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Biopsy with thermally-responsive untethered microtools

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Abstract

Thermally activated, untethered -grippers can reach narrow conduits in the body and be used to excise tissue for diagnostic analyses. As depicted in the figure, we show the feasibility of an *in vivo* biopsy of the porcine bile duct using untethered -grippers.

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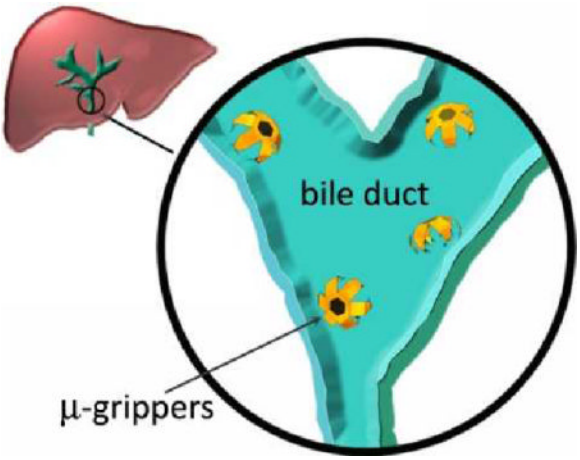
(Supporting Information is available online from Wiley InterScience or from the author)



Keywords

actuators; stimuli responsive materials; robotics; self-assembly; self-folding; microsurgery

With the development of minimally invasive techniques, there has been a push towards miniaturizing tools[1–4] to enable surgeries through natural orifices and with small incisions.[5–7] However, a dominant feature of present-day minimally invasive surgical tools is that the energy and signals required to operate them are transmitted through wires or tethers which connect them to controls located on the outside of the body. These tethers restrict the maneuverability and consequently access to hard to reach places in the body. The need to access miniature conduits in the body is no more evident than in a widely utilized surgical procedure, namely a biopsy. Surgical biopsies involve the retrieval of tissues or cells for histological, cytological or genetic examination and are the gold standard in establishing an accurate diagnosis for a wide range of diseases ranging from cancer to Alzheimer's disease[8]. Further, biopsies are required in a very wide range of organs including the liver, breast, lung, skin and there are a number of devices ranging from biopsy forceps to needle aspirators that are used to perform biopsies.

Since Feynman's seminal lecture[9] a number of researchers have suggested that advances in materials science as well as micro and nanotechnology could enable the creation of smart and integrated miniaturized devices that would open up new capabilities for medicine.[10–18] Indeed, miniature medical devices ranging from centimeter scale pill-sized cameras[19] to nanoparticles[20,21] and nanoprobes[22] have been developed and applied as imaging and measurement modalities for diagnostics. In addition to such wireless devices which do not contain any moving parts, untethered surgical robotic devices with moving parts have also been developed, including those with grasping manipulators[23–25] or inchworm-like robots.[26,27] Although these robotic manipulators have been applied *in vivo*, they have relatively large sizes, on the order of centimeters. One of the reasons for the relatively large size of these devices is the need to integrate commercial batteries to power the electromechanical actuators that facilitate motion in these devices. Wireless energy coupling strategies are also being pursued for medical devices but there is a limited wavelength range for transmission into the body and the antenna size ultimately limits miniaturization.[28–31] Hence, an important challenge that needs to be overcome to achieve the vision of micro and nanomedicine is the development of strategies to enable functional motion at small size scales and under *in vivo* conditions. For biopsy applications, since the sizes of cells are in the range of 10–100 microns, ideal sizes would be in the range of 10 microns to 1 mm.

