

SUPPORTING INFORMATION FOR

Continuous Flow Delivery System for the Perfusion of Scaffold-based 3D Cultures

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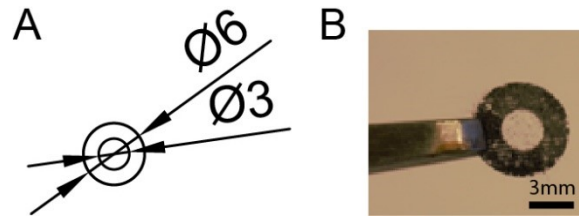


Figure S1. A) Schematic with dimensions listed in millimeters and B) photograph of the paper scaffolds used in this work. Each scaffold had a 3 mm diameter region of exposed cellulose fibers surrounded by a solid ring of wax.

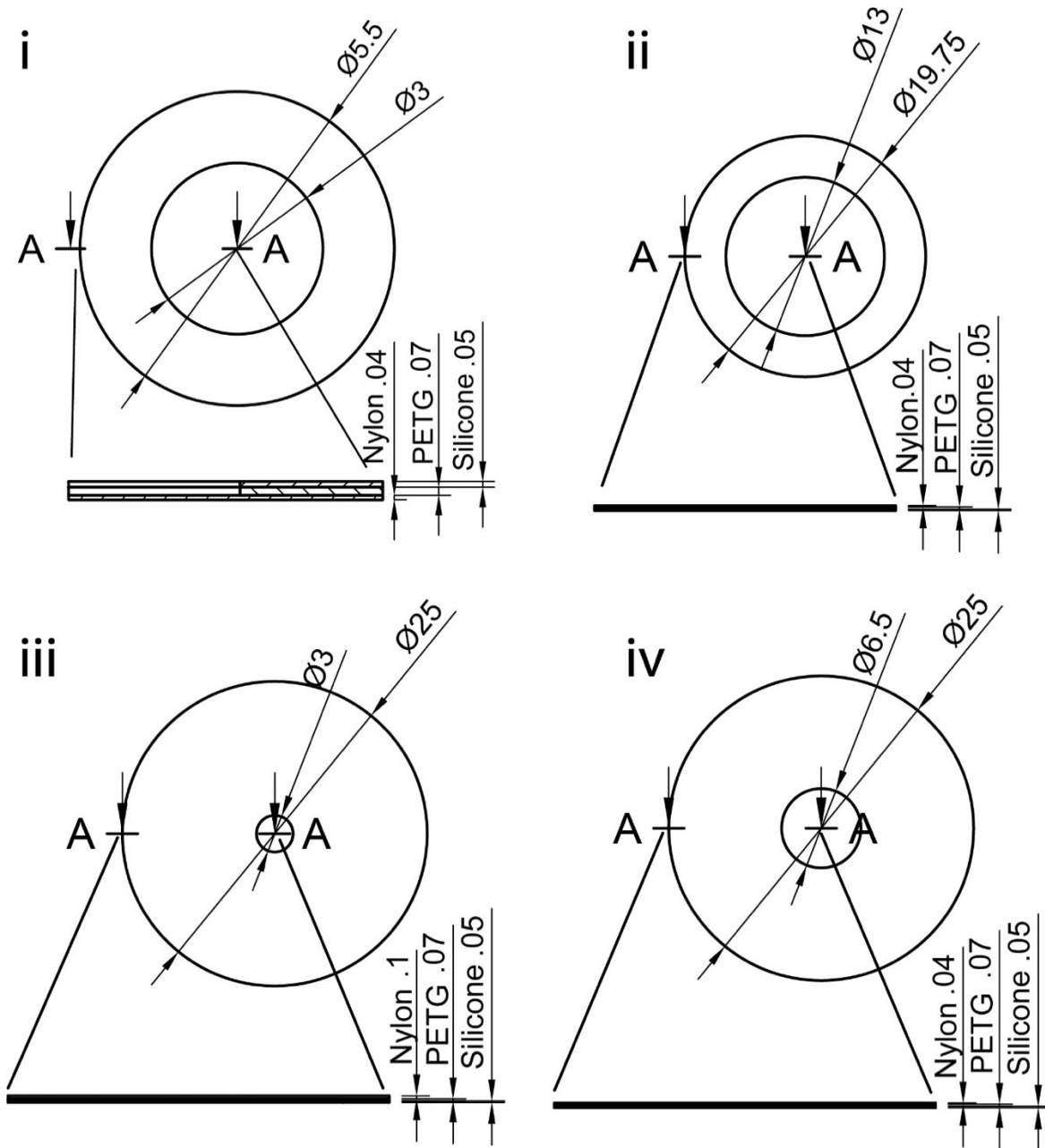


Figure S2. Schematics of the supported gel slab (SGS) scaffolds used in this work, which i) fit directly into a 96-well plate, ii) fit directly into a 12-well plate, iii) supported the culture of HUV-EC-C cells, and iv) were stacked to form a spheroid-on-demand structure. Scaffolds i), ii), and iv) had a nylon mesh bottom piece with an average pore size of 5.0 μm . These scaffolds were deposited with cells suspended in ECM. Scaffold iii) had a nylon mesh with an average pore size of 0.1 μm . The HUV-EC-C cells were deposited directly on these nylon bottom pieces. The scale of the schematics is mm.

Deposition of the HUV-EC-C cells.

The HUV-EC-C cells were deposited directly onto the nylon bottom piece (0.1 μm pore size) of an SGS scaffold. The scaffolds were placed in a custom-designed holder, whose components were laser cut from a sheet of cast acrylic or 3D printed with BioMed Clear resin on a Form 3B+ 3DP (FormLabs). **Figure S3A** contains photographs of the individual components. The (left) baseplate of the holder was filled with 3 mL of culture medium. The SGS scaffold was then sandwiched between (right) two acrylic plates, stacked atop the baseplate, and the components held in place with four stainless steel screws. Next, 50 μL of culture