Video Article

Visualization of Vascular and Parenchymal Regeneration after 70% Partial Hepatectomy in Normal Mice

Chichi Xie[1], Weiwei Wei[1], Andrea Schenk[3], Lars Ole Schwen[3], Sara Zafarnia[2], Michael Schwier[3], Felix Gremse[2], Isabel Jank[1], Olaf Dirsch[4], Uta Dahmen[1]

[1]Department of General, Visceral and Vascular Surgery, Jena University Hospital

[2]Experimental Molecular Imaging, RWTH Aachen University

[3]Fraunhofer Institute for Medical Image Computing MEVIS

[4]Institute of Pathology, Klinikum Chemnitz gGmbH

Correspondence to: Uta Dahmen at uta.dahmen@med.uni-jena.de

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Abstract

A modified silicone injection procedure was used for visualization of the hepatic vascular tree. This procedure consisted of *in-vivo* injection of the silicone compound, via a 26 G catheter, into the portal or hepatic vein. After silicone injection, organs were explanted and prepared for *ex-vivo* micro-CT (µCT) scanning. The silicone injection procedure is technically challenging. Achieving a successful outcome requires extensive microsurgical experience from the surgeon. One of the challenges of this procedure involves determining the adequate perfusion rate for the silicone compound. The perfusion rate for the silicone compound needs to be defined based on the hemodynamic of the vascular system of interest. Inappropriate perfusion rate can lead to an incomplete perfusion, artificial dilation and rupturing of vascular trees.

The 3D reconstruction of the vascular system was based on CT scans and was achieved using preclinical software such as HepaVision. The quality of the reconstructed vascular tree was directly related to the quality of silicone perfusion. Subsequently computed vascular parameters indicative of vascular growth, such as total vascular volume, were calculated based on the vascular reconstructions. Contrasting the vascular tree with silicone allowed for subsequent histological work-up of the specimen after µCT scanning. The specimen can be subjected to serial sectioning, histological analysis and whole slide scanning, and thereafter to 3D reconstruction of the vascular trees based on histological images. This is the prerequisite for the detection of molecular events and their distribution with respect to the vascular tree. This modified silicone injection procedure can also be used to visualize and reconstruct the vascular systems of other organs. This technique has the potential to be extensively applied to studies concerning vascular anatomy and growth in various animal and disease models.

Video Link

The video component of this article can be found at https://www.jove.com/video/53935/

Introduction

Liver regeneration is often determined by measuring the increase of liver weight and volume and by assessing the hepatocyte proliferation rate[16]. However, liver regeneration is not only inducing parenchymal regeneration but also vascular regeneration[6]. Therefore, vascular growth should be further investigated with respect to its role in the progression of liver regeneration. Visualization of the hepatic vascular system is critical to advancing our understanding of vascular regeneration. Numerous indirect methods have been developed to study the underlying molecular mechanisms of hepatic vascular regeneration. Traditionally, detection of cytokines (vascular endothelial growth factor, VEGF)[14], chemokines and their receptors (CXCR4/CXCR7/CXCL12)[4] have been the mainstay for studying vascular regeneration. However, a 3D model together with quantitative analysis of the vasculature would add critical anatomic information to gain a better understanding of the important relationship between hepatic parenchymal and vascular regeneration.

To visualize the hepatic vascular system, which requires contrasting the vascular trees, mice were injected with a radiopaque silicone rubber contrast agent directly into the portal or hepatic venous vascular tree. After polymerization of the silicone and explantation of the organ, the liver samples were subjected to µCT scanning using a CT scanner. The scans resulted in voxel image representations of the silicone-injection specimens[9].

For quality control, the vascular system was first visualized in 3D using preclinical software. Segmentation was performed by setting a threshold between the soft tissue intensity and the vessel intensity. The resulting vessel mask was visualized using surface rendering. The software also allowed for manual determination of two parameters of vascular growth: maximal vessel length and radius.

