micromachines

# Supplementary Information: Novel Platform for Regulation of Extracellular Vesicles and Metabolites Secretion from Cells Using a Multi-Linkable Horizontal Co-Culture Plate

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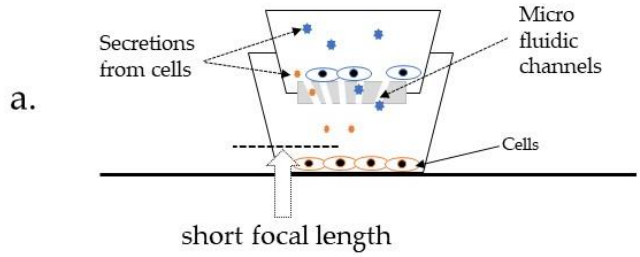
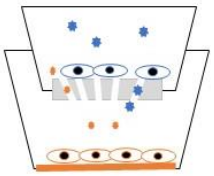
**Supplementary information**



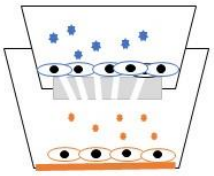




Figure S1. The three main problems associated with a vertical-type co-culture plate (VTCP). (a) The upper container cannot be observed because of the short focal length of the microscope. (b) The surface material to which the cells adhere differs, meaning that the cells may respond differently. (c) The high density of cells may prevent the exchange of materials.

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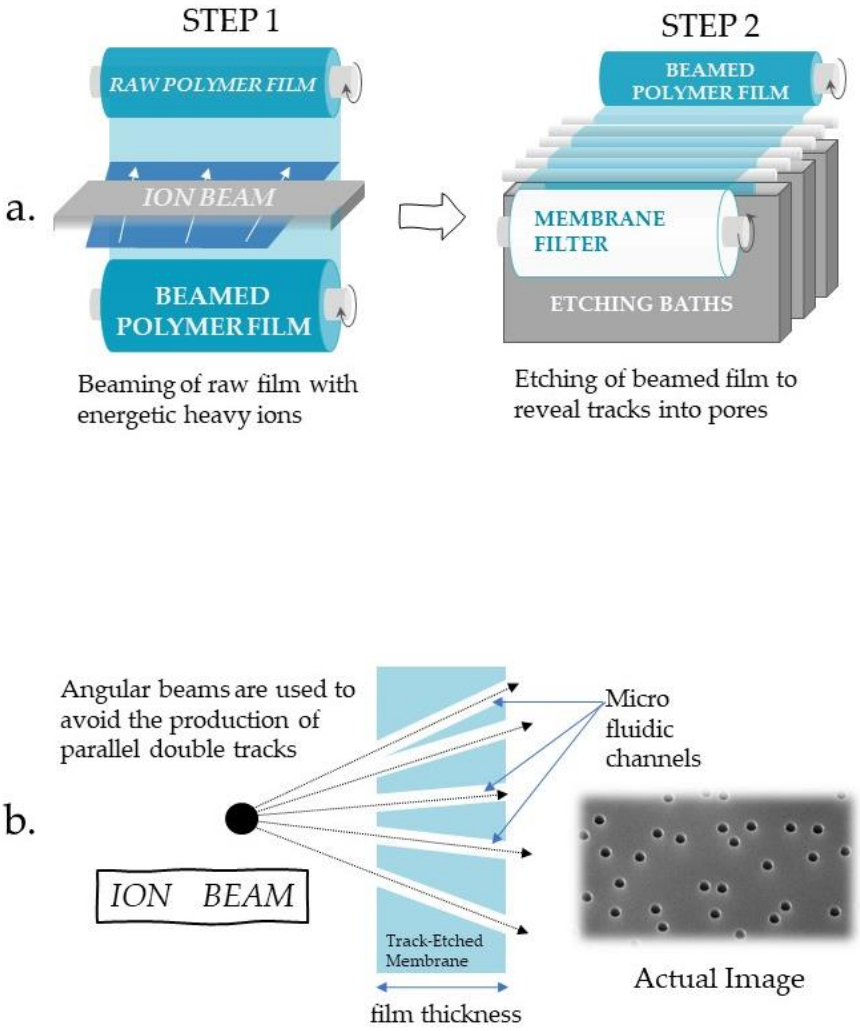



Figure S2. (a) Track-etched membranes were fabricated by irradiating a raw polymer film with energetic heavy ions. (b) The beamed film was then chemically etched to make uniform and precise pores. Pore density and pore orientation were determined during the beaming step, while the etching conditions defined the pore size.