medium containing 1.0×10^5 HUV-EC-C cells was introduced through the hole in the top acrylic plate and then incubated under standard culture conditions to allow the cells to attach to the nylon mesh. After a 4-h incubation period, the holder was disassembled, and the SGS scaffold was placed in the culture chamber of the CFDS. **Figure S3B** is a photograph of the assembled holder.

HUV-EC-C Membrane Permeability Assay.

A custom-designed device was 3D printed from BioMed Clear resin on a Form 3B+ 3DP (FormLabs) to quantify membrane permeability of SGS scaffolds containing a monolayer of HUVEC cells. **Figure S4** contains photographs of each device component. The assembled holder placed the cell-containing scaffold between two reservoirs, which could be easily filled as the scaffold was perpendicular to the benchtop. The reservoir in contact with the HUV-EC-C cells was filled with culture medium containing sodium fluoride (NaFlu, 8 $\mu\text{g}/\text{mL}$) or a fluorescein-labeled dextran with an average nominal mass of 10 kDa (0.1 mg/mL), 40 kDa (0.2 mg/mL), or 150 kDa (0.4 mg/mL). The reservoir in contact with the cell-free nylon side of the SGS scaffold was filled with culture medium. The holder was maintained under standard culture conditions, with 50 μL aliquots from each reservoir transferred to a 96-well plate every 15 min. The fluorescence intensity of these solutions was measured on a SpectraMax i3x Multimode microplate reader ($\lambda_{\text{ex}} = 488 \text{ nm}$ and $\lambda_{\text{em}} = 520 \text{ nm}$). The culture medium used in these experiments was phenol-free DMEM/F12 supplemented with 10% FBS, 25 mM HEPES, 1% PenStrep, 0.1 mg/mL heparin, and 30 $\mu\text{g}/\text{mL}$ endothelial cell growth factor.

