A binary approach to the colony forming assay: reliable and reproducible read-outs using 3D CoSeedis™

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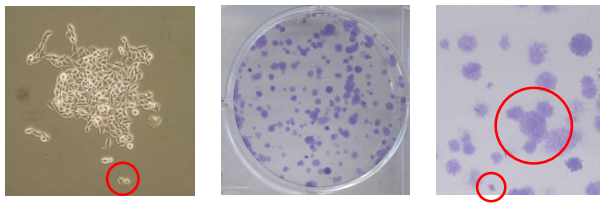
Introduction

The colony forming assay (CFA) or clonogenic assay has its origin back in the 1950's[1,2] using adherent HeLa cells. The CFA has since gained wide acceptance as the "gold standard assay" to assess tumour and normal cell response to cytostatic treatment, especially in radiation biology[3]. First attempts to expand this principle into anchorage-independent colonies were done in methylcellulose and agar-based assays using primary tumour cells, initially of mouse[4], later also of human hematopoietic and myeloma cells as well as other tumour types[5,6].

The CFA generally describes an *in vitro* cell survival assay based on the capability of single cells to grow into colonies[7]. It therefore defines a purely functional assay, which is in contrast to the widespread alternative metabolic assays, such as the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid), MTS (3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), or Alamar Blue.

A colony is defined to consist of at least 50 cells and the CFA is particularly useful to determine the effect of chemical or radiation treatments on the proliferation of cancerous cells, which explains its widespread use in cancer research[1,8].

Since only a fraction of treated cells retains the capacity to produce colonies, the current protocol requires seeding of appropriate dilutions of cells before and after exposure to treatment. Colony formation normally occurs within 1 to 3 weeks after seeding and individual colonies are fixed, stained and counted using a stereomicroscope[6].



Difficulties with current CFA
Primary vs. secondary colonies
Colony size > 50 cells
Overlapping colonies

However, current methods, such as assays in 2-dimensional (2D) or in agar, to perform a CFA are reported to be:

- Cumbersome
- Prone to bias due to inter-observer variations (or even intra-observer!)
- Lacking the possibility to sequentially administer drugs
- Missing critical cell-cell interactions and three-dimensionality, important for drug response[1,5,9,10] in 2D assays.

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Furthermore, for the classical CFA (e.g. in 2D or soft agar), it is important to seed a cell density that allows colony counting. Too high a cell seeding density leads to overlapping colonies making an accurate determination of colony forming efficiencies difficult if not impossible. Even though inexpensive as such, the current CFA nevertheless consumes a lot of resources in terms of labour mainly caused by necessary repetitions to compensate for inaccurate individual read-outs. A more reliable and reproducible assay would therefore be desirable.

A new approach to the colony forming assay

3D CoSeedis™ from abc biopply, a conical agarose-based microwell array, offers a new approach to the CFA. The system was initially designed to open up 3-dimensional (3D) organoid formation to a broader range of cells in cancer research. To do so, the 3D CoSeedis™ matrix shows a unique and proprietary topography and geometry that facilitates cells to aggregate in 3D constructs. The modular set-up of the system also allows using feeder cells to support 3D formation. Feeders can either be grown conventionally with test cells (in direct contact and intermingled), or they grow in distance to the test cells, meaning physically separated by the matrix. In the latter condition, the feeder cells can be excluded for subsequent treatment exposure and analysis (distance co-culture technique).

It is also possible to supplement the medium or matrix with extracellular matrix (ECM) components to push 3D growth of certain cell types.

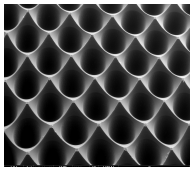
Taken together, 3D CoSeedis™ opens 3D cell culture to a wide range of cells that have so far been elusive to this kind of assay.

In the context of the CFA, 3D CoSeedis™ is ideally suited for suspension cells, adhesion-independent cells and cells that form colonies in the soft agar assay or in methylcellulose. 3D CoSeedis™ offers an elegant new way to look into colony forming efficiency for a broad range of healthy as well as malignant cancer cells. Since anchorage-independence is a criterion for malignancy of normally adherent cells (cf. anoikis resistance)[11], 3D CoSeedis™ can be used to detect and determine colony formation of healthy adherent cells vs. their malignant equivalent. For example, endothelial cells or keratinocytes go into apoptosis under 3D conditions when healthy, but may form adherent-independent colonies if cancerous.

Together with abc biopply's validated protocols, 3D CoSeedis™ eliminates bias related to the CFA by fixing colonies in position and focal plane, hence allowing (semi-) automatic binary analysis. Furthermore, sequential administration of chemical treatments is possible, since all colonies have a 360° supply with medium.

