

# Using biased image analysis for improving unbiased stereological number estimation – a pilot simulation study of the smooth fractionator

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**Key words.** Simple random, stereology, systematic uniform random.

## Summary

The smooth fractionator was introduced in 2002. The combination of a smoothing protocol with a computer-aided stereology tool provides better precision and a lighter workload. This study uses simulation to compare fractionator sampling based on the smooth design, the commonly used systematic uniformly random sampling design and the ordinary simple random sampling design. The smooth protocol is performed using biased information from crude (but fully automatic) image analysis of the fields of view. The different design paradigms are compared using simulation in three different cell distributions with reference to sample size, noise and counting frame position. Regardless of clustering, sample size or noise, the fractionator based on a smooth design is more efficient than the fractionator based on a systematic uniform random design, which is more efficient than a fractionator based on simple random design. The fractionator based on a smooth design is up to four times more efficient than a simple random design.

## Introduction

The viewing, sampling and estimation process in tissue with sparse or nonhomogeneous cell distributions can be improved by introducing computerized image analysis into existing stereology procedures. In most commonly available computer-aided sampling systems, the computer is used for dividing the image into units to be sampled (the fields of view – see Fig. 1) in a uniform, random way. Most commonly, the fields of view are then sampled in a systematic, uniformly random sampling (SURS) design, which gives each field of view the same probability of being sampled. The smooth fractionator (Gundersen, 2002) uses a smoothing protocol to greatly improve the SURS efficiency. The smoothing protocol reorders the units before

SURS whilst keeping the same constant sampling probability for each unit. Gundersen's (2002) paper (see fig. 6 therein) illustrates the use of biased information from, for example, image analysis as an associated variable used for reordering into the smoothed sequence. The present study combines the smoothing protocol and a computer-aided stereology tool to provide better precision and a lighter workload. Using simulation one can investigate the advantages and disadvantages, as well as the possible applications and setups so that the combination of smoothing protocol, stereology and image analysis is most favourable. The associated variable for the smoothing protocol is the crude (but automatically obtained) information about the amount of a specific colour in each field of view.

## Methods

The smoothing protocol is understood in the present context as the reordering of units according to an arbitrary associated variable. The associated variable should have a potential positive correlation between its value in each unit and the count in the unit. One may say that the associated variable is a weight (a number) given to each unit. This weight should be higher when the expected count in a unit is higher. When counting cells, one might assume that the bigger the physical size a unit has, the more cells will be counted. In this case, a bigger physical unit will be assigned with a higher weight value. The weight assignment can be based on any possible correlation between the unit's potential count and its physical size, colour or even based on human intuition (see Fig. 2). When the correlation between the weight and the eventual count is high, the advantage of the smoothing protocol is greater. Even in the worst scenario with a negative correlation, the estimator is still unconditionally unbiased – but may evidently be rather inefficient (Gundersen, 2002).

The methods have been implemented in a computer-aided stereology tool (CAST software; Visiopharm, Hørsholm,

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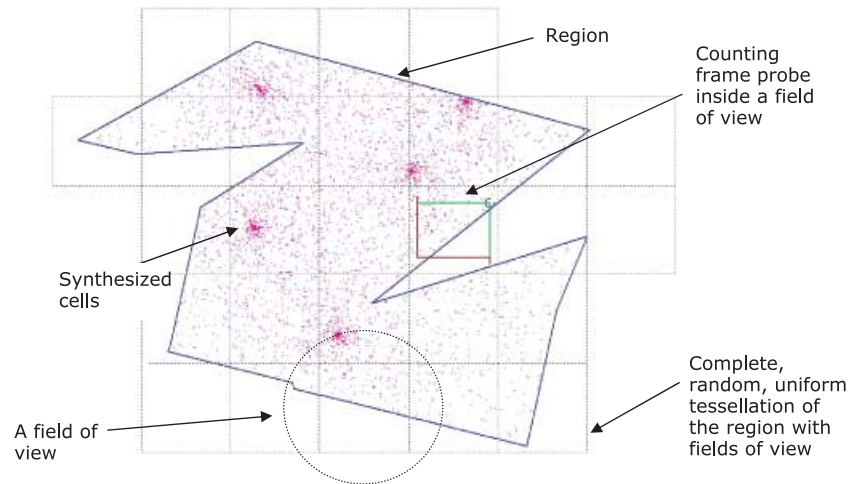


Fig. 1. Basic objects during computer-aided sampling. Note the uniform (and random) tessellation of fields of view completely covering the region.

