

Bioprinting User Manual

TissuePrint - HV

CELLINK BIO X

Document: User Manual

Last Update: Feb 23, 2021

Version: 1.0

Contents

[TABLE OF CONTENTS](#)

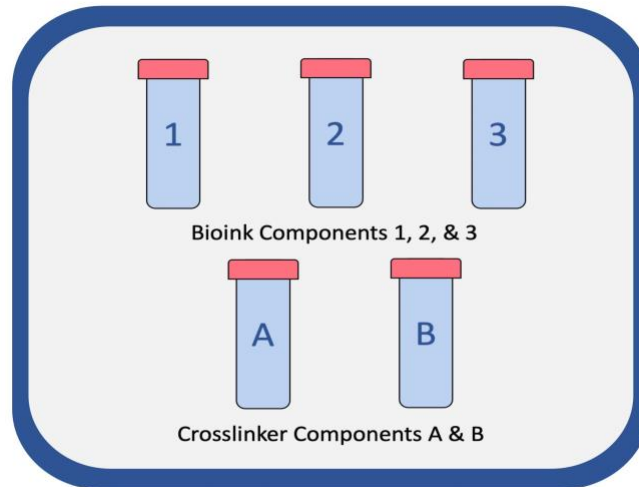
Welcome!

Axolotl Biosciences is driven by innovation, integrity, and collaboration, and we strive to deliver the best bioprinting experience to our users. This manual will be your guide to printing with Axolotl Biosciences' TissuePrint bioink using the CELLINK BIO X printer. This ink is a xeno-free fibrin-based bioink which supports multiple cell lines such as human induced pluripotent stem cells (HiPSCs), neural progenitor cells (NPCs), and mesenchymal stem cells (MSCs). TissuePrint comes in both high viscosity (HV) and low viscosity (LV) formulations. This user manual is designed specifically for the TissuePrint-HV formulation, which is optimized for extrusion based bioprinting. TissuePrint-LV, which is optimized for microfluidic based bioprinting, is also available, visit www.axotlbiosciences.com for more information.

1.0 Materials, Reagents, and Equipment

1.1 Product Map

What's inside the box:



1.2 Material and Equipment Required for Printing with the Cellink BioX

Purchased from Axolotl Biosciences

- TissuePrint Component 1
- TissuePrint Component 2
- TissuePrint Component 3
- Crosslinker Component A
- Crosslinker Component B

Purchased from CELLINK

- BIO X 3D Bioprinter*
- Clear cartridges, 3mL
- Sterile conical bioprinting nozzles
- Luer lock adaptor
- Nozzle tips (recommended 22 gauge)

Material Supplied by User

- Culture plate
- Cells + cell culture media
- 3mL luer lock syringes (preferred supplier?)
- Wide bore p1000 pipette tips (supplier?) (or cut off ends of standard p1000 tips)
- P200 pipette tips

*This protocol can be applied to extrusion based bioprinters. Modify based on specifications of your printer.

2.0 Protocol

The following procedure describes bioink preparation, bioprinting, and tips for culturing and analyzing 3D scaffolds. Troubleshooting information is included at the end of each protocol step where it may apply. A graphical overview of the procedure is included at the end of this section. For additional support and resources please visit www.axolotlbiosciences.com.

2.1 Preparation

Any mixing steps should be conducted in a BSC if you require aseptic conditions. ****make note for aliquoting different volume**

2.1.1 Preparing Bioink:

1. Let Component 1-HV and Component 3-HV thaw at 4°C (approximately 3 hours for 5 mL solution). Once completely thawed, thaw Component 2-HV at room temperature.
2. Using a p200 pipette, pipette Component 2-HV into Component 1-HV. Component 2-HV may clump upon addition to Component 1-HV. If this occurs, use a p1000 pipette with a wide bore pipette tip and slowly pipette up and down to disperse the clump.
3. Using a p1000 pipette, pipette Component 3-HV into solution. Slowly mix into the bioink by stirring with the pipette or pipetting up and down. Avoid generating air bubbles.