A preclinical software was then used for 3D reconstruction of vascular trees and subsequent calculation of the supplying or draining vascular territories[13]. In addition, this software automatically determined certain parameters of vascular growth, such as the total length of all visible vascular structures also known as the total edge length or total vessel volume.

The silicone perfusion procedure was performed in naive mice and in mice that underwent 70% partial hepatectomy (PH). Livers were collected at different observation time points after resection for analyzing vascular and parenchymal liver regeneration using the aforementioned visualization and quantification technique.

The main goals of this film are to: (1) demonstrate the delicate injection-technique required to achieve optimal contrasting and (2) show the potential benefit resulting from a detailed analysis of the resulting specimen using µCT and histological serial sections. After watching this film, the reader should have a better understanding of how to inject silicone compound into a specific vascular system and of the usefulness and applicability of the technique.

Protocol

Procedures involving animal subjects have been approved by Thüringer Landesamt für Verbraucherschutz Abteilung Tiergesundheit und Tierschutz, Germany. Because the portal venous system was visualized separately from the hepatic venous system, separate animals were needed for the different vascular trees.

1. Reagents Preparation

1. Heparin-saline solution
    1. Add 0.1 ml heparin into 10 ml saline (5 IU/ml).

2. Silicone compound mixture
    1. Add 2 ml MV-120 in one 5 ml tube. Dilute MV-120 by adding 3 ml MV diluent resulting in a 40% solution.

2. Portal Venous System Silicone Injection

1. Laparotomy
    1. Place the mouse in the anesthesia induction chamber and anaesthetize it with 2% isoflurane and 0.3 L/min oxygen.
    2. Fix the anaesthetized mouse on the operating table using tape with continuous inhalation of 2% isoflurane and 0.3 L/min oxygen. Check the toe-pinch withdrawal reflex of the mouse and start operation if the reflex is absent.
    3. Make a transverse incision on the abdomen using scissors for the skin layer and an electric coagulator for the muscle layer. Move out the intestines to the left side using cotton tips and cover the intestines with saline soaked gauze.

2. Catheter insertion
    1. Dissect portal vein under the microscope using micro-forceps. Place one 6-0 silk suture underneath the extrahepatic portal vein, in approximately 1 mm distance to its bifurcation and tie it loosely for later use.
    2. Inject prepared heparin-saline solution via penile vein (male) or inferior vena cava (female) for systemic heparinization for 5 min.
    3. Insert the 26-gauge (26 G) catheter with needle into portal vein and fix it with a clamp

3. Heparin-saline solution and silicone compound perfusion
    1. Load heparin-saline solution in 5 ml syringe and turn on perfusion device. Fill the catheter fully with heparin-saline solution to avoid air bubbles.
    2. Connect extension tube to the catheter and fix them tightly. Start heparin-saline perfusion with a perfusion rate of 0.4 ml/min.
    3. Ligate pre-placed 6-0 silk suture for double fixing the catheter and blocking blood flow from splenic vein and mesenteric vein. Euthanatize the mouse by exsanguination via perfusion under anesthesia.
    4. Rinse the liver using saline during the whole perfusion procedure in order to keep it moist.
    5. Add 0.1 ml curing agent into the MV-120 tube. Change heparin-saline syringe to silicone syringe.
    6. Start silicone perfusion with a perfusion rate of 0.2 ml/min for approximately 1 min to prefill the catheter system and to fill the portal vein system. Stop silicone perfusion when the vessels on the surface turn blue.

4. Sampling
    1. Keep the liver *in-situ* until the silicone is fully polymerized after approximately 15 to 30 min. Dissect ligaments connecting liver and adjacent organs with care to keep liver intact. Explant the liver and put it into formalin for fixation.