Procedure

The 3D CoSeedis™ matrix is an agarose-based scaffold-free carrier matrix that consists of up to 100 microcavities per cm^2. Its unique and proprietary topography ensures that cells localise at the bottom of the cavities and that they are induced to form 3D constructs. A defined geometric positioning of each microcavity is responsible for the array-like arrangement of the microcavities leading to a precise allocation of coordinates to each subsequent 3D construct. Furthermore, the uniform topography of the microcavities arranges each 3D construct in the same focal plane. An additional feature of 3D CoSeedis™ is its capability to induce 3D aggregation in cells that would normally not do so in agar. Therefore, the matrix expands the range of healthy and tumour cells that can be tested in a 3D CFA and consequently broadens the applicability of this assay in 3D.

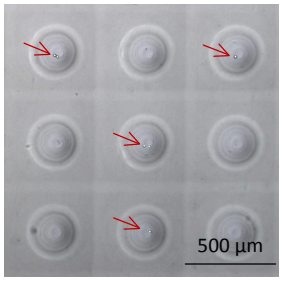


Electron-microscopic image of 3D CoSeedis™ surface.

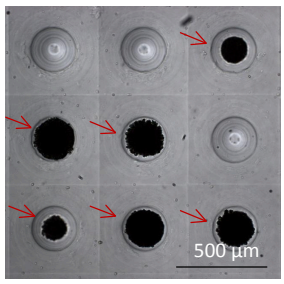
In a classical CFA, the test cells are plated at a density that allows the identification of individual clones deriving from single cells. In 3D CoSeedis™ this would be equivalent to microcavities

containing a single cell only. However, in reality, microcavities may contain more than just one single colony forming cell, which is reflected by the use of the statistical Poisson equation (see below).

Colony formation time is cell type-dependent but normally takes between 10 to 14 days. After treatment, not all cells will form a colony (indicating that some may have lost their colony forming capacity), nor will all colonies grow at the same rate. A representative example with HT-29 cells, a human colon cancer cell line, is shown to the right.



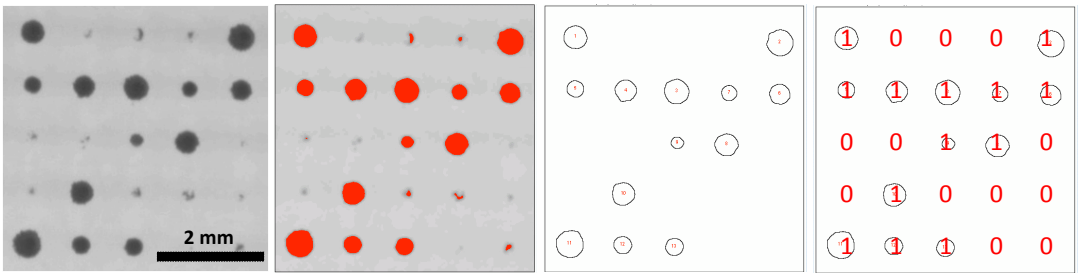
HT-29 cells
Seeded at about 1.5 cells/microcavity (cpm) day 0 after seeding



HT-29 cells
3D colonies of cells day 14 after seeding

After colony formation and growth phase are terminated, the 3D CoSeedis™ matrix can be analysed using a standard transillumination scanner. abc biopply developed a fully validated protocol that uses freely available software to scan and freeware to determine the colony size. A detailed step-by-step protocol is available from www.biopply.com.

Scanning and size determination of colonies within a certain section of the 3D CoSeedis™ matrix will ultimately lead to a "yes/no" or binary read-out, whereby "1" will be assigned to microcavities containing a colony (3D construct) and "0" to microcavities in which no colony (no 3D construct) can be detected (see image below).



A representative section of a scan in CFA with 3D CoSeedis™
Generally, a matrix area of 2 by 2 cm is used for the analysis. In the example shown, such a square would contain 400 microcavities (only 25 of them are depicted here). Colonies above a certain size limit (normally equivalent to > 50 cells/colony) are counted positively ("1"). Smaller colonies are determined as negative ("0").
Experimental data:
HT-29 cells were seeded into a 1x1 matrix at a density of 1.6 cpm. Analysis was done at day 12 after seeding.
Results:
Number of microcavities showing colony growth: 198
Number of microcavities without colony growth: 202
Total number of microcavities measured: 400

Statistical analysis

The colony forming efficiency is determined using a Poisson distribution. This statistical analysis takes into account that, depending on the seeding density, there might be more than one cell per microcavity that is capable to form colonies. According to Poisson, the probability $Px=k$ to have k colony forming cells in a microcavity is given by:

$$Px=k=\lambda k k!*e-\lambda$$

where λ defines the average number of colony forming cells per microcavity and e is Euler's number. Particularly, the probability of an empty microcavity corresponds to k = 0 and is given by

$$Px=0=\lambda 00!*e-\lambda=11*e-\lambda=e-\lambda$$

In the example above ("A representative section of a scan in CFA with 3D CoSeedis™), P equals the relative number of microcavities in which no colonies can be detected:

$$P=number\ of\ microcavities\ "0"total\ number\ of\ microcavities\ ("1"+"0")=202400=0.505=50.5\%$$