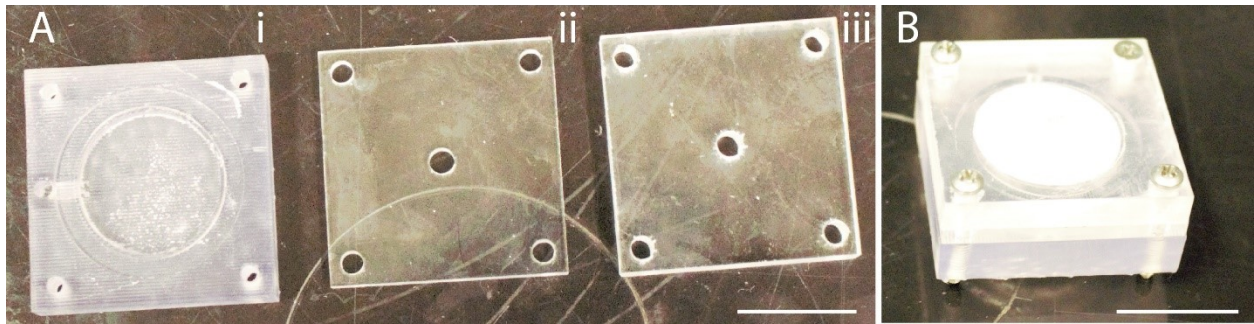


Figure S3. Photographs of A) the components and B) the assembled holder used to deposit the HUV-EC-C cells onto the nylon mesh bottom piece of the SGS scaffold. Component Ai) is the baseplate of the holder, and it was printed on a Form Labs 3B+ 3D printer following the fabrication procedure below. Components in Aii) and Aiii) were used to hold the SGS scaffold in place and ensure that both sides of the nylon mesh bottom piece were in contact with culture medium and the cell containing culture medium was localized to above the culture region. These components were laser cut from cast acrylic (McMaster Carr) on an Omtech 55W CO₂ laser cutter (35% power, 8 mm/sec). Component Aii) was 3.175 mm thick and required two passes of the laser cutter. Component Aiii) was 6.35 mm thick and required 5 passes of the laser cutter. The top plate, Aiii, was chosen to be 6.35 mm thick to allow the center hole above the culture region to hold the entire 50 μ L of cell suspension, while the bottom plate, Aii, was chosen to be 3.125 mm thick to reduce the distance fresh medium had to diffuse to reach the culture, while maintaining a stable structure to hold the cell-containing medium above the scaffold and prevent leakage. The scale bar in both photographs represents 20 mm.



Figure S4. Photographs of A) the components and B) the assembled holder were used to evaluate the membrane permeability of the HUVEC cells deposited in the SGS scaffolds. A) Each component was printed on a Form Labs 3B+ 3D printer. The components from left to right include the lid, to maintain sterility, side 1 of the reservoirs, side 2 of the reservoirs, a 25 mm (inner diameter) silicone O-ring (McMaser-Carr), to prevent leakage, the scaffold that gets placed between the two reservoirs, either cell-free or cell-containing, and the 20 mm 4-40 316ss screws used to hold the device together (McMaster-Carr). The screw holes in side 2 of the reservoir were threaded with a 4 - 40 tap (McMaster). B) The assembled holder, which places the cell containing membrane perpendicular to the benchtop. The scale bars in both photographs represent 40 mm.

Device Fabrication and Culture Assembly.

Figure S5 is a photograph of each component used to assemble the CFDS. The baseplate for the CFDS and the single-compartment culture chambers were cut from cast acrylic sheets (McMaster) with a an Omtech 55W CO₂ laser cutter (35% power, 8 mm/sec, 5 passes). The culture medium reservoir and the culture chambers were printed with BioMed Clear resin on a Form 3B+ 3DP (FormLabs). These parts were washed two times in 99% isopropyl alcohol in a FormLabs Form Wash, soaked in fresh isopropyl alcohol for 20 min, air-dried for 3 h, and then cured in the FormLabs Form Cure for 60 min at 60 °C. The screw holes in the culture chambers were threaded with a 4 - 40 tap (McMaster). The remainder of the components were fused deposition printed with ColorFabb_XT filament (MatterHackers) with no post-processing on an Ultimaker 3 3DP (Ultimaker). Each component was sterilized in an Anprolene AN74i sterilizer (Andersen Sterilizers) or via a 2-minute soak in 70% ethanol before use.

