Supplementary Material to accompany the manuscript entitled:

Quantitative assessment of anti-cancer drug efficacy from coregistered mass-spectrometry and fluorescence-microscopy images of multicellular tumor spheroids

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Spheroid cultivation.

HT-29 cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Sigma Aldrich; MO, USA), 2 mM L

glutamine (Eastport, Czech Republic), 100 U/mL penicillin and 100 U/mL streptomycin (Lonza, Switzerland) in humidified 5% CO_2 atmosphere at 37 °C. For multicellular spheroid formation, the HT-29 cells were seeded at a density of 50 000 cells/mL on the 12-well plate covered by film of 1% agar in 1× phosphate buffered saline (PBS) (Sigma Aldrich; MO, USA) in DMEM supplemented with 2 mM L-glutamine and no FBS. Cells were incubated on a rotary shaker Orbital Shaker, NB-101SRC (N-BIOTEK, Korea), at 75 rpm, in humidified 5% CO_2 atmosphere at 37 °C for 3 hours and after that FBS was added at 1% concentration. Next day, FBS was supplied to final concentration of 10 %, and the spheroids were cultivated for five more days at 60 rpm.

Sample preparation for MALDI MSI and LSCM analysis

The protocol of spheroid preparation for MALDI MSI was adopted according to Li et al.(2011), with a few modifications. The spheroids were washed three times with 1× PBS and transferred to plastic cryomolds (Tissue-Tek® Cryomold®; Sakura Finetek, CA, USA) with warm gelatine solution (180 mg/mL in 1× PBS, 40 °C) and frozen at -80 °C. The gelatine blocks were cut on a cryostat (Microtome CM1850, Leica Microsystems, Germany) at -22 ºC, and 12-µm thick equatorial cross sections were collected on indium tin oxide (ITO) conductive slides (Delta Technologies, Ltd; USA) by the thaw-mounting method. Subsequently, a protocol for multimodal MALDI MSI and LSCM analysis was applied. To this end, fiducial markers visible in both MALDI MSI and LSCM transmission brightfield scanning mode were created on the slide. After extensive experimentation, we found that a solution of a white marker paint (Centropen White Permanent 2686, Centropen, a. s., Czech Republic) was optimal for the fiducial markers. Three dots around each analyzed spheroid were created manually using a fused silica capillary (outer diameter 360 µm; BGB, Switzerland) immersed in the colorant solution.

Photos of the spheroid sections surrounded by the fiducials were taken on a stereo microscope (model STM 823; INTRACO MICRO, Czech Republic) equipped with a digital camera (Nikon D5100). After desiccation, the ITO slides were covered by the DHB matrix (98%; Sigma Aldrich, MO, USA) using a sublimation apparatus (GPE-1207-030PS; GPE Scientific Ltd, United Kingdom). A heating nest was warmed up to 130 °C while the vacuum in the sublimation chamber was 65–75 mTorr. The density of the sublimed matrix was approximately 0.5 mg/cm2. The samples were analyzed by MALDI MSI on the same day and stored at -18 °C overnight. The next day, the matrix was removed to allow for immunohistochemical (IHC) staining.

Fluorescent immunohistochemistry (IHC) of spheroid sections

After MALDI matrix removal, the spheroid sections were washed three times with cold 1× PBS and incubated with 0.1% Triton X-100 (Sigma Aldrich; MO, USA) in PBS for 6 min. Then a washing step with cold 1× PBS and blocking step with 1% bovine serum albumin (BSA) (Sigma Aldrich; MO, USA) in 1× PBS for 30 min followed. The sections were incubated with primary antibodies against Ki-67 protein (Abcam, United Kingdom), cleaved caspase 8 (Cell Signaling; MA, USA)) and SNAIL/SLUG (Abcam, United Kingdom), all of them diluted 1:200 by 1% BSA in 1× PBS and left overnight at 4 °C. Another three washing steps with cold 1× PBS followed. Secondary fluorescently labeled antibody (Alexa 546; Invitrogen, CA, USA) at 1:600 dilution (1% BSA in 1× PBS) and TO-PRO staining (1 mM stock solution) in 1:1000 dilution were applied for 1-2 hours in dark. Then, three more washing rounds with cold PBS and one round in cold Milli-Q water followed. Excess water was removed and a mounting medium (Dako fluorescent mounting medium; Agilent, CA, USA) with a coverslip (Menzel Gläser; Thermo Scientific, MA, USA) was applied to the sections. Samples were kept in dark and cold till the analysis by laser scanning confocal microscopy (LSCM) .

Why is Spearman's correlation coefficient preferable to Pearson'coefficient.