An attractive strategy to enable motion at such smaller size scales is to utilize residual stress, that is generated using deposition of thin films.[32–37] Further, the incorporation of an appropriately stiff polymeric trigger atop differentially stressed bilayers can arrest their spontaneous curving and render it stimuli responsive. [38] Then, by the appropriate choice of polymer, tools can be created that are responsive to a wide range of stimuli including temperature, biochemicals and even specific enzymes.[38,39]

In this study, we describe the use of microgrippers (μgrippers) with much smaller sizes as compared to previously utilized biopsy forceps or wireless robotic grasping devices. They derive mechanical energy from residual stress powered microactuators and respond to thermal environmental cues. In our previous studies, we demonstrated the feasibility of similar devices for grasping and retrieving cells from pieces of tissue placed in glass capillaries or acrylic organ models, under static fluid conditions,[38,39] but their utilization in real organs and especially under *in vivo* conditions remained unclear. Here, we show for the first time that μgrippers can excise tissue samples from real organs and hard-to-reach places within a live animal. This capability is enabled by the small size, ease of parallel fabrication, and tether-free actuation, incorporation of stimuli-responsive reconfigurable modalities and ferromagnetic nature of the μgrippers. We demonstrate these attributes in gastroenterological procedures in *ex vivo* tissue excision of a porcine liver and *in vivo* tissue retrieval from a porcine biliary tree. We show that it is possible to extract both intact cells as well as high quality RNA and DNA from the retrieved tissue which form the basis for cytologic and molecular biology analyses to establish diagnoses of cancer, inflammatory or other conditions.[40–42]

We designed the μgrippers as star-shaped devices resembling biological appendages, such as hands, and fabricated them using conventional multilayer microfabrication (Figure 1a–f) with sizes (tip-to-tip) ranging from 300 μm to 1.5 mm. The μgrippers contain residual stress powered actuators engineered within chromium and gold bilayer hinges. The rigid regions of the μgrippers are composed of nickel, a ferromagnetic material; hence they can be guided by an applied magnetic field.[43] The residual stress in the hinges was kept constrained by a polymer trigger layer so that the μgrippers remained flat until the polymer was softened. The wafer-scale fabrication process is cost-effective and scalable, and both fabrication and actuation can be achieved in a highly parallel manner. For the tissue excision experiments in this paper, we utilized μgrippers with sizes slightly less than 1mm, so that they were small enough to pass through endoscopic catheters but large enough to be characterized using conventional imaging methods. It is noteworthy that their size is ten to hundred times smaller than conventional biopsy forceps and robotic grasping manipulators. To enable feasibility with practical endoscopic procedures, the μgrippers were specifically designed to stay open at cold temperatures (Figure 1g), and close within 10 minutes when exposed to body temperature (Figure 1h–j), by the appropriate choice and thickness of the polymeric trigger (See supplementary note on choice of the polymer trigger). The actuation time of 10 minutes was chosen so that there was enough time for the μgrippers to reach the region of interest and also to avoid unnecessary delays during the procedures.

We, first, proved the feasibility of tissue excision from real organs by utilizing μgrippers in *ex vivo* studies, using porcine liver. We kept the liver in water bath to represent the body conditions and we randomly distributed the μgrippers using a pipette over the bile duct opening (Figure 2a, b). After 10 minutes, we collected the closed μgrippers using a magnetic catheter (Figure 2c). The tissue obtained by the μgrippers was visible under optical microscopes (Figure 2d). Our experiments prove that the μgrippers can respond selectively to thermal cues and additionally be used to excise tissue samples without any external controls, wires or tethers.

It is noteworthy that there is a significant leap between the operation of such tools under *ex vivo* versus *in vivo* conditions due to, (a) significant agitation within the animal due to muscular movements and fluid flow, (b) inability to fully visualize the procedure within the animal, and (c) the constraint of utilizing already existing minimally invasive technologies to deploy and retrieve the tools. As a proof-of-feasibility, we utilized the μgrippers to biopsy tissue within the biliary tree, an example of a hard-to-reach area in the human body. We performed *in vivo* endoscopic retrograde cholangiopancreatography (ERCP) in a porcine model. The porcine model was chosen since its gastrointestinal anatomy (Figure 3a) is similar to humans.[44] We inserted a standard adult ERCP endoscope through the mouth of the animal and advanced it to the duodenum. The porcine biliary orifice was identified and cannulated using a standard ERCP catheter preloaded with a guidewire under fluoroscopic guidance (Figure 3b). With the catheter in the common bile duct, we injected up to 1560 μgrippers through the catheter and visualized them as they entered into the biliary orifice via the endoscopic camera (Figure 3c, Supporting Video S1). We kept the deployment catheter at the biliary orifice for 10 minutes to allow the μgrippers to close in response to body temperature. Subsequently, we replaced the deployment catheter with the retrieval catheter featuring a magnetic tip. Figure 3d shows an endoscopic image of retrieved μgrippers on the magnetic tip while the catheter is exiting the biliary orifice (Supporting Video S2). Since there was no endoscopic image guidance after the biliary orifice, the movement of the magnetic catheter in the bile duct was observed fluoroscopically (Figure 3e–g).