2.1.2 Preparing Bioink to include Cells:

1. Let Component 1-HV thaw at 4°C (approximately 3 hours for 3mL solution).
2. Once Component 1-HV is completely thawed, thaw Component 2-HV at room temperature and Component 3-HV at 37°C. **(5-10 minutes)**
3. To formulate bioink: Using a p200 pipette, pipette Component 2-HV into Component 1-HV. Component 2-HV may clump upon addition to Component 1-HV. If this occurs, use a p1000 pipette with a wide bore pipette tip and slowly pipette up and down to disperse the clump, avoiding the introduction of bubbles. You can also slowly stir the solution with the pipette tip to disperse any clumps.
4. Prepare desired cells for resuspension in Component 3-HV. Either thaw or harvest from culture dishes as per cell specific harvesting protocols, into a conical tube and use cell specific media to obtain a final volume of 10 mL.
5. Centrifuge at 300g for 5 minutes, or according to culturing protocols.

6. Remove supernatant from conical using an aspirator or 10mL pipette and gently resuspend the cell pellet **the whole volume of** Component 3-HV using a p1000 and regular tip (or 5mL pipette etc), avoiding the introduction of air bubbles.
7. Slowly add cells in Component 3-HV solution to the bioink using a p1000 pipette. Slowly mix into the bioink by stirring with the pipette or pipetting up and down. Avoid generating air bubbles.

2.1.3 Preparing Crosslinker

1. Let Component A-HV and Component B-HV thaw at 4°C (approximately 2 hours for a 3mL solution).
2. **Pipette** Component B-HV to Component A-HV. Slowly mix by stirring with the pipette or pipetting up and down. Avoid generating air bubbles.

2.1.4 Troubleshooting

Avoiding Air Bubbles

Minimizing the formation of air bubbles when preparing bioink will yield the best result. When preparing the bioink and incorporating cells, avoid over dispensing the pipette while pipetting and mixing components, as this can introduce air pockets. For more viscous solutions, using a wide bore pipette when mixing may help decrease air bubble formation. If there are air bubbles in the bioink, centrifuging the bioink conical may help decrease the amount of air bubbles.

2.2 Bioprinting

NOTE: Please consult the CELLINK BIO X User Manual before printing and for any concerns directly related printer mechanics or software.

The bioprinter should be placed in the proper environment according to your application. If aseptic conditions are required, the bioprinter should be placed inside a biosafety cabinet (BSC). Everything should be properly sterilized (autoclave, UV, and/or 70% ethanol) before moving it into the BSC. Extreme care is required when manipulating the printer cartridges and the culture dish.

***List of things that should be in the BSC?? Things to autoclave the day before? Printing prep section?**

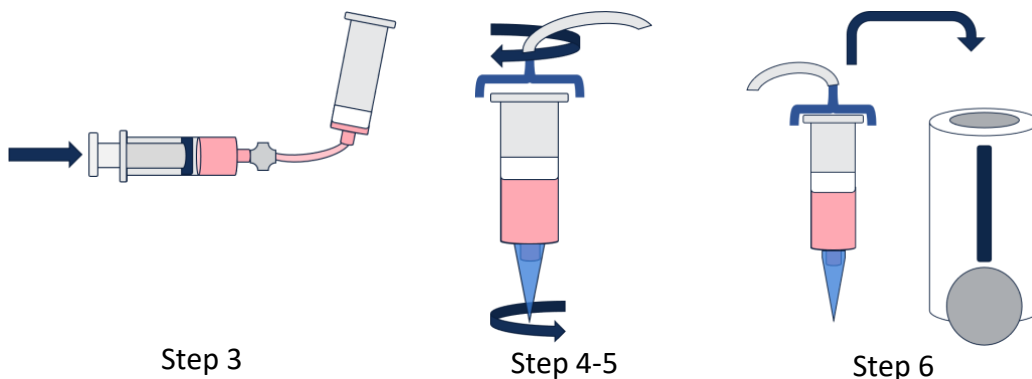
2.2.1 Preparing the Printhead

The following items should be properly sterilized and ready to use in the BSC before proceeding:

<ul style="list-style-type: none">• Crosslinker• Bioink• 3mL syringe, luer-lock female/female adapter, and 3mL printer cartridge with piston• 22 gauge nozzle	<ul style="list-style-type: none">• Culture dish• P1000 pipette and tips• Waste beaker• Conical rack
--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------	-----------------------------------------------------------------------------------------------------------------------------------------------

These steps should be done in the BSC if you require aseptic conditions.

1. Pour the bioink into a 5mL syringe. This may take a few moments due to viscosity of the solution. You may also use a needle to draw the solution up. Use a wide gauge needle to avoid damaging your cells.
2. Attach a luer-lock female/female adaptor to the syringe. Attach an empty 5mL print cartridge to the other end of the luer-lock adaptor. Ensure a piston is inside the print cartridge.
3. Transfer the bioink into the cartridge by slowly depressing the plunger of the syringe. Be careful not to introduce air bubbles. Detach the cartridge from the syringe.
4. Screw on a printing nozzle (recommended 22 gauge) to the cartridge.
5. Screw on the cartridge adaptor.
6. Load the print cartridge into the printhead and secure it.