3. Hepatic Venous System Silicone Injection

1. Perform laparotomy as performed in step 2.1 and expose operative field fully.
2. Catheter insertion
    1. Dissect portal vein under the microscope using micro-forceps. Place one 6-0 silk suture underneath the extrahepatic portal vein, in approximately 1 mm distance to its bifurcation and tie it loosely for later use.
    2. Inject prepared heparin-saline solution via penile vein (male) or inferior vena cava (female) for systemic heparinization for 5 min.
    3. Insert one 26 G catheter (catheter 1) with needle into portal vein and fix it with a clamp. Insert another 26 G catheter (catheter 2) with needle into inferior vena cava and fix it with a clamp.

4. Ligate the branches of inferior vena cava (including left and right renal veins) and its distal end using 6-0 synthetic, monofilament, nonabsorbable polypropylene suture.

3. Heparin-saline solution and silicone compound perfusion
    1. Load heparin-saline solution in 5 ml syringe and turn on perfusion device. Fill catheter 1 completely with heparin-saline solution to avoid air bubbles. Connect extension tube to catheter 1 and fix it tightly. Start heparin-saline perfusion at a rate of 0.4 ml/min.
    2. Ligate pre-placed 6-0 silk suture for double fixing the catheter and blocking blood flow from splenic vein and mesenteric vein. Euthanatize the mouse by exsanguination via perfusion under anesthesia.
    3. Rinse the liver using saline during the whole perfusion procedure in order to keep it moist. Add 0.1 ml curing agent into the MV-120 tube. Exchange heparin-saline syringe with silicone syringe.
    4. Place a clamp on the suprahepatic inferior vena cava to obstruct the outflow of the liver.
    5. Connect the extension tube to catheter 2 and start silicone perfusion with a perfusion rate of 0.2 ml/min for approximately 2 min to reach an objective hepatic vascular volume as reported. Stop silicone perfusion when the vessels on the surface turn blue.

4. Sampling
    1. Dissect hepatic ligaments avoiding any injury to the liver. Explant the liver and put it into formalin for fixation.

4. Micro-CT (µCT) Scanning

To scan the explanted liver sample using µCT, the following steps are needed.

1. Take the liver sample out of the fixation solution. Place the liver onto the µCT bed. Put the µCT bed with the liver sample into the µCT.
2. Acquire topogram before starting the scan. Use one subscan for the small liver sample and two subscans for large samples.
3. Select a µCT protocol with a high resolution (*e.g.*, HQD-6565-390-90). This protocol acquires 720 projections with 1,032 x 1,012 pixels during one full rotation with the scanning time of 90 sec per sub-scan. Start the µCT scan.

5. Histological Serial Sections

1. Embed the liver specimen in paraffin as a whole after µCT scanning. Cut whole paraffin sample into 4µm sections, resulting in a series of 2,000 to 2,500 sections.
2. Stain sections with appropriate staining technique to visualize molecular events of interest such as Ki-67 as proliferation marker and HMGB1 as a marker of ischemic damage. Determine sequence of staining in respect to scientific question.
3. Use a whole slide scanner to digitalize the stained sections.
4. Perform 3D-reconstruction of vascular tree(s) (already feasible) and visualize 3D-distribution of molecular events in respect to vascular tree (research in progress).

Representative Results

Quality Criteria

The quality of silicone injection can be judged with the naked eye during the procedure. The small vessels on liver surface fill gradually with the blue compound. If the normal vascular structure was observed on the liver surface, the silicone rubber injection quality was good. If the perfusion volume was insufficient, the small vessels on the liver surface were not fully filled. In contrast, over filling caused vascular rupture as indicated by irregular blue spots on the surface of the organ. Both are causing difficulties upon 3D-reconstruction ultimately rendering a procedure a failure.