Since the μgrippers are much smaller than conventional forceps, further characterization was necessary to address concerns regarding the quality and quantity of the retrieved tissue by them. We collected the retrieved μgrippers (Figure 4a) and separated them from the magnet by scraping them off. Then, we separated the tissue pieces from the μgrippers and stained them to be able to visualize them optically. Figure 4b and 4c show examples of fluorescently stained tissue samples retrieved in the procedure. In addition, microtome sections of fixed tissue samples treated with the hematoxylin and eosin (H&E) stain (Figure 4c) shows individual cells with dark purple nuclei demonstrating that cytological tissue analysis is possible.

In terms of the volume of tissue retrieved, our experiments suggest considerable variation depending on the number of μgrippers utilized and the specific details of how the μgrippers are deployed and retrieved. While the larger clusters could be analyzed using cytological analysis as discussed above, genetic or epigenetic diagnoses would be required for smaller samples. To demonstrate such a genetic diagnostic approach, we extracted deoxyribonucleic acid (DNA) from the retrieved tissue for gene identification. We employed two DNA primers designed for pig DNA.[45] Figure 4d demonstrates that we were able to amplify these genes and that the amplified DNA had the expected size. Hence, the tissue retrieved by the μgrippers is of sufficient quality and quantity to allow DNA extraction, as well as polymerase chain reaction (PCR) amplification in an effort to look for previously identified disease-diagnostic markers.

In summary, we have demonstrated how residual stress powered microactuators and origami inspired self-folding principles can be utilized to create microtools of relevance to minimally invasive surgery. It is worth mentioning that although the bilayer hinges are very thin (thickness ~ 160nm), the integrated μgrippers were strong enough to excise tissue even under *in vivo* conditions. While we have shown that untethered μgrippers provide attractive traits in terms of small sizes, parallel deployment and better accessibility, there is a concern that some devices may be left behind after the surgical procedure. We note that our results in the biliary tree are intended as a proof-of-concept that untethered μgrippers can be indeed used to retrieve tissue from a hard to reach place in the body. Nevertheless, in order to

characterize the number of μ-grippers that were left behind, we euthanized the animals once ERCP procedures were completed and then removed their hepatobiliary tree for direct visual and MRI inspection. The μ-grippers can be readily identified on MRI due to their metallic properties. Based on the MRI results, we calculated the average retrieval rate of the μ-grippers as 95%.

Despite this high retrieval rate, further improvements in guidance and retrieval methods are needed to ensure that all of the μ-grippers are recovered, thereby completely eliminating any risk in such procedures. For example, we envision that three dimensional magentic manipulation systems including MRI based guidance methods[46–48] could be utilized to steer the ferromagnetic μ-grippers. The use of biodegradable materials in the construction of such tools could also alleviate these concerns. It is noteworthy that our fabrication approach utilizes conventional planar lithographic patterning methods that are well-developed, versatile, precise and applicable across a range of length scales and with a variety of materials. Hence, it is conceivable that smaller devices could also be constructed, which would allow them to be utilized in even smaller conduits in the body. Further, this surgical concept could also be used with other materials, such as shape memory alloys[49] or polymers[50] and alternate device designs for enabling more complex surgical tasks, such as cutting or stapling.

