is not highlighted.

### **Responses to Additional comments**

1. *Line 152: This is repeated in the Discussion. It can be deleted here.*

**Response:** This paragraph has been deleted.

2. *Line 181: What settings are used?*

**Response:** The information about the settings has been added (-50°C, Vacuum pump with a displacement of at least 98 L/min, 0.002 mBar)

3. *Line 183: Homogenize how? Mortar and pestle?*

**Response:** Homogenize the dried cubes into powder by homogenizer (Silverson High Shear lab mixer SL2T).

4. *Line 188-191: This is unclear. Please revise.*



**Response:** It has been rewritten as Put closures on the PTFE bottles, but leave the closures untightened. Note: it allows the brown fume to be formed in the PTFE bottles and reflux inside the bottle for digestion till the brown fume disappear and turn clear.

5. *Line 201: Until RT? How long does it take?*

**Response: About one hour.**

6. *Line 203-204: 25 mL final volume?*

**Response: Yes, it is final volume.**

7. *Line 225-226: Trim to what size? How many tissue sections are being worked with?*

**Response:** The information on the size has been added: The size of each tissue sections should be approximately 2 x 1 cm and the thickness of each tissue section should not exceed 3 mm. Put the liver and kidney tissues from the same individual in the same cassette.

8. *Line 234-235: Immerse*

**Response:** Yes, immerse is a better term. We have modified the sentence as “Dehydrate the trimmed tissue sections with a tissue processor through a series of graded ethanol (70% for 1 h, 80% for 1 h, 95% for 1 h, 95% for 2 h, 100% for 1 h x 2 staining dishes, and 100% for 2 h), non-xylene (for 1 h and 2 h in different staining dishes), and immerse the dehydrated tissue samples in paraffin (for 1 h and 2 h in different staining dishes).”

9. *Line 245-246: Onto a microscope slide?*

**Response: Yes, onto a microscope slide.**

10. *Line 251-252: What concentrations?*

**Response: 100% (Pure).**

11. *Line 274: At RT?*

**Response: Yes.**

12. *Line 525-602: Please spell out journal names.*

**Response:** We have spelt out journal names.

We thank you and the reviewers very much for providing us so many valuable suggestions. We hope the changes that we have made in the revision meet the reviewer's and your requests. We are looking forward to having our revised manuscript published in your distinguished journal "*JoVE*". In addition, we need to update the new grant number in this manuscript, and the number is usually given to us in early August. We hope that we can update the new grant number during or after video production.

Sincerely yours,

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