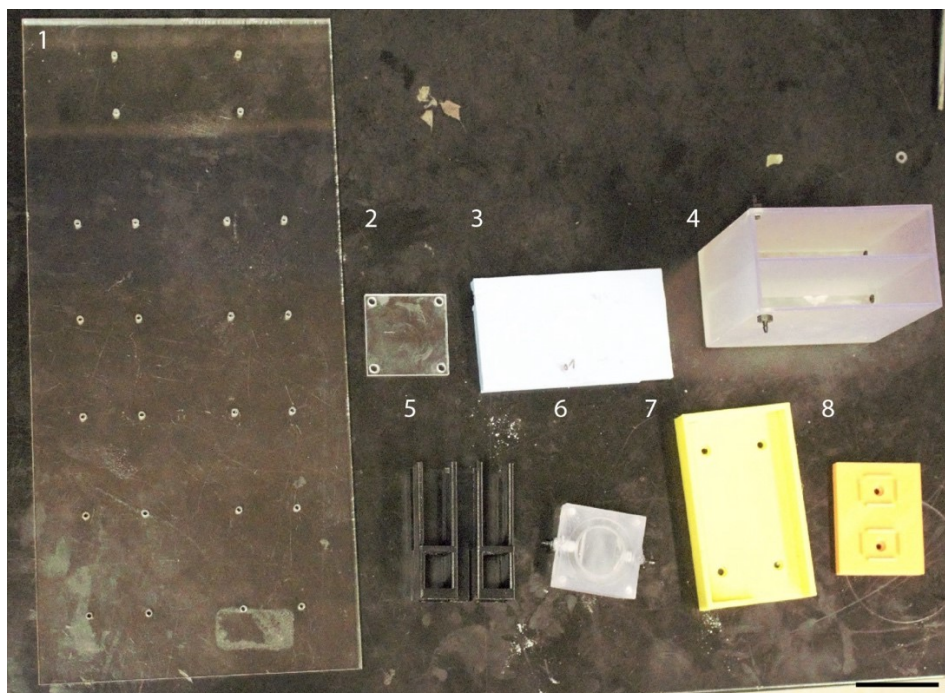


Figure S5. A photograph of the components used to construct the CFDS. 1) The base plate of the CFDS is laser cut from 6.25 mm thick cast acrylic on an Omtech 55W CO₂ laser cutter (35% power, 8 mm/sec, 5 passes). 2) Baseplate to the single-compartment culture chambers, laser cut from 3.175 mm cast acrylic on an Omtech 55W CO₂ laser cutter (35% power, 8 mm/sec, 2 passes). 3) Lid for the culture medium reservoir, printed with ColorFabb_XT filament on an Ultimaker S3 3D printer. 4) Culture medium reservoir capable of holding at least 50 mL, printed from FormLabs Biomed clear resin on a FormLabs 3B+ 3D printer. 5) Stands, which were attached to the baseplate of the CFDS and held the assembled culture chambers. These stands were printed with ColorFabb_XT filament on an Ultimaker S3 3D printer. 6) The portion of the culture chamber that held the cell-laden scaffolds and through which the culture medium passed. The chambers were printed from FormLabs Biomed clear resin on a FormLabs 3B+ 3D printer. 7) The base of the culture medium reservoir, which was printed with ColorFabb_XT filament on an Ultimaker S3 3D printer and attached to the baseplate of the CFDS. 8) The base of the culture chamber stands, which was printed with ColorFabb_XT filament on an Ultimaker S3 3D printer and attached to the baseplate of the CFDS. The screw holes in the culture chambers were threaded with a 4 - 40 tap (McMaster). Detailed manufacturing procedures can be found above. The scale bar represents 40 mm.

The associated 3D printing (STL) files for these components are part of a Mendeley Data set, which can be found at: DOI: 10.17632/3ts7hjj2tn.1

Consumables used to prepare the components needed for the CFDS, paper scaffolds, and SGS scaffolds.

	Material	Company	Catalog Number	Notes	Cost ^a
1	Silpuran Film	Hisco	60099747-54764	20x15 cm sheets, 20 total	\$1,201.40
	Nylon Mesh (7 um)	Component Supply	U-CMN-7	1 yard	\$279.69
	Nylon Mesh (0.1 um)	Sterlitech	NY012005	200 x 200 mm	\$154.72
	LensX 90 paper	Berkshire Corp.	LensX90	23x23 cm sheets, 1000 total	\$65.99
2	Cast acrylic sheet	McMaster Carr	8560K354	12x12x0.25"	\$18.38
	High-temperature soft silicone O-ring	McMaster Carr	1173N214	pack of 10	\$15.32
	Metric 316 stainless steel pan head Phillips screw	McMaster Carr	90116A022	pack of 100	\$14.29
	Tight-seal plastic tube fittings	McMaster Carr	5463K2	pack of 10	\$6.38
	BioMed Clear resin (Form 3 printer)	FormLabs	RS-F2-BMCL-01	1L of resin	\$349
	ColorFabb_XT copolyester filament	ColorFabb USA	XT-Black	1.755 mm diameter, 750 g	\$26.75

¹ Materials used to prepare the paper and SGS scaffolds, excluding the Xerox Wax Printer and Omtech Laser Cutter