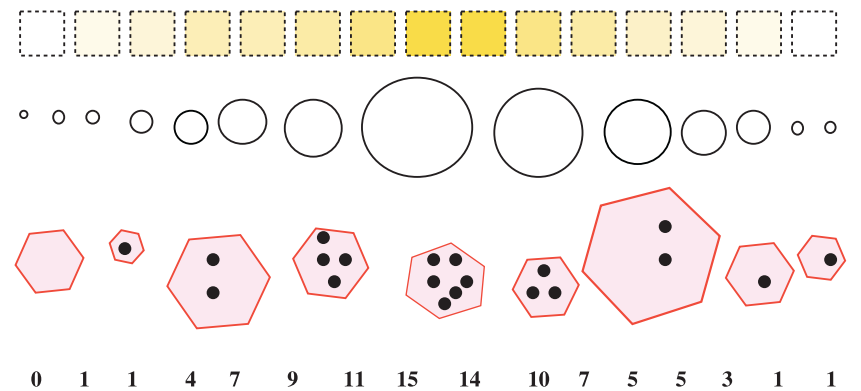


Fig. 2. Example of different smoothing protocols based on colour intensity, size, content (the number of dots inside each hexagon) and a numeric value.

Denmark). Using CAST, all the units to be sampled (the fields of view) have the same size. CAST ensures a random tessellation into fields of view by selecting a random top left corner position for the first field of view. The random tessellation, as well as the uniformity and completeness, ensures an unbiased estimate (Sterio, 1984). The computer samples a subset of the fields of view of the whole tessellation. A human user looks carefully at each of the sampled fields of view and performs the correct cell counting using a counting probe, i.e. the disector (Sterio, 1984).

The smoothing protocol requires a weight (the associated variable) to be assigned to each field of view. The weight assignment is based on the image of each field of view. The weight is assigned using an image analysis result, and should be higher when a potential cell count is higher. For example, when looking for red stained cells on a white background, the image analysis component should give a higher weight to a field of view with higher average red colour intensity (see Fig. 3). The computer does not perform the counting – it only gives a higher weight to a field of view with a potentially higher count of cells. The weight is only used for reordering according to the smoothing protocol before the actual SURS. Note that the smoothing protocol does not change the fact that all fields of view have a constant (uniform) sampling