Experimental

Fabrication of the μ-grippers

The μ-grippers were fabricated on silicon (Si) wafer substrates using conventional multilayer photolithography, thin film deposition and etching techniques. Approximately 750 planar μ-gripper templates (size of the open μ-gripper ~980 μm tip-to-tip) could be fabricated simultaneously on a three inch wafer substrate. The smallest feature size patterned, i.e. the gap between each μ-gripper appendage, was approximately 50 μm. To create the μ-gripper patterns, we designed photomasks using AutoCAD (Autodesk, Inc., San Rafael, CA) software, which were converted into commercial transparent masks (Fineline Imaging, Colorado Springs, CO).

Details of the methods used to fabricate such μ-grippers have been described elsewhere [51] with some differences. For the μ-grippers used in this study, we used 60nm-thick stressed Cr film, and 100nm of gold (Au). The rigid segments of the μ-grippers were composed of 8 μm of Ni and 1 μm of Au; both were deposited by electrodeposition using commercial electroplating solutions (Technic Inc, Cranston, RI). The trigger layer of the μ-grippers was composed of a mixture (5:1 (v/v)) of SC1805 and SC1813 (MicroChem Corp., Newton, MA), specifically selected to enable the required temperature and temporal response. After the μ-grippers were released from the substrate, they were rinsed several times in DI water and stored in an ice bath until their deployment.

In vivo ERCP

ERCP was performed with a standard side-viewing duodenoscope (PENTAX, Tokyo, Japan). The porcine biliary orifice was identified and cannulated using a standard ERCP catheter preloaded with a guidewire (Jagwire, Microvasive Endoscopy, Boston Scientific, Natick, MA) under fluoroscopic guidance. Using a catheter, we deployed the μ-grippers in an infusion into the bile duct while monitoring with the endoscopic camera until they reached the porcine biliary orifice (Supporting Video S1). After the μ-grippers exited the field of vision, we slowly flushed the catheter with 3mL of water to push the μ-grippers further into the common hepatic duct and allowed the μ-grippers to close to obtain tissue samples from the bile duct. In every deployment, we deployed approximately 150 μ-

grippers. For each animal, we repeated the deployment and retrieval sequence at least three times.

After the procedures, the animals were euthanized using Pentobarbital (100 mg/kg) IV followed by a (20cc) potassium chloride injection until no heart rate was recorded. Following euthanasia, we extracted the hepatobiliary system in its entirety during the necropsy examination. Experimental protocols were approved by the Johns Hopkins University IACUC and meet guidelines of the National Institutes of Health guide for the Care and Use of Laboratory Animals.

Retrieval procedure for the μ-grippers

We used a 1/16 inch diameter, grade N52, Neodymium magnet (K&J Magnetics Inc, Jamison, PA) to build a magnetic catheter for retrieval of the μgrippers with the help of endoscopic visualization (Supporting Video S2). The retrieval procedure was repeated with fresh magnetic tips, until the catheter returned with no retrieved μgrippers.

Characterization of the fraction of μ-grippers retrieved in the in vivo ERCP procedure

After the ERCP, we removed and scanned the hepatobiliary system of the animals with a 3T MRI scanner (MAGNETOM Trio, Siemens Med. Soln., Malvern, PA). A control vial with the μgrippers was also scanned together with the hepatobiliary system. Due to their metallic content, the μgrippers can easily be detected in an MRI because the distortion they cause is significantly larger than their physical size, Supporting Figure S1.

The number of μgrippers left inside the liver was determined from MR images. We calculated the percent retrieval by $\frac{N_d - N_l}{N_d} \times 100$ where N_d is the number of deployed μgrippers and N_l is the number of μgrippers left inside the liver, Supporting Table S1. For Animal 1, we used $\frac{N_r}{N_r - N_l} \times 100$ to calculate the percent retrieval, where N_r is the number of retrieved μgrippers. The average percent retrieval for all three animal experiments was, then, calculated as 97%.

Tissue analysis

To obtain Figure 4b, we stained a group of retrieved μgrippers with trypan blue (Sigma-Aldrich, St. Louis, MO) to image the cellular material under the optical microscope.