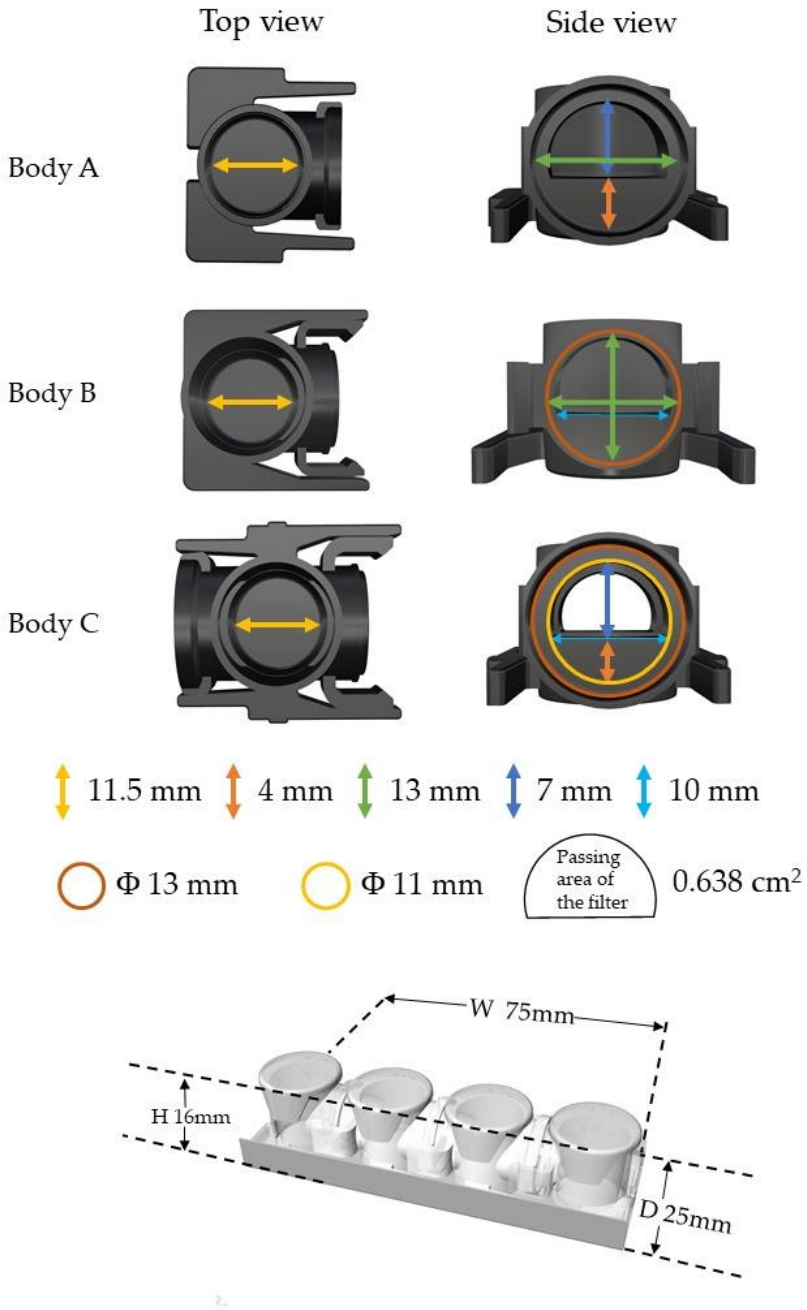



Figure S3. Size of each component. The size of each component is described in the figure. The top cover of the main body was made of polystyrene, the common sidewall cover was constructed using low-density polyethylene, and the O-ring was made of silicone. The O-ring was hydrophilized via corona discharge. All materials used for building the main body of the HTCP were sterilized with electron beams.

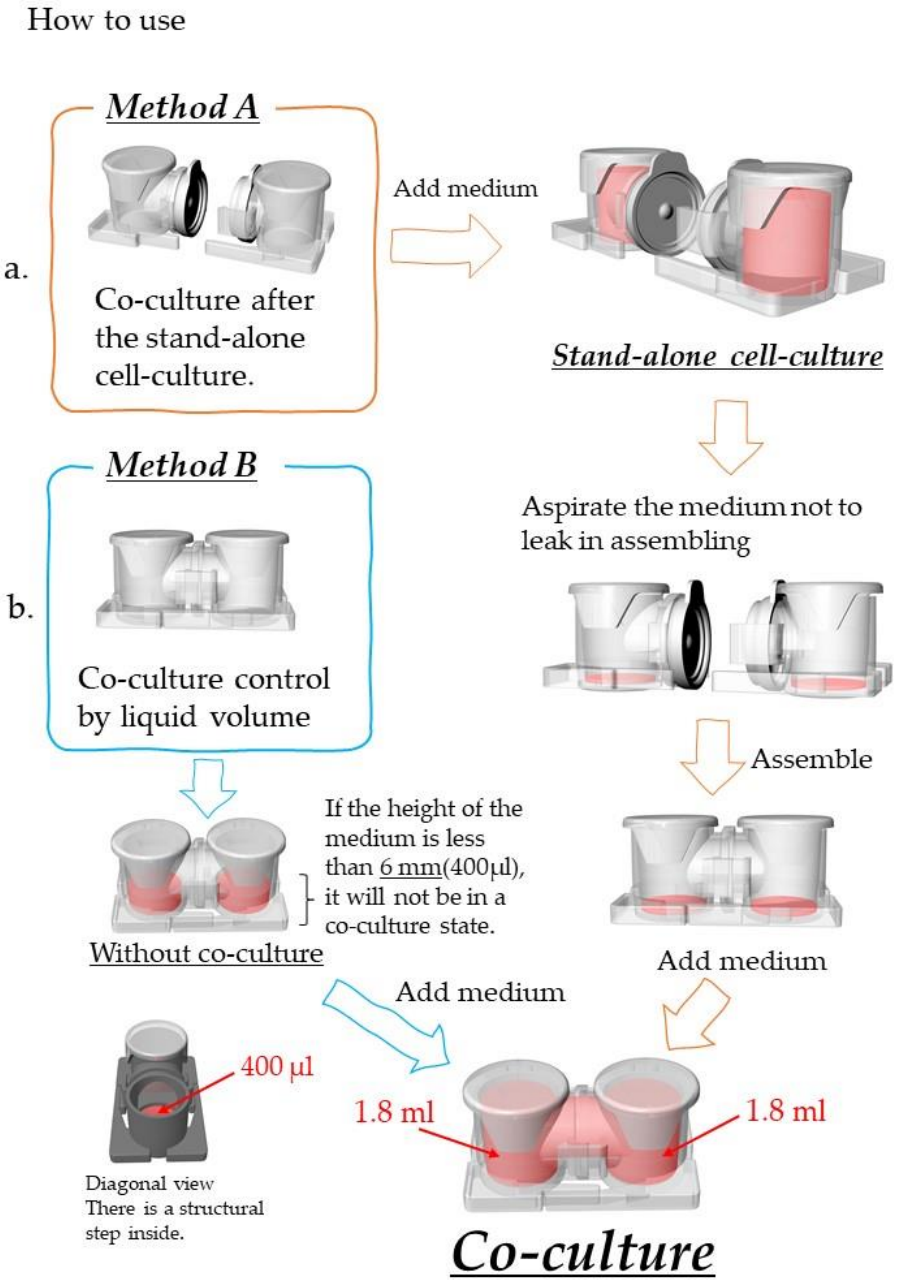



Figure S4. How to use. Methods A (Initial monoculture mode) and B (Con-trolling co-culture with medium volume). (a) In a stand-alone way, the cell types are cultured separately and then co-cultured by connecting the two containers (combined state) without dam-aging the cells. Bodies A and B are designed to allow the use of separated cell culturing. (b) In the connected mode, the two containers are joined from the beginning of cell culture. The height (volume) of the culture medium is used to control the amount of liquid that traverses the two containers, controlling the extent of co-culturing. Re-separation is possible. After reducing the amount of culture medium, one can separate bodies A and B by applying force to pull them apart to the left and right, and then cover the sides with a side cover and increase the amount of culture medium to create a monoculture. However, as this is a plastic product, recombination after separation could be problematic.