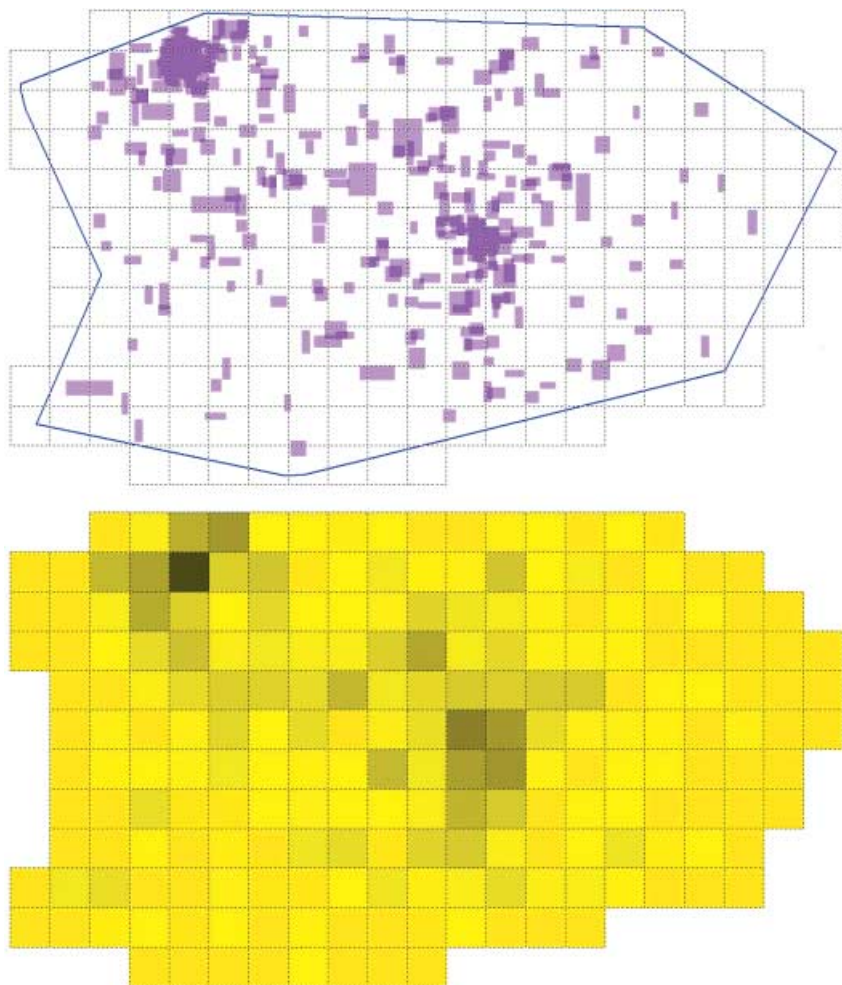
probability. The smoothing protocol ensures that the variability within sets (i.e. potential samples) of fields of view is smaller than in other sampling paradigms. The comparison between the different stereology paradigms was performed using simulation.

*Implemented stereological methods*

The CAST system supports a fractionator design based on SURS. When fixing the size of the counting frame and region of interest, one may increase the sampling period (thereby decreasing the sampling size) by increasing the physical distance between the counting frames (i.e. increasing the size of a field of view). In order to implement a fractionator design based on the smoothing protocol and independent (simple) uniform random (SR) sampling, the concept of selecting a subset of the fields of view (i.e. corresponds to a subset of counting frames) has been introduced and implemented into CAST (see Appendix).

The following sampling paradigms are compared:

- 1 *Simple random (SR) sampling (independent uniform sampling).* A predetermined number of fields of view are sampled randomly and independently, with replacements (i.e. a field of view may be chosen more than once).



**Fig. 3.** A region partitioned into fields of view in the upper image. According to the intensity of the pink cells, each field of view is assigned a weight. The weight for each field of view is shown in the lower image. The darker the field of view is, the higher the weight assigned to it.

- 2 *Systematic uniformly random sampling (SURS)* (Gundersen & Jensen, 1987). The selected fields of view have approximately the same spatial distance from each other (see Fig. 4). This is the most commonly used stereological sampling method. The grid is approximately hexagonal – every field of view has approximately the same distance from all its closest neighbouring fields of view (see Fig. 5).
- 3 *Smooth sampling based on smooth design and automatic image analysis.* The smoothing protocol is applied to all (or a large subset) of the fields of view before sampling. Each field of view is assigned a weight according to its colour content. The smoothing protocol is performed according to the assigned weight. See Gundersen (2002) and Fig. 6. SURS is performed on the rearranged fields of view after the smoothing protocol. It is possible to sample only a subset of the fields of view, assign weights only to this subset, smooth it, and then carry out SURS on it. The advantage of choosing a subset for the weight analysis is technical, because the weight and image analysis are performed only on a part of the region. In this study the weight assignment

was always performed on the entire tessellation of the fields of view.

See Fig. 7 for a visual example of fractionator sampling based on SURS and smooth sampling.