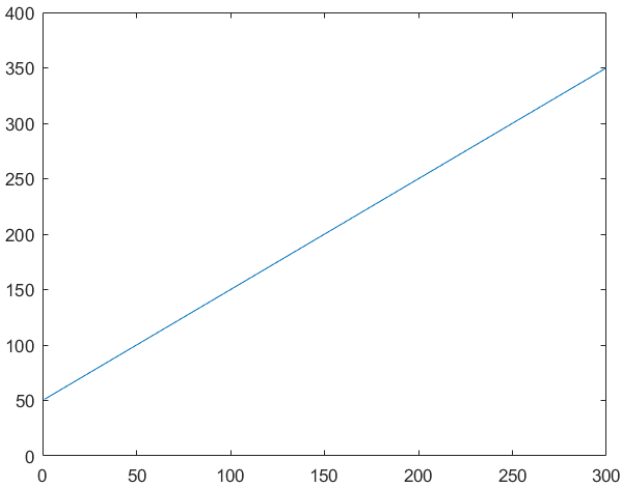
If we need to detect dependency between two discrete sets of data X and Y, we can do it by calculating some measure of dependence, such as the Pearson correlation coefficient.

Example:

Consider two discrete functions (i.e. sequences)

$$x = 1, 2, ..., 300;\ y = x + 5;$$

Obviously, x and y are uniquely dependent, which is also well illustrated by their graph:



i.e. a measure of dependence should achieve its maximum, say 1. We can take the Pearson correlation coefficient as the measure of dependence:

$$\rho(x, y) = \frac{E\left[\left(X - \mu_X\right)\left(Y - \mu_Y\right)\right]}{\sigma_X \sigma_Y}$$

μ_X, μ_Y are average values of X and Y

σ_X, σ_Y are standard deviations of X and Y

E is the expectation of the product inside the brackets

The value of the Pearson coefficient for $x = 1, 2, ..., 300;\ y = x + 5;$, is easily calculated using MATLAB:

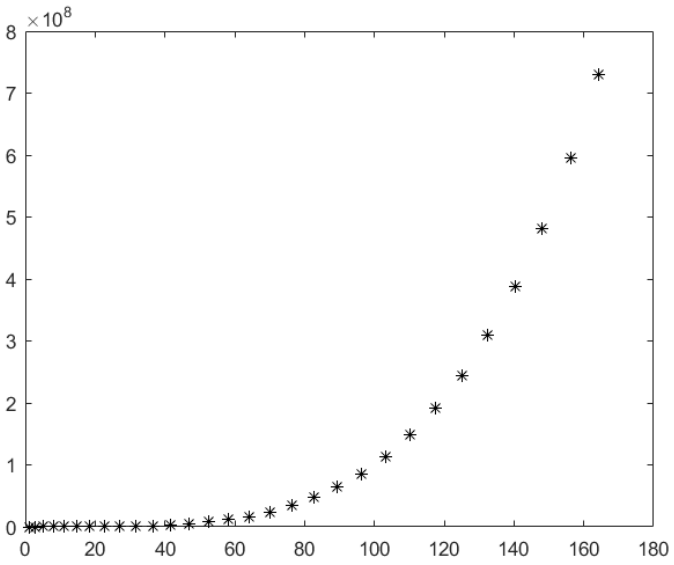
$$\rho = corrcoeff\left(x, y\right) = 1$$

i.e. in the case of *linear dependence,* the Pearson coefficient has the value of 1, as required for unique dependence.

If the two data sequences *x*, *y* are *uniquely*, but *not linearly*, dependent:

$$t = 1, 2, .., 30;\ x = t^{\frac{3}{2}};\ y = x^4$$

we still have a *unique* dependence between x and y:



which should imply the measure of depencence =1, but the Pearson coefficient has a value lower than 1:

$$\rho = corrcoeff\left(x, y\right) = 0.8510$$

i.e. the unique dependence is not correctly detected. Such *nonlinear*, yet *monotonic unique* dependencies between *x* and *y* are correctly detected by the Spearman correlation coefficient. The Spearman coefficient is computed as follows:

- sort the values of x by their magnitude= 1.0000 2.8284 5.1962 8.0000 11.1803 ...
- replace the values of x by their rank 1 2 3 4 5

- sort the values of y by magnitude =(0.8577 1.1338 1.4804 1.9110 2.4414...)* 1.0e+08
- replace the values of y by their rank 1 2 3 4 5
- Spearman coefficient=Pearson coefficient between corresponding pairs of ranks instead of values

When the Spearman algorithm is applied to the dataset $t = 1, 2, .., 30;\ x = t^{\frac{3}{2}};\ y = x^4$, we get the value

$$\rho = corrcoeff\left(rank\left(x\right), rank\left(y\right)\right) = 1$$

i.e. the unique dependence between *x* and *y* has been detected correctly.

Of course, the Spearman coefficient also correctly detects the *linear* unique dependence between datasets such as $x = 1, 2, ..., 300;\ y = x + 5;$. In this case, too, it has the value

$$\rho = corrcoeff\left(rank\left(x\right), rank\left(y\right)\right) = 1$$

To sum up: for *monotonic* dependence between two datasets the following holds

- the Pearson coefficient detects *correctly* unique dependence between *linearly dependent* datasets
- the Pearson coefficient assesses *wrongly* unique dependence between *nonlinearly dependent* datasets
- the Spearman coefficient detects correctly unique dependence between *both linearly and nonlinearly* dependent datasets

This is the reason why we prefer the Spearman coefficient to the Pearson coefficient as a measure of dependence between two datasets.

LI, H. AND HUMMON AB (2011) Imaging Mass Spectrometry of Three-Dimensional Cell Culture Systems. Anal Chem. **83**(22), 8794-8801.