2.2.2 Printer Setup and Settings

1. Set up the printer according to the CELLINK User Manual

2. Recommended* settings are:
 - Printhead: Pneumatic
 - Nozzle: 22 gauge
 - Pressure: 3kPa
 - Speed: 10mm/s
 - Temperature: 20°C
3. Place desired culture dish on the build plate and calibrate the printer

*Printing parameters may vary from printer to printer, sometimes depending on seeding density and environmental conditions. See Troubleshooting for more information.

2.2.3 Printing

1. Pipette crosslinker into the culture dish (1mL for a 6-well plate, 500uL for a 12-well plate). **Remove after 60s.** The surface of the culture dish should remain wet.
2. Print the desired file.
3. Once printing is complete, pipette 1mL of crosslinker into the culture dish **and remove after 1 minute.**
4. Pipette appropriate volume of application specific cell culture media **into the culture dish so the construct is covered.** Pipette in the corner furthest away from the construct to avoid disturbing it.
5. For a construct with cells, incubate the construct at 37°C and 5% CO₂ (or according to experiment application). For a construct without cells, store as desired in either 4°C, 37°C, or according to experiment application.

2.2.4 Troubleshooting

Bioink does not extrude from nozzle tip:

- First time using nozzle:
 - Try increasing the system pressure slightly (by 1kPa increments) and retesting extrusion. If 20kPa is reached and bioink still does not extrude, move on to the next step.
 - Try inserting a sterile, thin metal wire or thin needle into the tip to dislodge a potential clog.
 - Try inspecting the nozzle. If the tip appears bent or damaged, replace it.
- After previously successful prints:
 - Try increasing the system pressure slightly (by 1kPa) and retesting extrusion. As the cartridge empties, more pressure may be required to extrude the bioink.
 - If some time has elapsed between prints, try increasing pressure by 5kPa and retesting extrusion. The bioink may have dried out in the tip of the nozzle,

causing a clog. Increasing the pressure by 5kPa or 10kPa should be enough to release the dried bioink. Avoid leaving long gaps between prints to prevent this.

- Clog during printing:
 - Try using a pipette tip to remove the clog from the nozzle tip.
 - Try increasing the system pressure slightly until the clog dislodges or the bioink extrudes. This may also make it easier to remove the clog with a pipette tip if previous attempts were unsuccessful.
 - If the nozzle tip was too close to the build plate, this may have caused pre-crosslinking inside the nozzle tip. For future prints, try printing with the nozzle **tip further away from the build plate.**

Bioink drips from the nozzle between prints

- Try decreasing the system pressure slightly until it ceases.
- Try periodically wiping the nozzle with a Kimwipe. If the dripping occurs infrequently, it will not affect printing.
- Try detaching the pressure cap from the syringe between prints to decrease the amount of pressure pushing on the bioink.

Bioink fibers run together before crosslinking can occur:

- Try pipetting 1mL of crosslinker onto the culture dish and leaving it for a few minutes. Remove excess crosslinker (leaving a thin coating approximately 100uL). This will act as a crosslinker coating that can help pre-crosslink the bioink.
- Try decreasing the print speed or pressure. Printing slower, thinner lines can help pre-crosslink the bioink faster and prevent the fibers from running together.

The construct is dragged along the print bed

- The printhead may be too low. Try raising the printhead so the nozzle does not make contact with the structure, but not too high that the ink can't deposit.
- There may be build-up of bioink in the nozzle. Cancel the print and try increasing the system pressure to dislodge the clog.