² Materials used to prepare the CFDS, excluding the FormLabs 3 STL printer, the QidiTech extrusion printer, and Omtech Laser Cutter

^a Cost of items based on website prices, July 2024

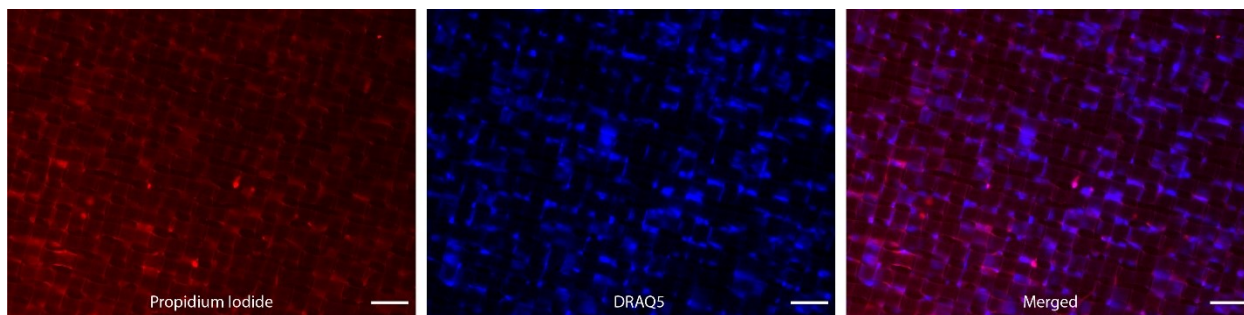


Figure S6. Representative widefield fluorescence micrographs of SGS scaffolds with a nylon mesh bottom piece (5.0 μm pore size) deposited with 1.0×10^4 M231 cells suspended in ECM. The cells were stained with propidium iodide (PI) and DRAQ5. These stains were used to evaluate the relative viability of the cells, maintained for 72 h under continuous perfusion (0.46 mm/sec). The micrographs were collected on a Nikon TE-2000 inverted fluorescent microscope with a 10X/0.8 Plan Apo objective: $\lambda_{\text{ex, PI}} = 560$ BP40 nm, $\lambda_{\text{em, PI}} = 630$ BP70 nm; $\lambda_{\text{ex, DRAQ5}} = 620$ BP50 nm, $\lambda_{\text{em, DRAQ5}} = 690$ BP50 nm. The cells were counted manually using the multipoint tool in ImageJ. The scale bars represent 100 μm .