To obtain Figure 4c, the retrieved tissue was fixed with 10% formalin (Sigma-Aldrich, Saint Luis, MO), embedded in paraffin and then sectioned 2–3 μm thick films. We placed the sections on glass slides and stained with hematoxylin and eosin (H&E) (Sigma-Aldrich, Saint Luis, MO) as per a standard protocol [52].

DNA Extraction and PCR

We separated a group of retrieved μgrippers specifically for the genetic analysis. DNA was extracted with the Viagen solution (Catalog number 102-T) (Viagen Biotech, Los Angeles, CA), following the manufacturer's protocol. The concentration of DNA was measured, on average, to be 134 ng/μL. The primers used for PCR were as follows:

MUC4: FW-CAGGATGCCCAATGGCTCTAC, RV–CCCCGAAGTTGTGAAAGGAAG,

KLRN: FW -CTGTGTTTCTAAGACTTGACTG, RV – TGTTCAAGAAGAGCATCAAG.

The PCR reactions were performed using the HotStarTaq DNA Polymerase from QIAGEN (Catalog number: 203203) (Valencia, CA). The amplification was performed at 95°C for 15

min (initial denaturation) and at 95°C for 30 s (denaturation) for each pair of primers. The conditions for extension for all primers were 70°C for 30 s and 70°C for 10 min (final extension). For each pair of primers, 35 cycles of amplification were carried out.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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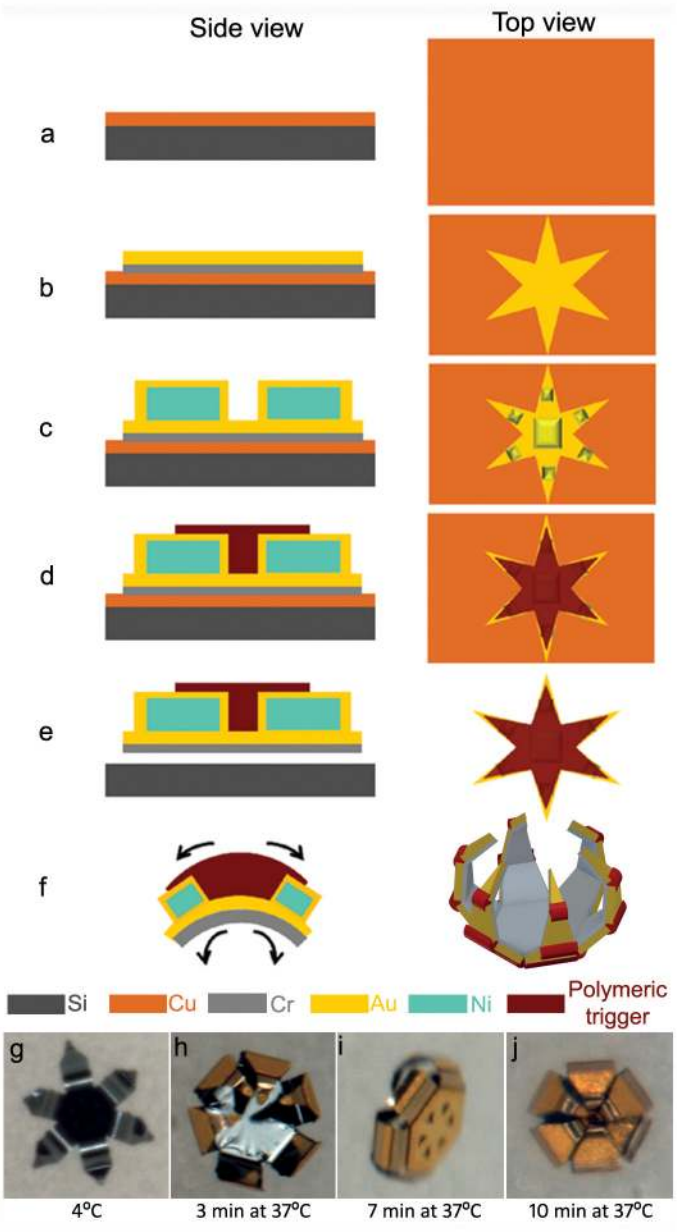