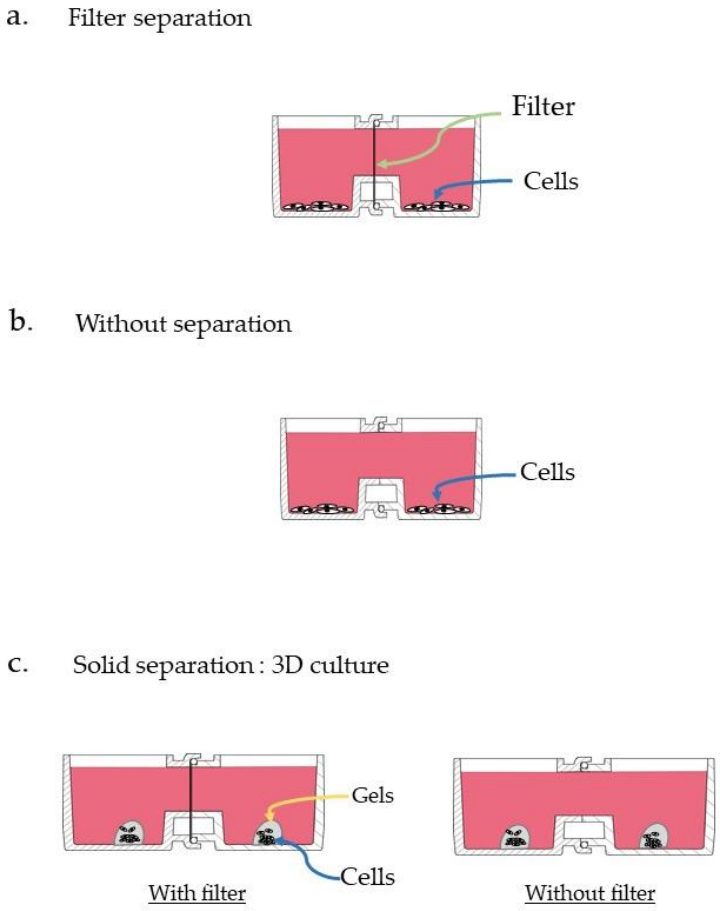



Figure S5. Schematic of various use methods: (a) filter separation, (b) without separation, and (c) solid separation with gels (three-dimensional culture).

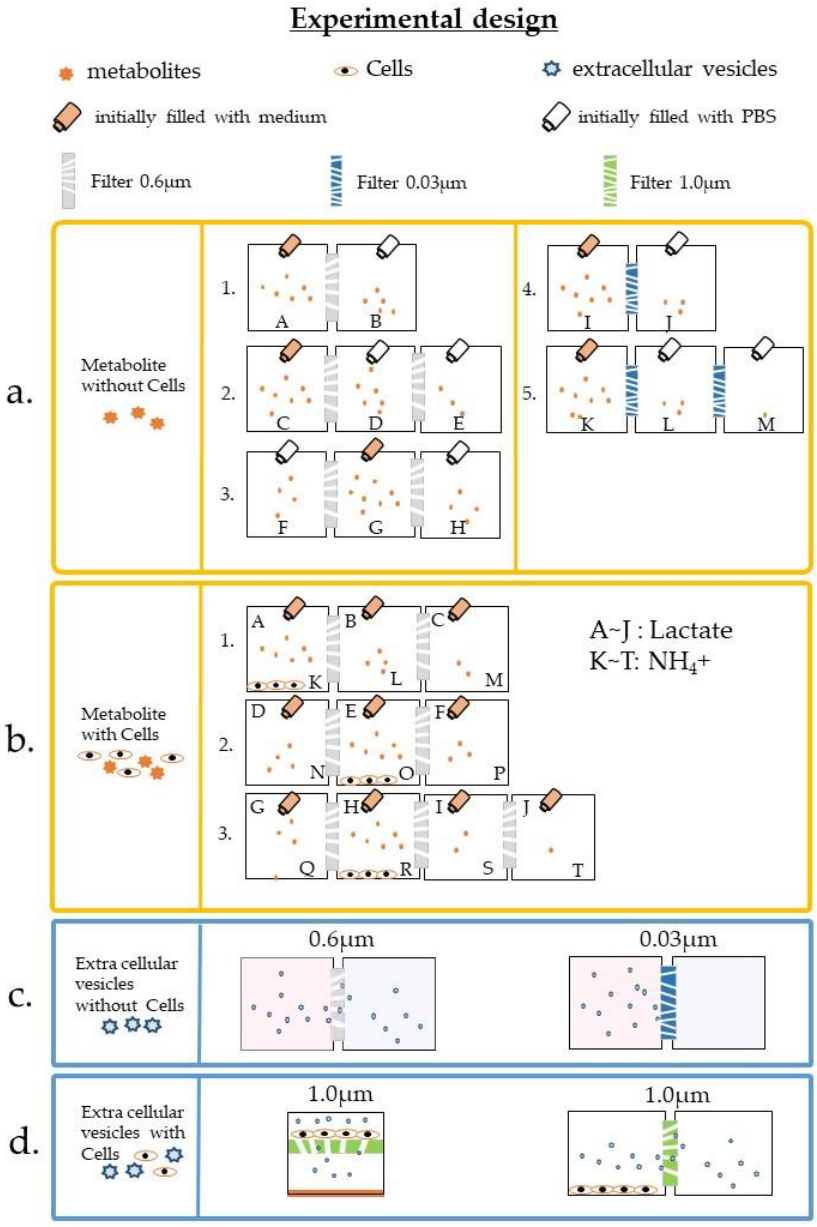



Figure S6. Summary of experimental design. (a) Differences in mass transfer due to the number of containers, position of the medium, and pore size of the filter were measured. All the measurements were performed under cell-free conditions. (b) Differences in metabolite concentrations due to differences in the positions of the containers in which the cells were placed were measured. (c) The filter passability of exosomes was measured. (d) Comparison of the passability of exosomes between VTCP and HTCP.