For better evaluation of the injection quality, µCT scanning followed by 3D vascular reconstruction using preclinical software was performed. Injection was determined to be successful when segmentation resulted in the visualization of an intact complete vascular tree without ruptured structures indicated by visible extravasation. If the vascular tree appeared incomplete (**Fig. 1A**), perfusion volume was insufficient. If more than one vascular tree appeared or extravasation was seen (**Fig. 1B**), perfusion volume or pressure was inadequate. This was the most likely reason for a failure. Perfusion pressure can theoretically vary slightly depending on the viscosity of the solution. Viscosity is dependent on the polymerization time elapsed between mixing the compound and injection into the vascular system. Since some manipulation is required for mixing and injection, time interval can slightly vary between 3 min to 5 min.

If the solution is of low viscosity, perfusion pressure is low. In this case the compound can transfer into the non-injected vascular tree via the sinusoidal system leading to a slightly higher perfusion volume. If the solution is further polymerized leading to a higher viscosity, the perfusion pressure will rise, causing disruption of the vessels and extravasation.

Determination of Objective Perfusion Volume

Since inadequate perfusion or hyperperfusion would cause failure of injection, standardizing the perfusion volume was considered. As reported[2], 6% of the hepatic volume is occupied by blood and 44% of the blood in the liver resides in the large vessels (*e.g.*, hepatic artery, portal vein). Liver volume in mice is about 1.3 ml[11]. Therefore, total vascular volume in portal venous system in normal mice was estimated to range between 0.03 ml and 0.04 ml. Flow rate was set to 0.2 ml/min and total perfusion time was set to approximately 1 minute resulting in a total injected volume of around 0.2 ml.

This seemingly high volume is needed, since the catheter system must be prefilled, which takes about 0.1-0.15 ml. An extra volume of 0.05 ml is needed to fill the proximal portal vein between liver hilum and the ligation of the catheter. Flow rate for hepatic vein injection was also set to 0.2 ml/min but total perfusion time was prolonged to 2 min, resulting in a higher total volume of about 0.4 ml. Total intrahepatic vascular volume was assumed to be similar to the total portal vein vascular volume. However, the volume needed to fill the intrahepatic vena cava between the infrahepatic ligation and the suprahepatic clamp was estimated with 0.2 ml.

Success Rate

A total of 49 animals were subjected to silicone injection: 22 animals into the PV system and 27 animals into the HV system. Our success rate of injection was 55% (12/22) in the PV group and 89% (24/27) in the HV group. Taking into account that the PV group was used to establish the silicone injection technique in the beginning (n=4), the success rate of injection for the PV system should effectively be higher than 55%. However, µCT images obtained from these injections were suitable for 3D reconstruction and quantitative analysis. Additionally, the vascular trees from either PV or HV of 36 mice could be reconstructed with the Imalytics Preclinical software.

Parenchymal and Vascular Regeneration

A time resolved µCT series of murine liver samples after hepatectomy was subjected to a qualitative analysis. Parenchymal regeneration consisted of 3D growth of the remnant liver. Vascular regeneration was defined as tissue that displayed an increase in the length and diameter of the vascular stem, with its main branches and outgrowth of additional terminal branches, in both the portal venous and hepatic venous tree.

Currently, several quantitative parameters suitable for describing vascular growth and the relationship between hepatic parenchymal regeneration and vascular regeneration are under investigation. These include parameters indicative of vascular growth, including maximal vascular length (**Fig. 2**), vascular radius and vascular density in terms of total vascular length/liver volume or vascular volume/liver volume.

Total liver volume and volume of selected liver lobes were calculated. In normal livers, total liver volume ranged from 1.2 ml to 1.6 ml (n=6, including both the PV group and HV group). It decreased to 0.6 to 0.7 ml after performing an extended liver resection by removing the left lateral and median lobe. The liver volume increased continuously during the liver growth. By post-operative day 7 (POD 7), the liver volume increased to approximately 88% of its original volume, *i.e.*, by 2.6-fold. The increase of liver volume correlated with liver weight recovery.