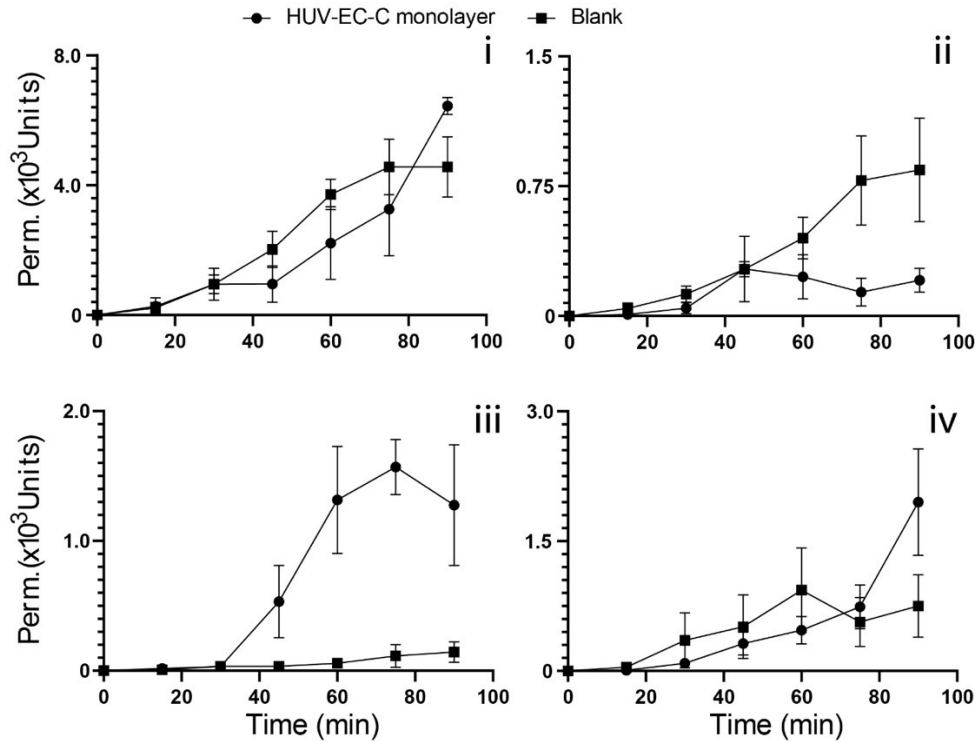


Figure S7. Plots assessing the average membrane permeability of the 0.1 μm nylon filter in an SGS scaffold, with (●) or without (■) HUV-EC-C cells. The scaffolds separated a 1X PBS solution from a solution of 1X PBS containing i) sodium fluorescein, ii) 10 kDa fluorescein-labeled dextran, iii) 40 kDa fluorescein-labeled dextran, or iv) 150 kDa fluorescein-labeled dextran. The fluorescence intensity of the 1X PBS solution was measured over 90 minutes. Each data point represents at least 4 scaffolds prepared from at least 2 cell passages.

R Studio code for comparing viability profiles in the tumor-on-demand stacks.

```
library(readxl)
source("pairtest.R")
invasion<-read_excel("3.xlsx",col_names = FALSE) # the excel name can be changed
here
#rowSums(invasion)
invasionper<-invasion/rowSums(invasion)
groupsize<-dim(invasionper)[1]/2
print(groupsize)

#Orthogonal transformation
invarionorthogonal1<-invasionper[,1]*(-3)+invasionper[,2]*(-
1)+invasionper[,3]*1+invasionper[,4]*3
invarionorthogonal2<-invasionper[,1]*1+invasionper[,2]*(-1)+invasionper[,3]*(-
1)+invasionper[,4]*1
invarionorthogonal3<-invasionper[,1]*(-1)+invasionper[,2]*3+invasionper[,3]*(-
3)+invasionper[,4]*1
invarionorthogonal<-
cbind(invarionorthogonal1,invarionorthogonal2,invarionorthogonal3)
tmpdata = invarionorthogonal[1:groupsize,]-
invarionorthogonal[(groupsize+1):(2*groupsize),]

for (i in 1:3){
  print(shapiro.test(tmpdata[,i])$p)
}

#original
pairalltest(tmpdata) #1st row, t test p-value; 2nd row, step-down p-value; 3rd, one-sample
Wilcoxon signed rank test; 4th, step-down p-value
#use 2nd row if normal holds for all variables

#power transform (if any of 3 orthogonal transformed variables is not normal)
addvalue=1.5 # make all data positive
indexlocal=c(rep(1,groupsize),rep(0,groupsize))
invasionorthogonallocal=cbind(as.numeric(unlist(invarionorthogonal[,1])),as.numeric(unli
st(invarionorthogonal[,2])),
as.numeric(unlist(invarionorthogonal[,3])))
translambda=powerTransform((invasionorthogonallocal+1.5) ~ indexlocal)$lambda
transtmp = invasionorthogonallocal+1.5
transnew = transtmp
for (i in 1:dim(transtmp)[2]){
  transnew[,i]=BoxCox(transtmp[,i],translambda[i])
}
tmpdata = transnew[1:groupsize,]-transnew[(groupsize+1):(2*groupsize),]
```

```

for (i in 1:3){
  print(shapiro.test(tmpdata[,i])$p)
}

```

```

pairalltest(tmpdata)

```

R Studio source code for “pairtest.R”.

```

stepdown<-function(orderedpvalues){
  pp1 = orderedpvalues[1]*3; pp2=max(pp1, (2*orderedpvalues[2])); pp3=max(pp2,
orderedpvalues[3])
  return(c(pp1,pp2,pp3))
}

```

```

pairalltest<-function(tmpdata){ #the difference size = (L, 3)
  ttestresults=c(t.test(tmpdata[,1])$p.value, t.test(tmpdata[,2])$p.value,
t.test(tmpdata[,3])$p.value)
  print(ttestresults)
  print(stepdown(ttestresults[order(ttestresults)]))
  wtestresults=c(wilcox.test(tmpdata[,1])$p.value, wilcox.test(tmpdata[,2])$p.value,
wilcox.test(tmpdata[,3])$p.value)
  print(wtestresults)
  print(stepdown(wtestresults[order(wtestresults)]))
}

```