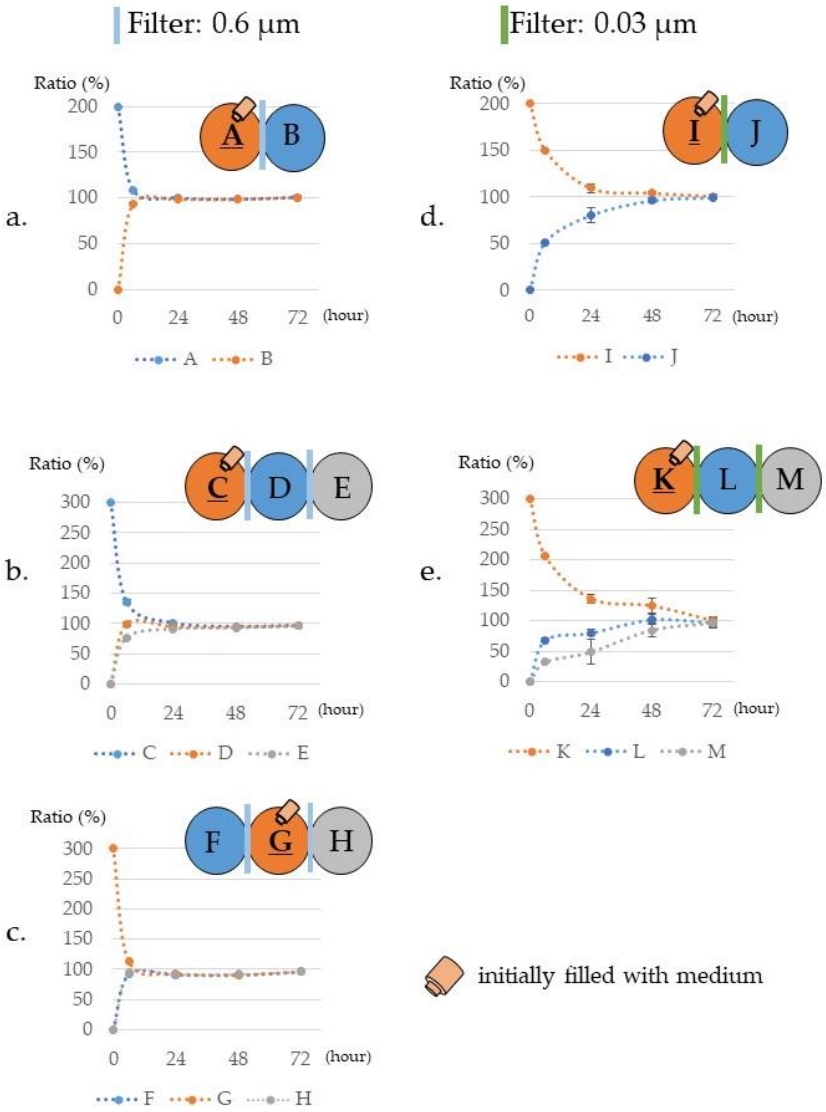
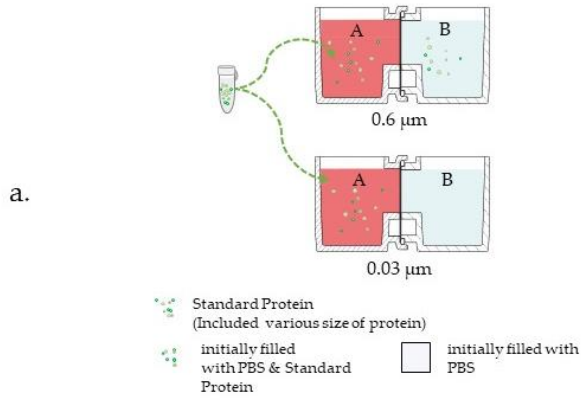



Figure S7. The results of NH4+ transfer without cells. Orange-colored wells with clip art of bottle were filled with medium including glucose. The remaining wells were filled with phosphate-buffered saline (PBS), which did not contain glucose. When all wells were mixed, the final concentration was converted to 100 %. The vertical axis shows the converted concentra-tion in %. Glucose concentration in the medium was measured after 6–72 h. The vertical axis is the concentration (%), and the horizontal axis is the time (h). Filter pore size is 0.6 μm or 0.03μm.



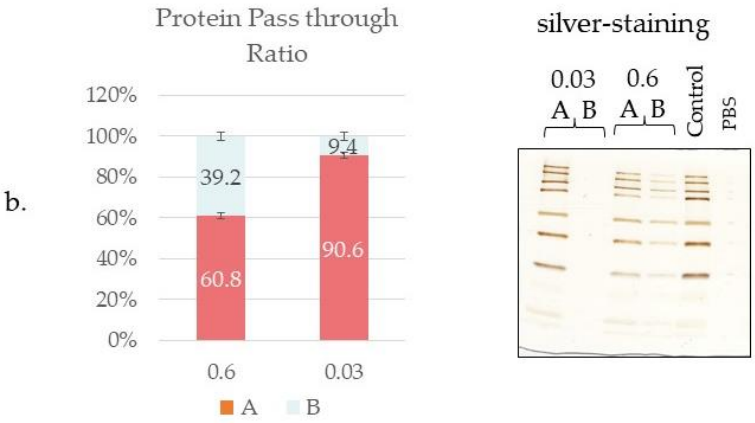




Figure S8. Migration of proteins through filters (A) Equal amounts of stand-ard proteins were added to one side of the horizontal type co-culture system (HTCP) to test the ability of proteins to pass through filters of different sizes. (B) Proteins passing through the 0.6 μm and 0.03 μm filters were measured with silver staining.