Total vascular volumes of the PV system and HV system were computed. Total vascular volume of the HV system was higher than in PV system, because part of the intrahepatic inferior vena cava was included. Total vascular volume of PV system ranged from 0.05 to 0.08 ml in normal mice. It decreased to 0.03 to 0.04 ml after 70% partial hepatectomy. By POD 7, total vascular volume of the remnant liver increased to 100% of original volume. Total vascular volume of HV ranged from 0.14 to 0.16 ml. Vascular volume decreased to 0.08 to 0.09 ml after resection. Within the first postoperative week, the total vascular volume of the remnant liver increased by 94% of original value.

As the relation between results, increase in vascular volume and parenchymal volume, a vascular density was calculated, more precisely the vascular volume fraction (vascular volume divided by liver volume). This revealed that vascular volume fraction remained relatively stable throughout the regeneration process.

3D Visualization of Molecular Events

After CT-scanning the selected specimens were subjected to serial sectioning, yielding to up to 2000 slides, which were fully digitalized and used to reconstruct the portal as well as the hepatic venous tree[12]. This is the prerequisite for the future 3D visualization of molecular events during regeneration in respect to the growing vascular tree.

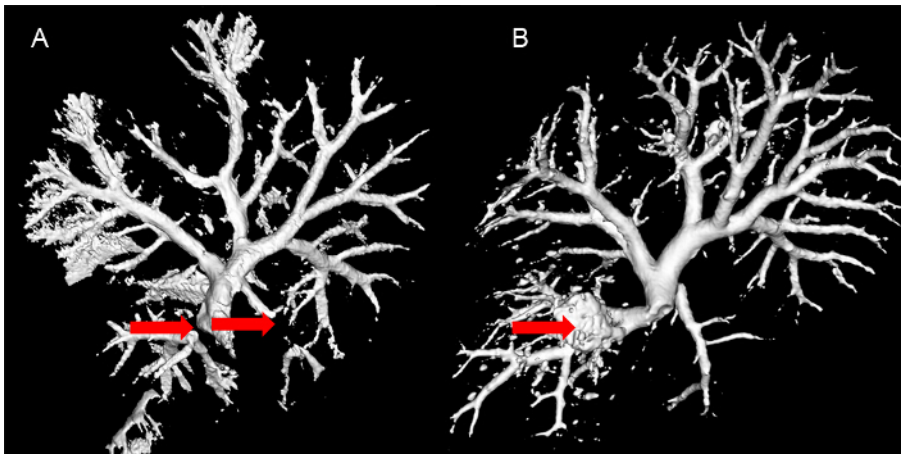

Figure 1. Monitoring the quality of silicone injection. A. Incomplete filling of right inferior portal vein (arrow in the left) and caudate portal vein (arrow in the right) were visualized in Imalytics Preclinical software as discontinuities of the vascular tree. This indicated an inadequate perfusion pressure or perfusion volume or the existence of air bubbles. **B**. Extravasation of right inferior portal vein was visualized which indicated disruption of a vessel due to inadequate pressure or hyperperfusion. Please click here to view a larger version of this figure.