2.3 Culturing 3D Constructs

Culturing protocols will vary based on the cell type and experiment. It is recommended to follow your lab's validated culturing protocols while keeping the following in mind:

- The tissue constructs will be delicate. When changing media on the constructs, it is recommended to use a 1000uL pipette (or be very careful with a serological pipette), and slowly pipette the media in the corner of the well furthest from the construct to avoid breaking it. Do not use an aspirator as this may aspirate pieces of the construct.
- With long term cultures, it is normal to see small pieces of the construct disperse from the construct. **Be careful not to suck these pieces into the pipette**, as they may still be attached to the main construct.
- Depending on the size of your construct, you may need more media than with a **2D cell culture**. **The construct should be completely covered by media.**

2.4 Analyzing 3D Constructs

The 3D constructs can be analyzed in a variety of ways: live/dead staining, immunocytochemistry, flow cytometry etc. Protocols can be found at www.axotlbiosciences.com. You might take into consideration the following for analyzing 3D tissues:

- The construct will display autofluorescence. We have found better results using ethidium homodimer-III instead of propidium iodide for staining dead cells.
- It might be necessary to increase washes and incubation times and use a shaker plate when staining the constructs.
- A benchtop instrument for tissue dissociation, such as the gentleMACS™ dissociator, can be used for breaking down the constructs for flow cytometry.

TissuePrint-HV Graphical Overview

Reagents Included

Bioink Components 1, 2, & 3

Crosslinker Components A & B

1 Storage

Upon receiving reagents, store at -20°C or below until use. Expiration indicated on packaging.

Store

-20°C

2 Thawing

Thaw reagents. For best results: Use bioink within 1hr and use crosslinker within 7 days of thawing.

4 - 8°C Components 1, 3, A & B

20 °C Component 2

To Include Cells

4 - 8°C Components 1, A & B

20 °C Component 2

37 °C Component 3

3a Bioink Prep

Pipette Component 2 into Component 1 to make the bioink.

Pipette Component 3 into the bioink

3b Bioink Prep To Include Cells

Pipette Component 2 into Component 1 to make the bioink.

Incorporate cells into Component 3 then pipette into the bioink.

4 X-Linker Prep

Add Component B to Component A.

5 Load Printer Cartridge

Transfer bioink into a 3mL syringe. Use the Cellink luer-lock adaptor to load print cartridge.

6a Load Print Head

Screw the nozzle onto the cartridge then secure the cartridge adaptor.

6b Load Print Head

Load the bioink cartridge into the printhead.

7 Print Settings

Recommended* settings are:

Printhead: Pneumatic
Nozzle: 22 gauge
Pressure: 3kPa
Speed: 10mm/s
Temperature: 20°C

8 Prep Print Bed

Place culture plate on build plate. Pipette 1mL of crosslinker onto plate and remove after 60s.

9 Print

Print desired file.

10 Crosslink

Pipette 1mL of crosslinker onto plate and remove after 1min

11 Culture

Pipette culture media onto plate, careful to not disturb construct. Incubate at 37°C.

12 Finish and Clean Up

Dispose of waste into appropriate hazardous waste bin.

Power off printer.

Wipe down surfaces with ethanol.

*Printing parameters may vary depending on seeding density and environmental conditions. See Troubleshooting for more information.

3.0 Terms and Conditions

AXOLOTL BIOINK™ LIMITED USE LICENSE AGREEMENT

Last Updated: [24 February 2021]

Please read this Limited Use License Agreement (“**Limited Use License**”) carefully before using Axolotl Biosciences, Inc.’s (“**Axolotl**”) fibrin-based biological ink products (“**BioInk**”). **By using BioInk, you (the “User”) are agreeing to be bound by the terms and conditions of this Limited Use License.** If User does not agree to the terms of this Limited Use License, do not purchase BioInk and do not use BioInk.