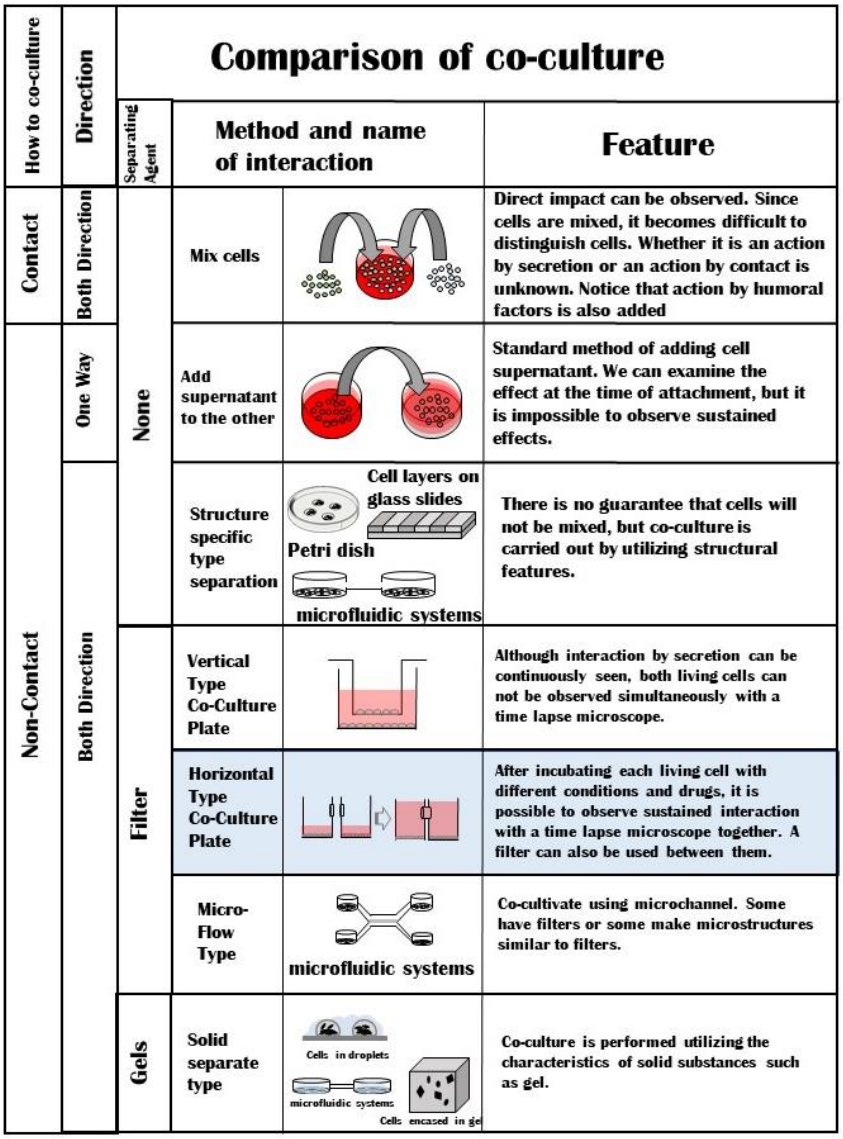



Figure S9. Various co-culture methods have been introduced in this study. Co-culture methods can be divided into two categories based on whether the cells are in direct contact or not. Next, they are divided by based on the nature of the material exchange i. e. bidi-rectional or unidirectional. Finally, different types of separation materials exist. Some use filters, while others use microfluidic channels, and some use no separating material. Modified from Shimasaki et al. [1] with permission.

Differences in Co-culture Vessels by Vertical and Horizontal Binding Directions.

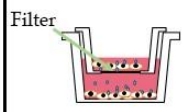
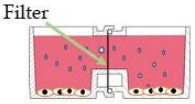
| **Type of vessels** **Connection type** | VTCP Vertical connection | HTCP Horizontal connection |
|---|---|---|
| **Image** |   |   |
| **The morphology of the cells in the two chambers can be easily visualized by microscope** | ✕ **morphology of the cells in upper chamber can not be visualized.** | ○ **Time-lapse video can be taken of the growing cell cultures in both chamber.** |
| **Both cell cultures grow on same type of plastic surface.** | ✕ | ○ |
| **A variety of different filter pore sizes can be used; changing the maximum size of molecules shared between the two culture vessels.** | △ | ○ |
| **Growth of cells do not prevent the exchange of liquid materials such as EVs by blocking the pore of filter** | △ | ○ |
| **Multi connection** | ✕ | ○ |

Figure S10. List of differences in features between VTCP and HTCP modified from Shimasaki et al. [1] with permission.