Figure 1. Untethered, thermo-sensitive and residual stress powered grippers

(**a–f**) The fabrication and actuation schematic of grippers. **(a)** A thin Cu layer was used as the sacrificial layer. (**b**) The pre-stressed Cr-Au bilayer was patterned. (**c**) Ferromagnetic Ni was electroplated as the rigid segments between the hinges and then covered with Au. (**d**) The thermo-sensitive polymeric trigger was patterned. (**e**) The grippers were released from the substrate by dissolving the sacrificial layer. (**f**) The grippers closed when exposed to the body temperature. **(g–j)** Bright field microscopy sequence showing thermal actuation of the grippers at 37 °C within 10 minutes.

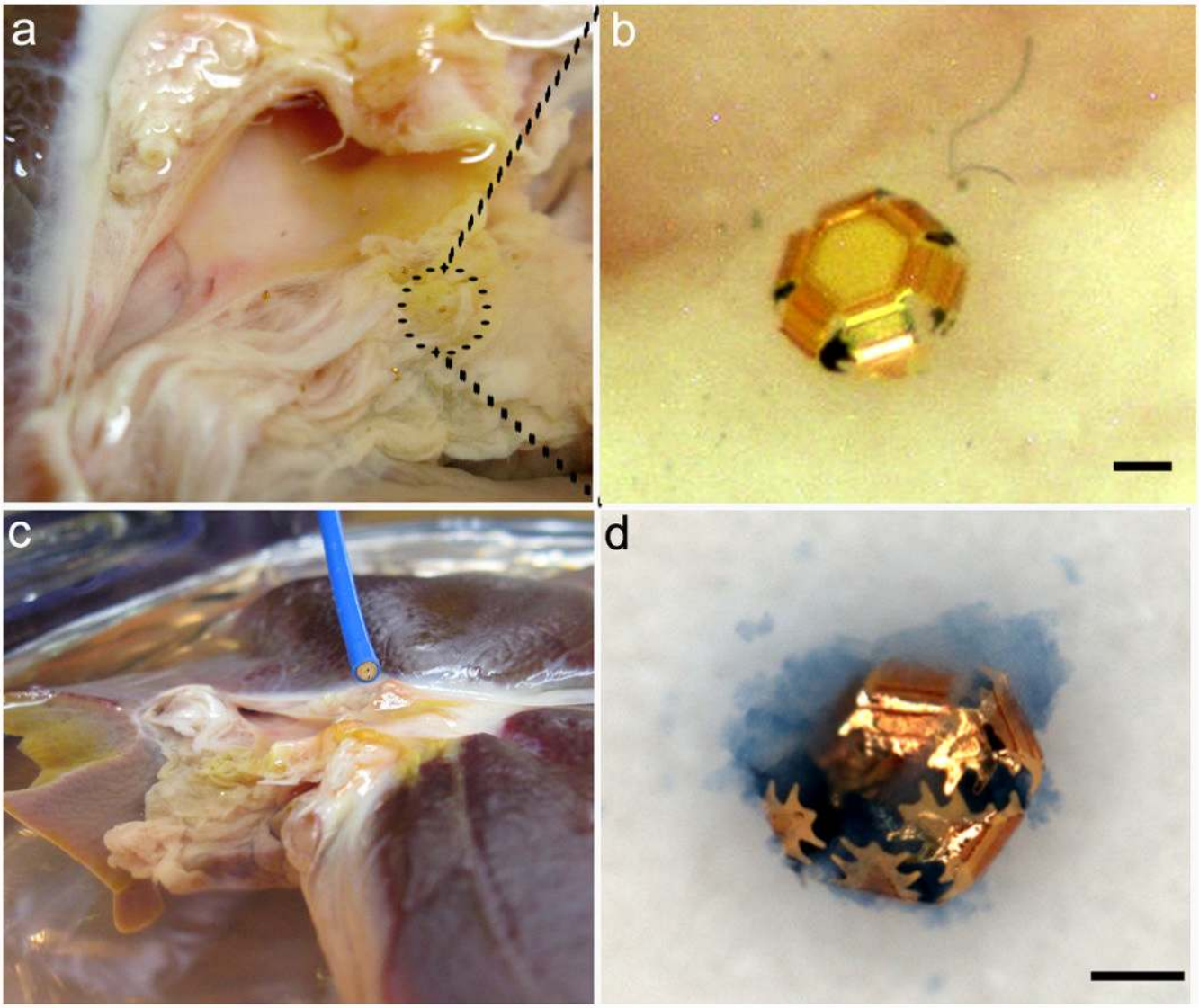

Figure 2. ***Ex vivo*** **tissue excision using untethered grippers**