1. **DESCRIPTION.** BioInk allows the User to support printing of multiple different cell lines, such as somatic cells, human induced pluripotent stem cells (hiPSCs), neural progenitor cells (NPCs), and mesenchymal stem cells (MSCs). BioInk generates stable and reproducible 3D-structures (constructs) that can remain in culture for over a month, maintain long-term cultures with high viability, and can support tissue differentiation and function. The BioInk products are experimental in nature and must be used with prudence and appropriate caution, since not all of their characteristics are known. The BioInk products have not been approved for use in humans by the U.S. Food and Drug Administration or any other regulatory body and may not be used in humans.
2. **USE AND LIMITED LICENSE.** Subject to User’s compliance with all terms of this Limited Use Agreement and all applicable laws and regulations, including, without limitation, applicable human health and animal welfare laws and regulations, Axolotl hereby grants to User a royalty free, non-exclusive, non-sublicensable, non-transferrable right to practice the technology covered by Axolotl’s published and pending patents solely for the purpose of using the amount of BioInk purchased by User for internal, non-clinical research purposes or non-clinical research and development purposes conducted by User. User agrees not to, and not to permit others to license, sell, rent, lease, assign, distribute, transmit, host, outsource, disclose, or otherwise commercially exploit BioInk, to make BioInk available to any third party, or to use BioInk for any purpose other than as permitted under this section. User may not delegate any duties nor assign any rights or claims hereunder without Axolotl’s prior written consent, and any such attempted delegation or assignment will be void. User will not characterize, sequence, resolve, copy, reverse engineer, or disassemble BioInk or make any derivatives thereof. Unless expressly permitted by Axolotl in writing, User will not modify, change, remove, cover or otherwise obscure any of Axolotl’s brands, trade or service marks on the BioInk products. Axolotl gives no rights to use BioInk in any commercial application, including manufacturing, quality control, commercial services such as reporting the results of User’s activities for a fee or other consideration, or *in vitro* diagnostic uses, *ex vivo* or *in vivo* therapeutic uses, or any type of consumption by or application to humans or animals. If User needs commercial use rights in respect of BioInk (including the right to perform fee-for services), please contact our out-licensing department at willerth@axolotlbiosciences.com. Where User’s use of BioInk is outside the scope of this Limited Use Agreement, it is solely User’s responsibility to acquire additional rights.
3. **INTELLECTUAL PROPERTY OWNERSHIP.** As between User and Axolotl, Axolotl exclusively owns all intellectual property rights relating to BioInk and in any inventions (patentable or otherwise), discoveries, improvements, data, know-how, or other results that are conceived, developed, discovered, reduced to practice, or generated by or for Axolotl, or jointly by User and Axolotl, in relation to processes, designs and methods utilized in manufacture of a custom product deriving from, similar to, or relating to BioInk. User agrees to transfer and assign, and hereby does transfer and assign, to us all of User’s right, title, and interest in and to any such joint intellectual property. At Axolotl’s request and at Axolotl’s expense, User will help Axolotl secure and record Axolotl’s rights in such joint intellectual property. Nothing in the Limited Use Agreement limits Axolotl’s ability to enforce its intellectual property rights.
4. **INTELLECTUAL PROPERTY INFRINGEMENT.** Axolotl wants to avoid claims of intellectual property infringement. If Axolotl believes a product it has sold to User may be subject to a claim for intellectual property infringement, User must allow Axolotl (at Axolotl’s option) to either (a) secure for User the right to continue using the product; (b) substitute the product with another suitable product with similar functionality; or (c) tell User to return the product to Axolotl and Axolotl will refund to User the price paid.
5. **CONFIDENTIALITY.** User agrees to keep confidential any non-public technical information, commercial information (including prices, without limitation) or instructions (including any sequences, chemical compositions or formulations) received from Axolotl as a result of discussions, negotiations and other communications between User and Axolotl in relation to Axolotl products and services.
6. **MODIFICATIONS.** Axolotl reserves the right to modify, suspend, or discontinue, temporarily or permanently, the supply of BioInk or any service to which it connects, with or without notice and without liability to User.
7. **TERM AND TERMINATION.** The rights contained in this Limited Use License shall remain in effect while the purchased quantity of BioInk is being used by User. User shall have no rights to use BioInk beyond the purchased quantity.
8. **AMENDMENTS TO LIMITED USE LICENSE.** Axolotl reserves the right, at its sole discretion, to modify or replace this Limited Use License at any time.