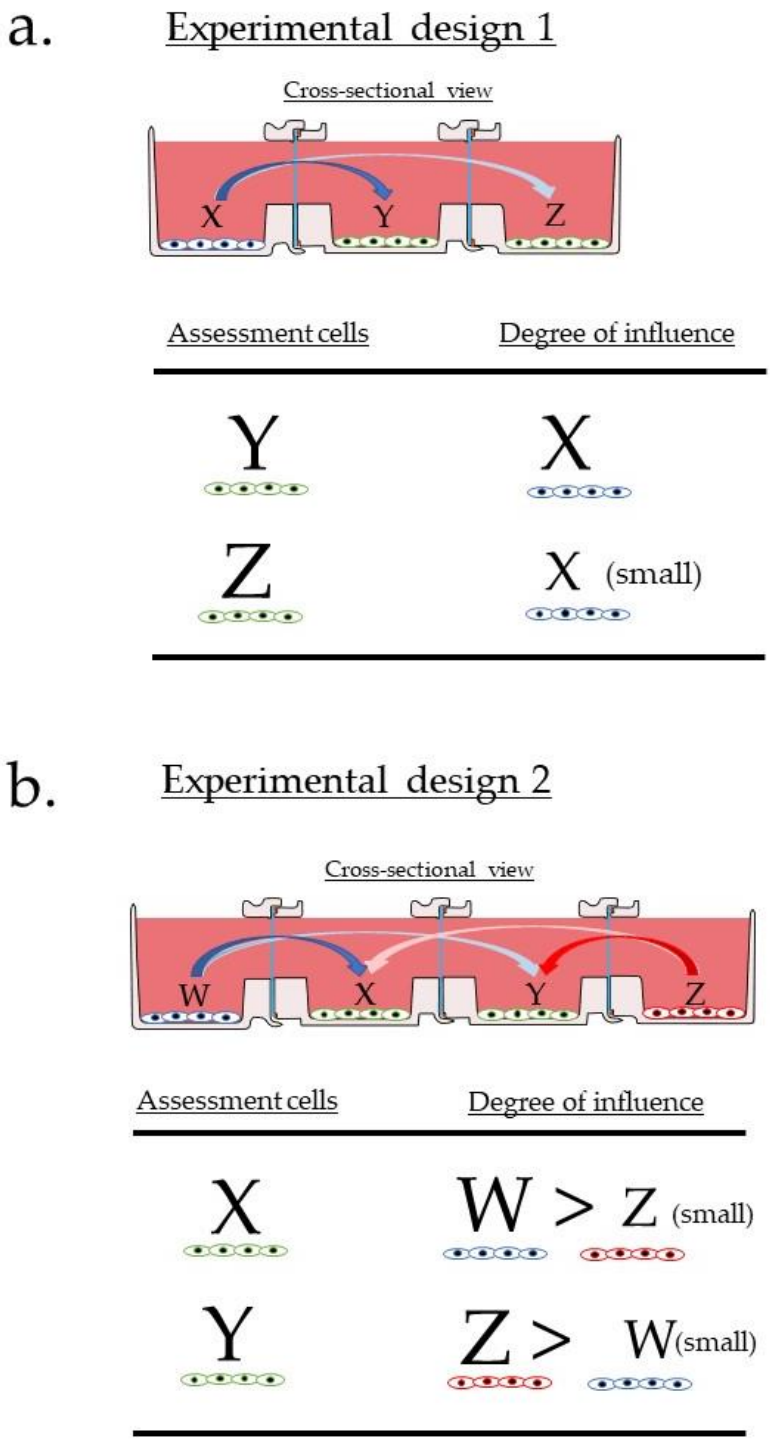



Figure S11. (a) Experimental design 1: A method to check for changes in the cells due to different degrees of influence. Different distances from the cells produce different concentrations, and changes due to different degrees of influence can be observed. (b) Experi-mental design 2: How to check the change in the two types of cells due to different degrees of in-fluence. For example, suppose X and Y are the same cells, and W and Z are different cells from X and Y. We can confirm that X and Y show different changes owing to the different influences of W and Z.

Filter material/ pore size/ density/ area

| Material | Filter Size(Φ) ( area ) | Pore Size(Φ) | Pore density (Number)/ $cm^2$ | Number of effective pores | Total area of effective pore area | Ratio of open pore area to filter area |
|---|---|---|---|---|---|---|
| Polycarbonate | 13mm (1.327 $cm^2$) | 0.03μm | $6.00x10^8$ | $4.00x10^8$ | $2.71x10^{-3}$ $cm^2$ | 0.424% |
| Polycarbonate | 13mm (1.327 $cm^2$) | 0.6μm | $4.00x10^8$ | $2.55x10^8$ | $7.22x10^{-1}$ $cm^2$ | 11.311% |

Table S1. The density and number of pores in each filter were described. The area of the filter pores as a percentage of the effective filter area in the passage was calculated and expressed as the percentage of open pores.

| | |
|---|---|
| (A) $y = 6E\text{-}05X^2 + 0.0027X + 0.0043$ | $R^2 = 0.9999$ |
| (B) $y = 5E\text{-}05X^2 + 0.0004X - 0.0014$ | $R^2 = 0.9999$ |
| (C) $y = 4E\text{-}05X^2 + 0.0001X - 0.0038$ | $R^2 = 0.9987$ |
| (D) $y = 4E\text{-}05X^2 + 0.0002X + 0.0062$ | $R^2 = 0.9992$ |
| (E) $y = 5E\text{-}05X^2 + 0.0005X + 0.0154$ | $R^2 = 1$ |
| (F) $y = 4E\text{-}05X^2 + 0.0004X + 0.0065$ | $R^2 = 1$ |
| (G) $y = -2E\text{-}06X^3 + 0.0003X^2 - 0.0065X + 0.0368$ | $R^2 = 1$ |
| (H) $y = -1E\text{-}06X^3 + 0.0002X^2 - 0.0046X + 0.04$ | $R^2 = 1$ |
| (I) $y = -2E\text{-}06X^3 + 0.0002X^2 - 0.0056X + 0.0325$ | $R^2 = 1$ |
| (J) $y = -2E\text{-}06X^3 + 0.0002X^2 - 0.007X + 0.0333$ | $R^2 = 1$ |
| (K) $y = -2E\text{-}06X^3 + 0.0002X^2 + 0.0003X + 0.139$ | $R^2 = 1$ |
| (L) $y = -3E\text{-}06X^3 + 0.0003X^2 - 0.004X + 0.1603$ | $R^2 = 1$ |
| (M) $y = -1E\text{-}06X^3 + 0.0001X^2 - 0.0002X + 0.1397$ | $R^2 = 1$ |
| (N) $y = -2E\text{-}06X^3 + 0.0002X^2 - 0.0029X + 0.1461$ | $R^2 = 1$ |
| (O) $y = -9E\text{-}07X^3 + 0.0001X^2 + 0.0013X + 0.1251$ | $R^2 = 1$ |
| (P) $y = -2E\text{-}06X^3 + 0.0002X^2 - 0.0029X + 0.149$ | $R^2 = 1$ |
| (Q) $y = -2E\text{-}06X^3 + 0.0003X^2 - 0.0045X + 0.1574$ | $R^2 = 1$ |
| (R) $y = -2E\text{-}06X^3 + 0.0003X^2 - 0.0041X + 0.1582$ | $R^2 = 1$ |
| (S) $y = -3E\text{-}06X^3 + 0.0003X^2 - 0.0053X + 0.1476$ | $R^2 = 1$ |
| (T) $y = -2E\text{-}06X^3 + 0.0003X^2 - 0.0046X + 0.1455$ | $R^2 = 1$ |