(**a–b**) Optical image of grippers distributed on the bile duct opening of the porcine liver. Scale bar represents 200 m. (**c**) Optical image of grippers during retreival using a magnetic catheter. (**d**) Image of a retrieved gripper with an excised tissue piece after staining with trypan blue. Scale bar represents 100 m.

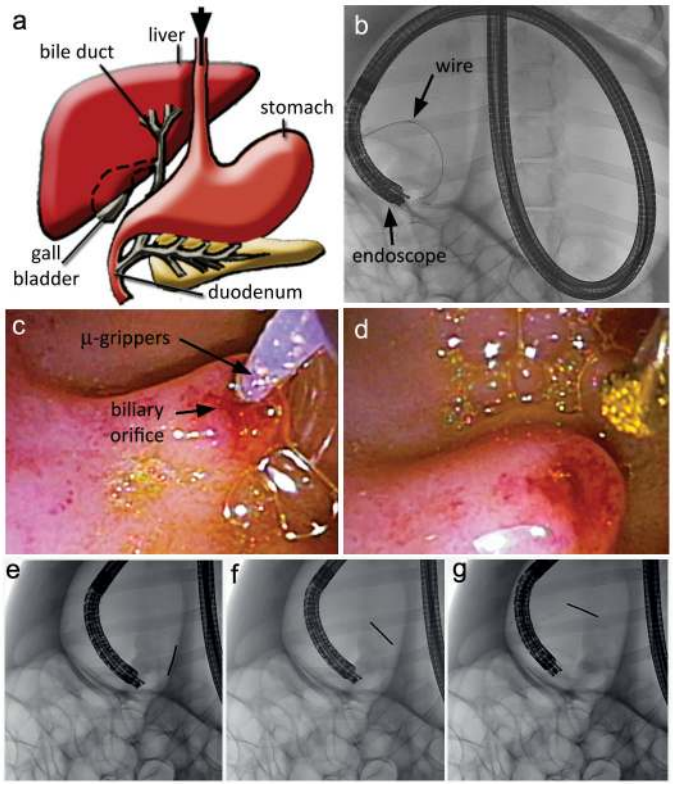

Figure 3. *In vivo* biopsy of the bile duct using untethered -grippers

(**a**) A schematic diagram of the porcine upper gastrointestinal track; the endoscope entry is depicted with an arrow. (**b**) Fluoroscopic image showing the endoscope entering from the mouth (top) and reaching the duodenum. The guide wire that was advanced into the bile duct is also visible. Endoscopic images of (**c**) the delivery of the -grippers through a catheter into the porcine biliary orifice, and (**d**) the retrieval of the -grippers via a magnetic catheter. (**e–g**) Fluoroscopic image sequence of the retrieval magnet being maneuvered inside the bile duct.