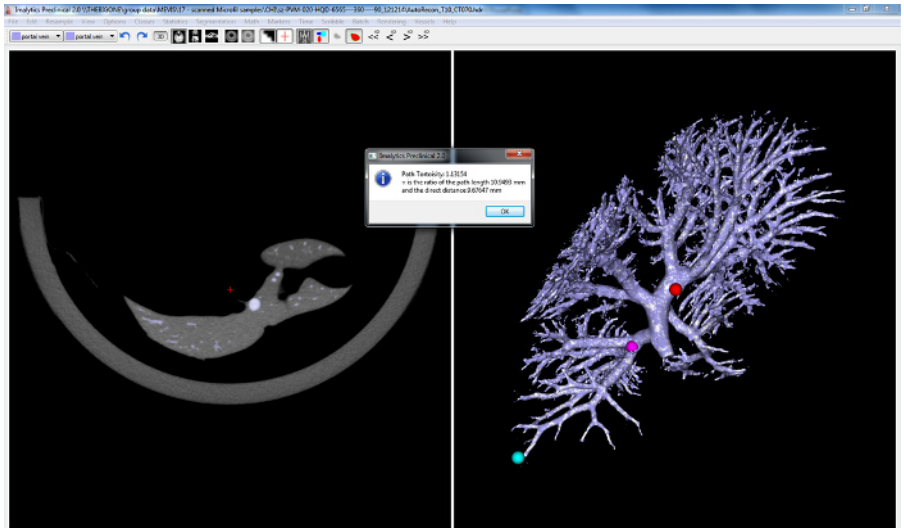

Figure 2. Measurement of maximal vessel length of right inferior portal vein. To determine the maximal vessel length, one start point and one end point were placed at the root and distal end of the right inferior portal vein (RIPV) of the vascular mask (blue and magenta ball markers) in preclinical software. The vascular path length of the RIPV (10.95 mm) was obtained as part of evaluating the "Path Tortuosity". Please click here to view a larger version of this figure.

Discussion

Contrasting the vascular tree by silicone injection and µCT scanning has been introduced in tumor models and neurological disease models frequently to study the angiogenic progression[5,7,8,10]. Improvements in methodology of silicone injection were made in the present study for visualizing and quantifying vascular growth after partial hepatectomy in mice.

There are a number of critical steps needing attention to achieve good perfusion quality. First of all, systemic heparinization is highly recommended before flushing the liver with heparin or saline to avoid blood clotting inside the liver. Elimination of air bubbles from the tubing is also important to achieve good quality of silicone perfusion. The determination of perfusion rate and pressure should first be based on physiological hemodynamic parameters[17]. Perfusion time reflects the total injected volume and is estimated taking the expected intravascular volume and the prefilling volume of the catheter system into account.

Silicone is a highly viscous compound which differs from blood or saline. Therefore, several trials are needed for adjusting perfusion rate and pressure. Maintaining constant injection flow rate and pressure is necessary to prevent severe dilation or even rupture of small vessels. Perfusion time is set according to the estimated needed injection volume. However, perfusion should be stopped immediately when the blue compound becomes visible in vessels on the surface of the liver. Otherwise, the silicone can drain into sinusoids and into another vascular system which will interfere with later reconstruction and analysis.

Typically, silicone is perfused systematically via the left ventricle in most published reports [1,3,15]. The left ventricle is easy to expose to enable free access to the injection site. However, the disadvantage is that this route is rather indirect because the contrast agent has to undergo circulation before reaching the site of interest. Therefore, the surgical procedure of silicone injection technique has been modified in this study. In contrast to the frequently selected indirect route of application performed by others, silicone compound was directly injected into the vascular system of interest, instead of contrasting the whole body. In this way, the velocity and volume of perfusion can be better controlled. The portal venous system and hepatic venous systems can be perfused and reconstructed separately for later individual analysis, as shown in the video.

The limitation of this modified procedure is the technical difficulty. It is challenging to successfully perform an intraportal injection using highly viscous compound in mice, because the vascular structure is so delicate. It is rather easy to destroy the vessels during the procedure. Thus, portal vein dissection and catheter insertion should be performed gently.

Contrasting of the vascular trees and subsequent µCT imaging of the explanted livers is a useful tool for visualizing and quantifying vascular regeneration after partial hepatectomy. Quantitative vascular parameters can be utilized for better understanding of the kinetic of vascular growth in the progression of liver regeneration. Furthermore,3D reconstruction of both hepatic vascular systems based on serial sections is technically feasible, albeit representing an enormous work load.