Y(g/L) X (Hour)

Table S2. The list of correlation approximation equations and correlation coef-ficients is shown in Figure 6.

| Condition | Figure No. | Objects | Location | Time | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 0 h | 6 h | 24 h | 48 h | 72 h | 6 h | 24 h | 48 h | 72 h |
| | | | | mean | mean | mean | mean | mean | S.E. | S.E. | S.E. | S.E. |
| | | Glucose 0.6μm | A | 200 | 119.54766 | 99.363057 | 97.160883 | 97.121212 | 1.6 | 1.0 | 0.3 | 0.5 |
| | | Glucose 0.6μm | B | 0 | 84.006462 | 94.267516 | 95.741325 | 96.969697 | 1.3990717 | 0.7297095 | 0.2731941 | 0.6943297 |
| | | Glucose 0.6μm | C | 300 | 169.67419 | 110 | 96.897375 | 93.693694 | 3.7089345 | 1.6898057 | 1.8018698 | 1.4065311 |
| | | Glucose 0.6μm | D | 0 | 93.233083 | 93.658537 | 93.556086 | 91.441441 | 4.186289 | 2.195122 | 1.6535091 | 0.7802031 |
| | | Glucose 0.6μm | E | 0 | 46.115288 | 82.682927 | 90.930788 | 89.864865 | 0.4340979 | 1.2673542 | 0.7159905 | 0.6756757 |
| | | Glucose 0.6μm | F | 0 | 84.210526 | 96.097561 | 97.852029 | 95.720721 | 0 | 1.1177014 | 1.0936935 | 1.4065311 |
| Medium | Figure 4. | Glucose 0.6μm | G | 300 | 139.84962 | 101.70732 | 98.806683 | 95.720721 | 1.5037594 | 1.2673542 | 1.4319809 | 1.7004132 |
| | | Glucose 0.6μm | H | 0 | 83.9599 | 96.341463 | 98.329356 | 95.495495 | 0.4340979 | 1.5231702 | 1.8018698 | 1.0321116 |
| | | | | 0 h | 6 h | 24 h | 48 h | 72 h | 6 h | 24 h | 48 h | 72 h |
| | | | | mean | mean | mean | mean | mean | S.E. | S.E. | S.E. | S.E. |
| | | Glucose 0.03μm | I | 200 | 192.73021 | 166.40127 | 129.49527 | 115.60606 | 1.008885 | 1.9888529 | 2.8522305 | 1.1439143 |
| | | Glucose 0.03μm | J | 0 | 11.470113 | 37.738854 | 65.457413 | 82.121212 | 0.2798143 | 0.8274128 | 4.8564051 | 3.4116152 |
| | | Glucose 0.03μm | K | 300 | 294.48622 | 247.07317 | 189.02148 | 156.53153 | 2.4169551 | 1.5231702 | 6.9047619 | 6.8016526 |
| | | Glucose 0.03μm | L | 0 | 18.045113 | 55.853659 | 80.668258 | 89.414414 | 2.2556391 | 10.094733 | 5.8314042 | 3.333029 |
| | | Glucose 0.03μm | M | 0 | 0 | 4.3902439 | 27.684964 | 43.918919 | 0 | 2.6382083 | 5.3739047 | 6.4455351 |
| | | | | | 6 h | 24 h | 48 h | 72 h | 6 h | 24 h | 48 h | 72 h |
| | | | | | mean | mean | mean | mean | S.E. | S.E. | S.E. | S.E. |
| | | Lactate 0.6μm | A | | 0.0233333 | 0.1 | 0.2666667 | 0.4933333 | 0.008165 | 0.0141421 | 0.0285774 | 0.0432049 |
| | | Lactate 0.6μm | B | | 0.0033333 | 0.0366667 | 0.14 | 0.3 | 0.0040825 | 0.008165 | 0.0254951 | 0.0374166 |
| | | Lactate 0.6μm | C | | 0 | 0.0166667 | 0.09 | 0.1933333 | 0 | 0.0040825 | 0.0254951 | 0.0530723 |
| | | Lactate 0.6μm | D | | 0.01 | 0.03 | 0.11 | 0.2266667 | 0 | 0 | 0.0070711 | 0.0040825 |
| | | Lactate 0.6μm | E | | 0.02 | 0.0566667 | 0.1566667 | 0.3166667 | 0 | 0.0040825 | 0.0108012 | 0.0177951 |
| | | Lactate 0.6μm | F | | 0.01 | 0.0366667 | 0.11 | 0.2266667 | 0 | 0.0040825 | 0.0122474 | 0.0248328 |
| | | Lactate 0.6μm | G | | 0.0066667 | 0.0066667 | 0.13 | 0.2566667 | 0.0040825 | 0.008165 | 0.0141421 | 0.0362859 |
| | | Lactate 0.6μm | H | | 0.02 | 0.0366667 | 0.1733333 | 0.34 | 0.0070711 | 0.0177951 | 0.0285774 | 0.0561249 |
| | | Lactate 0.6μm | I | | 0.0066667 | 0.0066667 | 0.11 | 0.21 | 0.0040825 | 0.0108012 | 0.0212132 | 0.0463681 |
| with Cell | Figure 5. | Lactate 0.6μm | J | | 0 | -0.016667 | 0.07 | 0.1466667 | 0 | 0.0040825 | 0.0141421 | 0.0294392 |
| | | | | | 6 h | 24 h | 48 h | 72 h | 6 h | 24 h | 48 h | 72 h |