Consequently, we can determine λ:

$$\lambda=-\ln(P)$$

Or in our example:

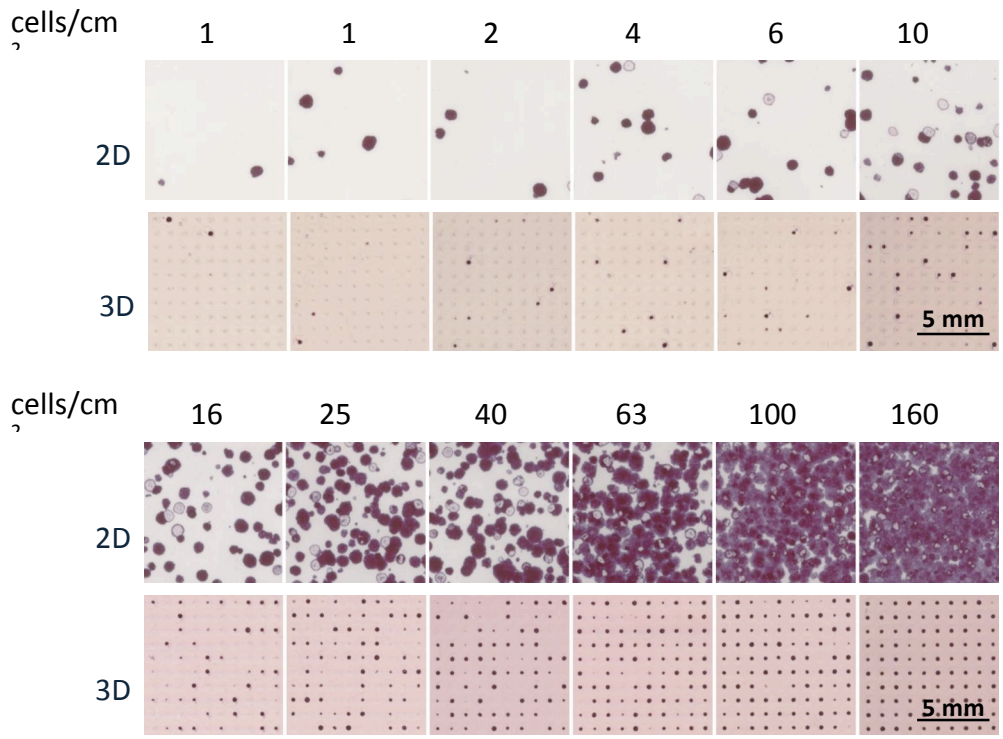
$$\lambda = -lnnumber\ of\ microcavities\ \ "0"number\ of\ microcavities\ "1"+"0"=-ln202400=0.683$$

Therefore, the colony forming efficiency E can be calculated by dividing the average number of colony forming cells per microcavity λ by the number of seeded cells per microcavity (cpm):

$$E=\lambda cpm=-\ln(P)cpm=0.6831.6=0.427=42.7\%$$

Statistical robustness

In contrast to the CFA in 2D, soft agar or methylcellulose, 3D CoSeedis™ shows a much higher tolerance to the number of seeded cells per cm^2 that can be analysed. Consequently, the assay becomes a lot more robust and the results are more reliable and reproducible. Bias-caused counting errors in the traditional CFA are eliminated by the binary analysis method enabling automated read-outs.



Seeding density

HT-29 cells were seeded at the indicated number of cells/cm^2.
At day 14 in culture, petri dishes and matrices were photographed and scanned, respectively.
Whereas the assay remains analysable with 3D CoSeedis™ over a large range of seeding densities (10 – 1000 cell/cm^2), in 2D individual colonies cannot be distinguished any longer at densities above 160 cells/cm^2.

Conclusion

3D CoSeedis™ offers a new way to do colony forming assays with non-adhesive cells by which individual colonies are localised in an array-type gridwork. Such an arrangement allows for (semi-) automatic analysis via a transillumination scanner and the allocation of positive/negative values to pre-defined colony sizes. The resulting analysis method is a lot more resistant to counting bias: individual colonies can easily be identified and do not overlap nor form daughter colonies and the assay makes the analysis over a broad range of cell seeding densities possible.

An additional advantage of the 3D CoSeedis™ matrix allows the analysis of cells that would normally elude 3D culturing in alternative systems. Furthermore, 3D growth can be stimulated and supported

by the use of either feeder cells (in distance co-culture), the addition of ECM components, either to the medium or matrix, or combinations thereof. It is therefore feasible that the 3D CoSeedis™ matrix may well open up the CFA to 3D analysis for cells that have so far eluded this approach. Consequently, the system may lead to more physiologically relevant test results in the CFA. This may be particularly the case as 3D growth and cell-cell-interaction have previously been implicated in the response to chemicals and drugs[9,10].

Taken together, 3D CoSeedis™ makes the CFA a lot more robust to experimental variations, hence providing a more reliable tool to generate reproducible results in cancer research.

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