#### *Simulation Setup*

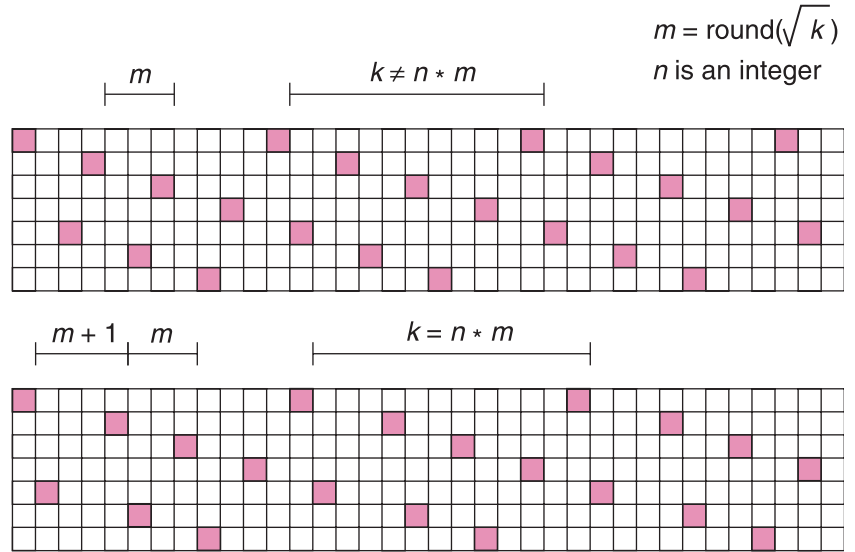
The study includes the following variation in the above sampling paradigms

- Presence of noise.
- Influence of the sampling intensity (different sample sizes).
- Location of the counting frame (central vs. the lower left corner of a field of view).

The following basic guidelines have been defined for all sampling paradigms:

- The region was a fixed, not oversimplified shape with one pronounced irregularity as seen in Fig. 8. The region had a fixed area of  $0.9375 \text{ mm}^2$ .
- Each set of tests was run on three spatial distribution patterns of cells: homogeneous, intermediary, and clustered. In each

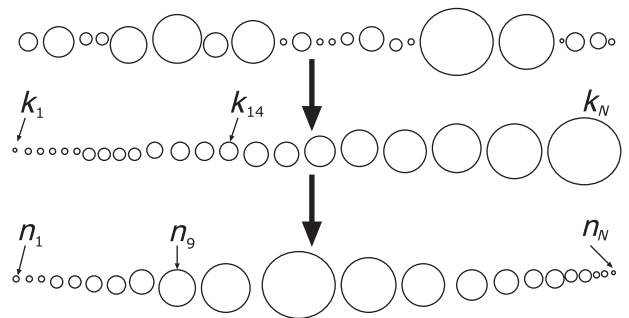
**Fig. 4.** Systematic uniformly random sampling protocol. The sampled fields of view are at an approximately constant distance from their closest neighbours. After  $k$  (sampling period) is set,  $m$  is calculated. If  $k$  is indivisible by  $m$  (upper illustration) the position of the selected field of view is shifted  $m$  units every consecutive row. If  $k$  is divisible by  $m$  (and  $k = n * m$ , where  $n$  is an integer, lower illustration), the position of the selected field of view is shifted  $m + 1$  units on the first row, and by  $m$  units the next  $n - 1$  consecutive rows. The process is repeated on all the fields of view covering the region. The separation into two cases is necessary for avoiding unintentionally aligned fields of view (which is the case when  $k$  is divisible by  $m$  and the position of the selected fields of view is shifted  $m$  units every consecutive rows).



**Fig. 5.** Example of systematic uniformly random sampling using the protocol described in Fig. 4.

distribution pattern, 2500 cells were distributed around three random origins (see Fig. 8). The cells were randomly redistributed before every sampling (whilst keeping the general pattern of homogeneous, intermediary and clustered).

- Field of view size was fixed at  $60 \mu\text{m}$  (width) and  $45 \mu\text{m}$  (height) –  $2700 \mu\text{m}^2$ . It had similar proportions as a computer monitor.
- The counting frame area was fixed at  $1404 \mu\text{m}^2$ , 52% of the field of view area.
- Average cell size was  $11 \mu\text{m}^2$  (standard deviation of  $0.05 \mu\text{m}^2$ ). All cells were rectangles.