| | | | | | mean | mean | mean | mean | S.E. | S.E. | S.E. | S.E. |
| | | NH4+ 0.6μm | K | | 0.1466667 | 0.2266667 | 0.3933333 | 0.5133333 | 0.0040825 | 0.0177951 | 0.0470815 | 0.0163299 |
| | | NH4+ 0.6μm | L | | 0.1466667 | 0.2 | 0.3566667 | 0.4 | 0.0040825 | 0.0141421 | 0.0204124 | 0.0187083 |
| | | NH4+ 0.6μm | M | | 0.1433333 | 0.1966667 | 0.3066667 | 0.3666667 | 0.0040825 | 0.0147196 | 0.0285774 | 0.0216025 |
| | | NH4+ 0.6μm | N | | 0.1366667 | 0.1833333 | 0.32 | 0.3833333 | 0.008165 | 0.0147196 | 0.0070711 | 0.0227303 |
| | | NH4+ 0.6μm | O | | 0.1366667 | 0.2066667 | 0.3366667 | 0.4366667 | 0.0108012 | 0.0227303 | 0.0040825 | 0.0147196 |
| | | NH4+ 0.6μm | P | | 0.14 | 0.19 | 0.33 | 0.3866667 | 0 | 0.0070711 | 0.0187083 | 0.0147196 |
| | | NH4+ 0.6μm | Q | | 0.14 | 0.18 | 0.3333333 | 0.42 | 0.0470815 | 0.0141421 | 0.0147196 | 0.0254951 |
| | | NH4+ 0.6μm | R | | 0.1433333 | 0.1933333 | 0.3633333 | 0.4666667 | 0.0734847 | 0.0070711 | 0.0318852 | 0.0815475 |
| | | NH4+ 0.6μm | S | | 0.1266667 | 0.1666667 | 0.3233333 | 0.3833333 | 0.0441588 | 0.0122474 | 0.0108012 | 0.0530723 |
| | | NH4+ 0.6μm | T | | 0.1266667 | 0.1533333 | 0.2766667 | 0.3333333 | 0.0681909 | 0.0040825 | 0.0326599 | 0.0177951 |
| | | | | | | 24 h | 48 h | 72 h | | 24 h | 48 h | 72 h |
| Medium | Figure 7. | | | | | mean | mean | mean | | S.E. | S.E. | S.E. |
| | | EV in VTCP 1.0μm | | | | 7% | 16% | 19% | | 0% | 6% | 3% |
| | | EV in HTCP 1.0μm | | | | 23% | 34% | 41% | | 3% | 2% | 6% |
| | | | | 0 h | 6 h | 24 h | 48 h | 72 h | 6 h | 24 h | 48 h | 72 h |
| | | | | mean | mean | mean | mean | mean | S.E. | S.E. | S.E. | S.E. |
| | | NH4+ 0.6μm | A | 200 | 108.49673 | 100 | 98.484848 | 100.72402 | 1.1320594 | 1.0188534 | 0 | 0.763018 |
| | | NH4+ 0.6μm | B | 0 | 93.464052 | 98.235294 | 98.484848 | 100.8092 | 1.1320594 | 1.0188534 | 1.5151515 | 1.526036 |
| | | NH4+ 0.6μm | C | 300 | 135.45455 | 100.76923 | 94.83871 | 97.086777 | 4.1659779 | 2.6646936 | 0 | 0.6855645 |
| | | NH4+ 0.6μm | D | 0 | 99.090909 | 94.615385 | 93.548387 | 95.971074 | 4.1659779 | 2.3076923 | 1.1174521 | 0.9116057 |
| | | NH4+ 0.6μm | E | 0 | 76.363636 | 90.769231 | 92.903226 | 95.929752 | 2.7272727 | 1.3323468 | 0 | 1.5789474 |
| | | NH4+ 0.6μm | F | 0 | 92.727273 | 91.538462 | 90.967742 | 96.714876 | 0 | 1.3323468 | 1.9354839 | 0.9116057 |
| Medium | Supplemental Figure A7 | NH4+ 0.6μm | G | 300 | 112.72727 | 93.076923 | 90.967742 | 96.301653 | 1.5745916 | 2.6646936 | 1.9354839 | 0.9116057 |
| | | NH4+ 0.6μm | H | 0 | 93.636364 | 91.538462 | 92.258065 | 96.528926 | 1.5745916 | 1.3323468 | 2.9565004 | 0.9116057 |
| | | | | 0 h | 6 h | 24 h | 48 h | 72 h | 6 h | 24 h | 48 h | 72 h |
| | | | | mean | mean | mean | mean | mean | S.E. | S.E. | S.E. | S.E. |
| | | NH4+ 0.03μm | I | 200 | 149.6732 | 109.41176 | 104.54545 | 100.91567 | 1.1320594 | 4.6689729 | 1.5151515 | 2.6431718 |
| | | NH4+ 0.03μm | J | 0 | 51.633987 | 80.588235 | 96.464646 | 99.63799 | 1.1320594 | 7.9574996 | 2.3144322 | 1.3215859 |
| | | NH4+ 0.03μm | K | 300 | 205.45455 | 135.38462 | 123.87097 | 99.380165 | 1.5745916 | 6.6617339 | 12.087093 | 7.4618141 |
| | | NH4+ 0.03μm | L | 0 | 68.181818 | 80 | 101.93548 | 97.004132 | 2.7272727 | 4.8038446 | 8.9396171 | 7.2928455 |
| | | NH4+ 0.03μm | M | 0 | 31.818182 | 48.461538 | 84.516129 | 95.929752 | 1.5745916 | 20.117995 | 10.659814 | 8.696164 |

Table S3. Raw data of the results An Excel file is available online from the MDPI.