This technique can be applied in multiple models where visualization and quantification of vascular growth is important. Moreover, 3D visualization of molecular events in respect to the underlying vascular tree is at reach. Examples of molecular events of interest could be the detection of proliferation marker such as Ki-67 or markers of ischemic damage such as HMGB1. Assessing the spatially resolved molecular events in the vicinity of the regenerating vascular tree within the regenerating hepatic parenchyma is the prerequisite for advanced multi-scale systems biology modeling. This silicone injection technique is one experimental step towards reaching this goal.

Disclosures

The authors declare that they have no competing financial interests.

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References

1. Bearden, S.E., & Segal, S.S. Neurovascular alignment in adult mouse skeletal muscles. *Microcirculation.* **12** (2), 161-167 (2005).
2. Brown, R.P., Delp, M.D., Lindstedt, S.L., Rhomberg, L.R., & Beliles, R.P. Physiological parameter values for physiologically based pharmacokinetic models. *Toxicol.Ind.Health.* **13** (4), 407-484 (1997).
3. Dai, D. *et al.* Elastase-Induced Intracranial Dolichoectasia Model in Mice. *Neurosurgery.* (2015).
4. Ding, B.S. *et al.* Inductive angiocrine signals from sinusoidal endothelium are required for liver regeneration. *Nature.* **468** (7321), 310-315 (2010).
5. Downey, C.M. *et al.* Quantitative ex-vivo micro-computed tomographic imaging of blood vessels and necrotic regions within tumors. *PLoS.One.* **7** (7), e41685 (2012).
6. Ehling, J. *et al.* CCL2-dependent infiltrating macrophages promote angiogenesis in progressive liver fibrosis. *Gut.* (2014).
7. Ehling, J. *et al.* Micro-CT imaging of tumor angiogenesis: quantitative measures describing micromorphology and vascularization. *Am.J.Pathol.* **184** (2), 431-441 (2014).
8. Ghanavati, S., Yu, L.X., Lerch, J.P., & Sled, J.G. A perfusion procedure for imaging of the mouse cerebral vasculature by X-ray micro-CT. *J.Neurosci.Methods.* **221** 70-77 (2014).
9. Gremse, F. *et al.* Hybrid microCT-FMT imaging and image analysis. *J.Vis.Exp.* (100) (2015).
10. Jing, X.L. *et al.* Radiomorphometric quantitative analysis of vasculature utilizing micro-computed tomography and vessel perfusion in the murine mandible. *Craniomaxillofac.Trauma Reconstr.* **5** (4), 223-230 (2012).
11. Melloul, E. *et al.* Small animal magnetic resonance imaging: an efficient tool to assess liver volume and intrahepatic vascular anatomy. *J.Surg.Res.* **187** (2), 458-465 (2014).
12. Schwier, M., Bohler, T., Hahn, H.K., Dahmen, U., & Dirsch, O. Registration of histological whole slide images guided by vessel structures. *J.Pathol.Inform.* **4** (Suppl), S10 (2013).
13. Selle, D., Preim, B., Schenk, A., & Peitgen, H.O. Analysis of vasculature for liver surgical planning. *IEEE Trans.Med.Imaging.* **21** (11), 1344-1357 (2002).
14. Shergill, U. *et al.* Inhibition of of VEGF- and NO-dependent angiogenesis does not impair liver regeneration. *Am.J.Physiol Regul.Integr.Comp Physiol.* **298** (5), R1279-R1287, (2010).
15. Sueyoshi, R., Ralls, M.W., & Teitelbaum, D.H. Glucagon-like peptide 2 increases efficacy of distraction enterogenesis. *J.Surg.Res.* **184** (1), 365-373 (2013).
16. Wei, W. *et al.* Rodent models and imaging techniques to study liver regeneration. *Eur.Surg.Res.* **54** (3-4), 97-113 (2015).
17. Xie, C., Wei, W., Zhang, T., Dirsch, O., & Dahmen, U. Monitoring of systemic and hepatic hemodynamic parameters in mice. *J.Vis.Exp.* (92), e51955 (2014).