**Fig. 6.** Illustration of smoothing protocol based on physical size. Number of elements is  $N$ . Let  $m$  be  $N/2$  or  $(N - 1)/2$  when  $N$  is odd. After sorted ascending according to physical size, the assignment of element  $n_i$  in the smoothed sequence is  $n_i = k_{2i}$  for  $i$  smaller or equal to  $m$ , or  $n_i = k_{2N-2i+1}$  for  $i$  bigger than  $m$ .

- The tessellation of the fields of view was randomly translated. The position of the upper left corner of the first field of view was randomized before every sampling.
- Cells were drawn as pink rectangles. Overlapping cells (two or more) were drawn as dark pink.
- Noise (which contributes to the weight but not to the count) was drawn as light blue rectangles. Overlapping noise rectangles (two or more) were drawn as dark blue.
- The weight assigned to a field of view was proportional to the number of pink (or blue for noise) pixels in that field of view. Dark pink or dark blue pixels contributed to the weight 50% more than light pink or light blue pixels. Consecutive overlapping cells contributed to the count, but did not make the weight consecutively higher. A final weight of a field of view was the percentage of its pixels' weight out of the potential maximum pixels weight if the entire field of view was dark pink.

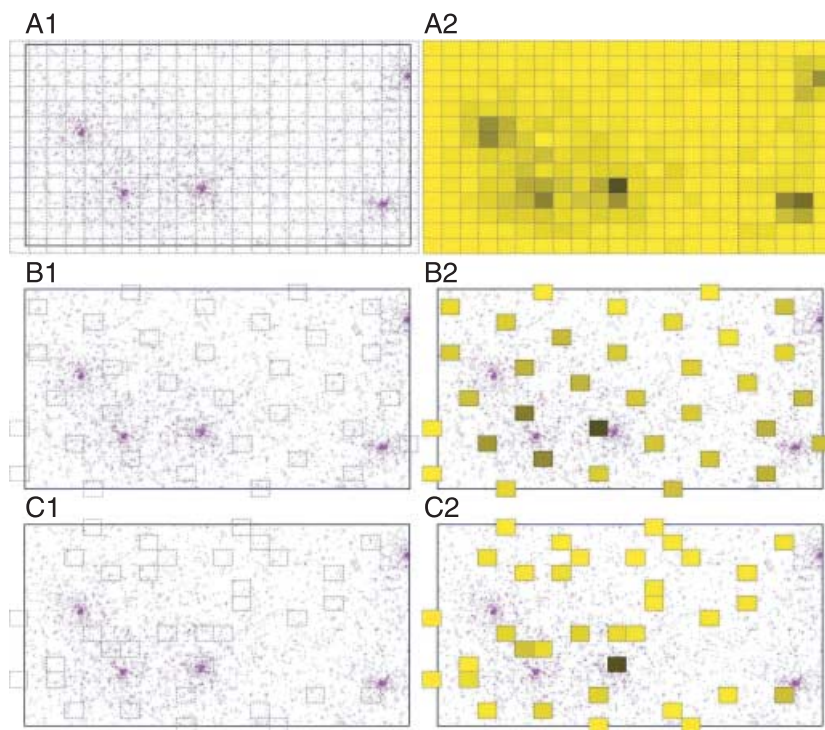


Fig. 7. (A1–2) A region with cells is divided into fields of view, each field of view is assigned a weight (shown as grades of yellow). (B1–2) Sampling using systematic, uniformly random sampling. (C1–2) Sampling using the smooth fractionator. On the left – the fields of view content, on the right – same fields of view with weight as a grade of yellow. The darker the field of view is, the higher the weight assigned to it.

The contribution from the simulation itself to the estimator coefficient of error (CE) was always monitored. By keeping the number of repetitions in the simulation significantly high, and, by that, making the simulation standard error of the mean (SEM of the count estimates) virtually zero, the simulation CE was ensured to have an insignificant impact on results and conclusions.