9. **NO WARRANTY.** USER EXPRESSLY ACKNOWLEDGES AND AGREES THAT USE OF BIOINK AND USER'S RELIANCE ON THE OPERATION, OUTPUT OR RESULTS OF BIOINK IS AT USER'S SOLE RISK AND THAT THE ENTIRE RISK AS TO SATISFACTORY QUALITY, PERFORMANCE, ACCURACY AND EFFORT IS WITH USER. TO THE MAXIMUM EXTENT PERMITTED BY APPLICABLE LAW, BIOINK IS PROVIDED "AS IS" AND "AS AVAILABLE," WITH ALL FAULTS AND WITHOUT WARRANTY OF ANY KIND, AND AXOLOTL HEREBY DISCLAIMS ALL WARRANTIES AND CONDITIONS OF ANY KIND, EITHER EXPRESS, IMPLIED, OR STATUTORY, INCLUDING, BUT NOT LIMITED TO, THE IMPLIED WARRANTIES AND/OR CONDITIONS OF MERCHANTABILITY, OF SATISFACTORY QUALITY, OF FITNESS FOR A PARTICULAR PURPOSE, OF ACCURACY, OF QUIET ENJOYMENT, AND OF NONINFRINGEMENT OF THIRD-PARTY RIGHTS.
10. **LIMITATION OF LIABILITY.** TO THE EXTENT NOT PROHIBITED BY LAW, IN NO EVENT SHALL AXOLOTL BE LIABLE FOR PERSONAL INJURY OR ANY INCIDENTAL, SPECIAL, INDIRECT, EXEMPLARY OR CONSEQUENTIAL DAMAGES WHATSOEVER, INCLUDING, WITHOUT LIMITATION, DAMAGES FOR LOSS OF PROFITS, LOST OR MODIFIED DATA, BUSINESS INTERRUPTION, OR ANY OTHER COMMERCIAL DAMAGES OR LOSSES, ARISING OUT OF OR RELATED TO (I) USER'S USE OF OR INABILITY TO USE BIOINK OR (II) ANY OTHER MATTER RELATING TO BIOINK, IN EACH CASE, HOWEVER CAUSED, REGARDLESS OF THE THEORY OF LIABILITY (CONTRACT, TORT, OR OTHERWISE) AND EVEN IF AXOLOTL HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. IN NO EVENT SHALL AXOLOTL'S TOTAL LIABILITY TO USER FOR ALL DAMAGES IN AGGREGATE (OTHER THAN AS MAY BE REQUIRED BY APPLICABLE LAW IN CASES INVOLVING PERSONAL INJURY) EXCEED THE AMOUNT OF TWO HUNDRED DOLLARS (\$200.00). THE FOREGOING LIMITATIONS WILL APPLY EVEN IF THE ABOVE STATED REMEDY FAILS OF ITS ESSENTIAL PURPOSE. IF USER IS DISSATISFIED WITH ANY PORTION OF BIOINK OR WITH THIS LICENSE, USER'S SOLE AND EXCLUSIVE REMEDY IS TO DISCONTINUE USE OF BIOINK. THE PROVISIONS ABOVE IN THIS SECTION 10 DO NOT LIMIT OUR LIABILITY THAT CANNOT BE LIMITED BY LAW, INCLUDING BUT NOT LIMITED TO LIABILITY FOR FRAUD AND DEATH OR PERSONAL INJURY CAUSED BY OUR NEGLIGENCE.
11. **INDEMNITY AND RELEASE.** User agrees to defend, release, indemnify and hold Axolotl and its affiliates and its officers, employees, directors and agents harmless from any from any and all losses, damages, expenses, including reasonable attorneys' fees, rights, claims, actions of any kind and injury (including death) arising out of or relating to (a) User's use of BioInk, (b) User's violation of or failure to comply with this Limited Use License, (c) User's violation of any rights (including intellectual property rights) of Axolotl or any third party, or (d) User's modification, use or resale of BioInk.
12. **EXPORT RESTRICTIONS.** User may not use or otherwise export or re-export BioInk except as authorized by United States law and the laws of the jurisdiction in which BioInk was obtained. In particular, but without limitation, BioInk may not be exported or re-exported (a) into any U.S. embargoed countries or (b) to anyone on the U.S. Treasury Department's Specially Designated Nationals List or the U.S. Department of Commerce Denied Persons List or Entity List. By using BioInk, User represents and warrants that User is not located in any such country or on any such list. User also agrees that User will not use these products for any purposes prohibited by United States law, including, without limitation, the development, design, manufacture, or production of chemical or biological weapons.
13. **CHOICE OF LAW.** This License, its construction, and User's use of BioInk will be governed by and construed in accordance with the laws of the State of Washington without regard to the conflict of laws rules thereof that would require the application of the law of a different jurisdiction.
14. **MISCELLANEOUS.** Axolotl's failure to exercise any rights under the Limited Use License is not a waiver of its rights to damages for User's breach of contract and is not a waiver of any subsequent breach. If any provision or part of the Agreement is found by any court of competent jurisdiction to be invalid or unenforceable, such invalidity or unenforceability will not affect the other provisions of the Agreement. No person other than User or Axolotl will have any rights under the Agreement. Headings are for convenience only and shall not be used in the interpretation of this Limited Use Agreement. Any notice or communication required or permitted under this Limited Use License must be in writing and will be deemed received when personally delivered, or three (3) business days after being sent by certified mail, postage prepaid, to a party's specified address. No waiver, consent, modification, amendment or changes to the terms of this Limited Use License will be binding unless in writing and signed by both parties. Axolotl's failure to object to terms contained in any subsequent communication from User will not be a waiver or modification of our Agreement.