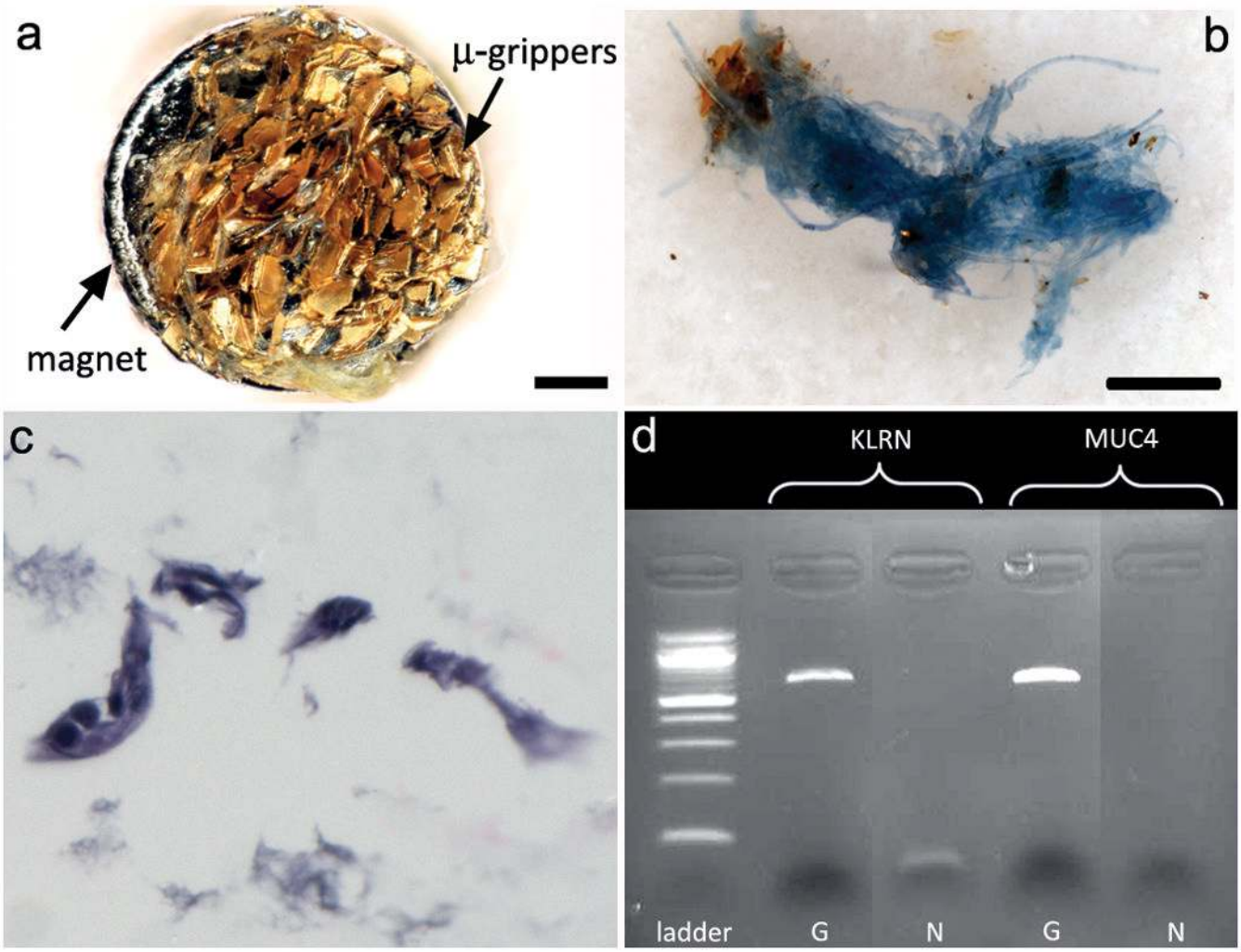

Figure 4. Optical microscopy images and PCR results of the biopsied tissue from *in vivo* experiments

(**a**) Optical image of the retrieved μgrippers on the magnetic catheter tip. (**b**) The retrieved tissue after staining with trypan blue. Scale bars represent 200 μm. (**c**) H&E stained section of cells retrieved by the μgrippers from the porcine bile duct. (**d**) Genomic DNA from the tissue obtained with the μgrippers (G) compared to the negative control (N).