## Results

SR sampling, SURS and smooth sampling (the smooth fractionator) were compared in three different cell distributions with reference to sample size, noise and counting frame position. Figure 9 shows the influence of the sample size on the estimator CE. Figure 10 shows the influence of noise on the estimator CE, and Fig. 11 shows the influence of the counting frame position within the field of view on the estimator CE.

## Discussion

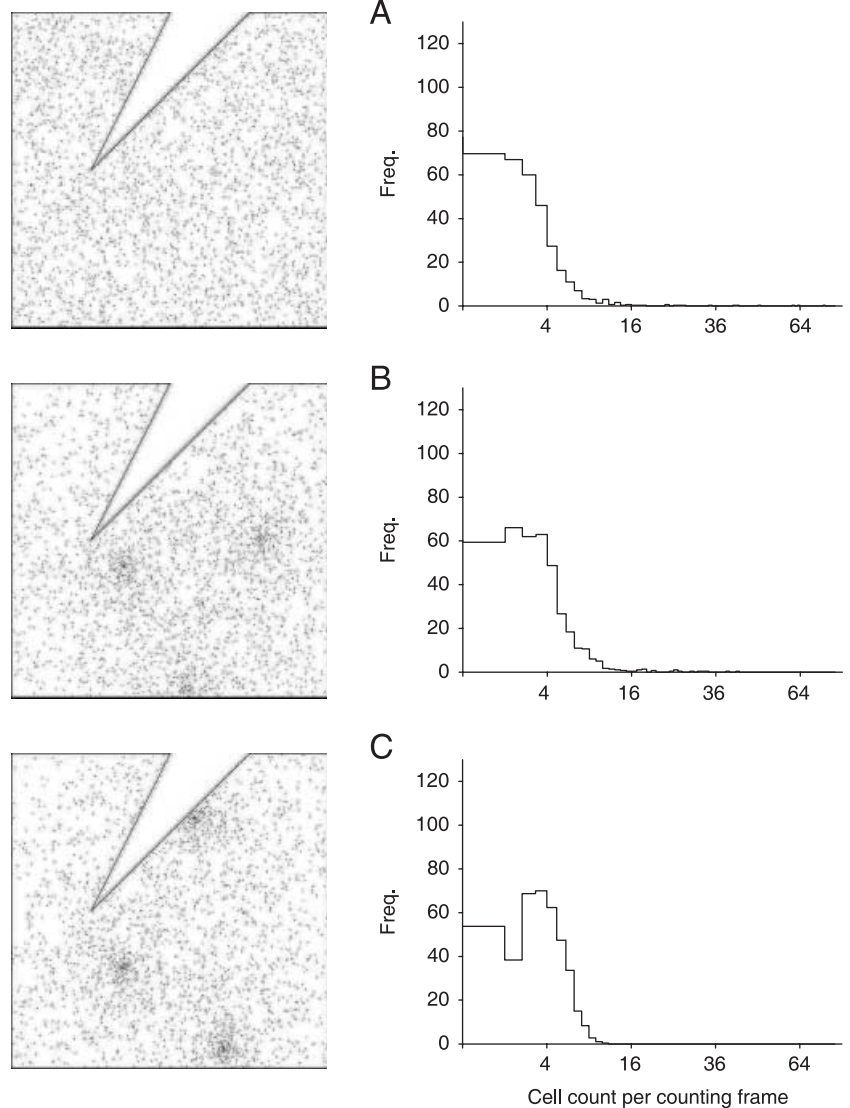
The general pattern (regardless of clustering or sample size) shows that the smooth fractionator is most efficient. The smooth fractionator is roughly twice as efficient as an ordinary fractionator based on a SURS design, which is roughly twice as efficient as a fractionator based on SR. The respective estimators  $CE^2$  decreases with increasing cell count (Fig. 9).

It is remarkable that even the minimal variation between random fields in a homogeneous distribution is enough to

make smooth sampling superior to SURS. The relative advantage is the same in nonhomogeneous and homogeneous cases (Fig. 9). In a homogeneous distribution, the smooth sampling is superior to SURS, which is superior to SR sampling. This is due to the edge effect, where the fields of view are only partially inside the section. In a nonhomogeneous distribution, the systematic spatial nature of the SURS and the arranged clustering of cells around the origins give less variation within the sampled fields of view when compared to SR sampling. Therefore, SURS efficiency in comparison to SR sampling increases with the degree of clustering.

Noise may deteriorate the performance of smooth sampling as illustrated in Fig. 10. In clustered and nonhomogeneous distributions, smooth sampling has the same problems as SR sampling and SURS with variance within the cell counts in the sampled fields of view. Therefore, in nonhomogeneous distributions, noise only has a little effect with respect to the uniformity of smooth sampling superiority over SR sampling and SURS. However, even with a noise of 100% (which only affects smooth sampling), it is still uniformly more efficient than SR sampling and SURS.

The efficiency of smooth sampling is improved when placing the counting frame to the lower left of the field of view (Fig. 11). This is due to the fact that the cells to the left and below the counting frame do not contribute to a count, but contribute to the weight of a field of view. Minimizing the area to the left and below the counting frame increases precision because the signal to count ratio is improved.



**Fig. 8.** Spatial distribution patterns used during the simulation. To the left – visual examples of homogenous (A), intermediary (B) and clustered (C) patterns. To the right – counted cells frequency per field of view (for each pattern). Each frequency value is the average of five random cell distributions (of the same spatial distribution pattern). Cell counts are equidistant on a square-root scale.

## Conclusion

The fractionator based on a smooth sampling design is roughly twice as efficient as an ordinary fractionator based on a SURS design which is again twice as efficient as a SR fractionator. This is regardless of cell spatial distribution, noise or counting frame position. Smooth sampling is never less efficient than SURS, and the gain is approximately a factor of two to three. The increased efficiency can be obtained without extra work. A computer can do all the smoothing and weight assignments for us – automatically.

## Acknowledgements

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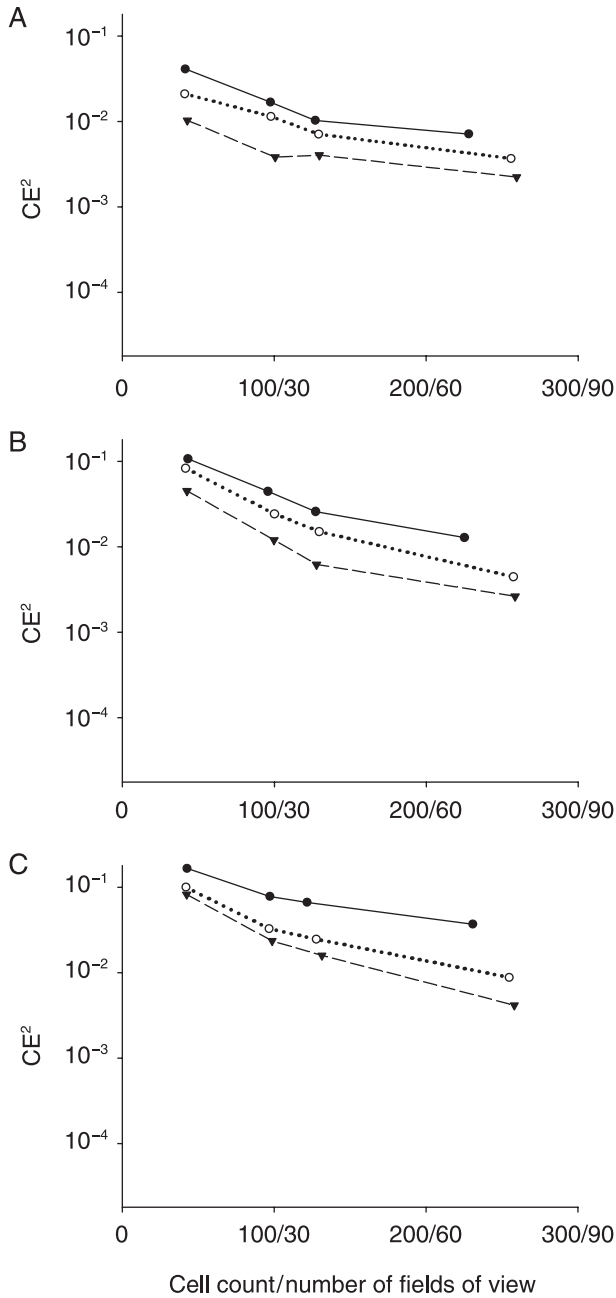
## References

- Gundersen, H.J.G. (2002) The smooth fractionator. *J. Microsc.* **207**, 191–210.
- Gundersen, H.J.G. & Jensen, E.B. (1987) The efficiency of systematic sampling in stereology and its prediction. *J. Microsc.* **147**, 229–263.
- Sterio, D.C. (1984) The unbiased estimation of number and sizes of arbitrary particles using the disector. *J. Microsc.* **134**, 127–136.

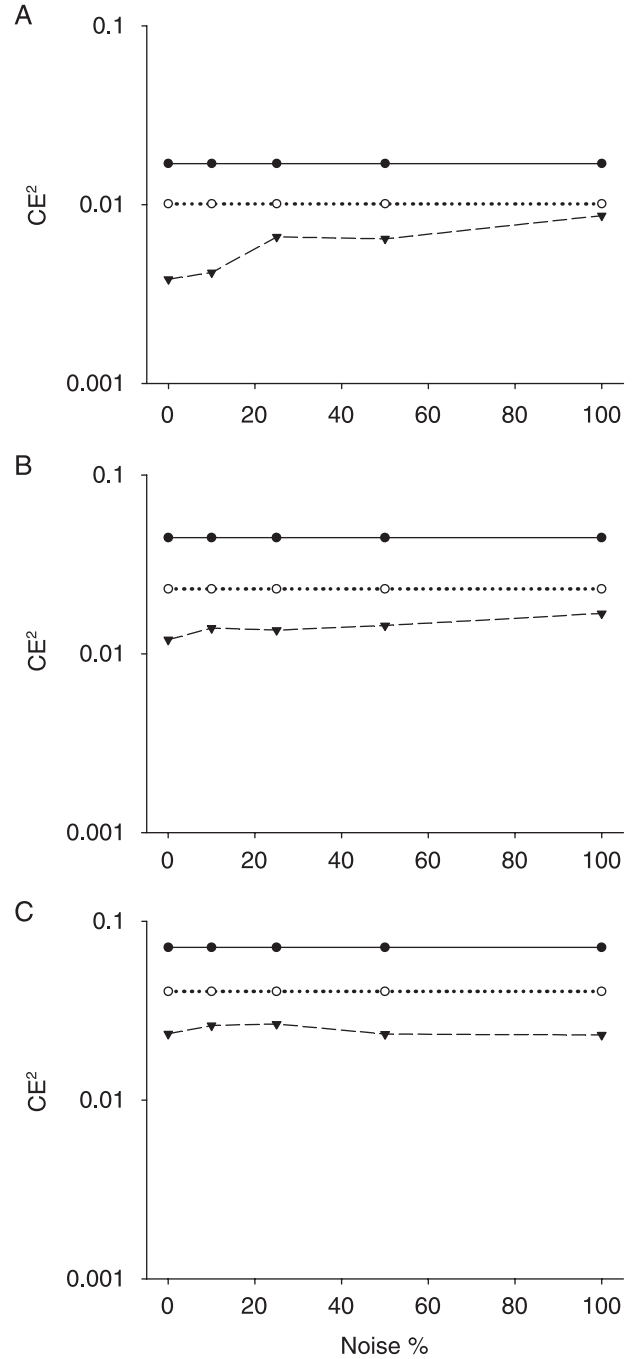
## Appendix – Simulation platform inside the computer-aided stereology tool

### *The basic steps of a simulation*

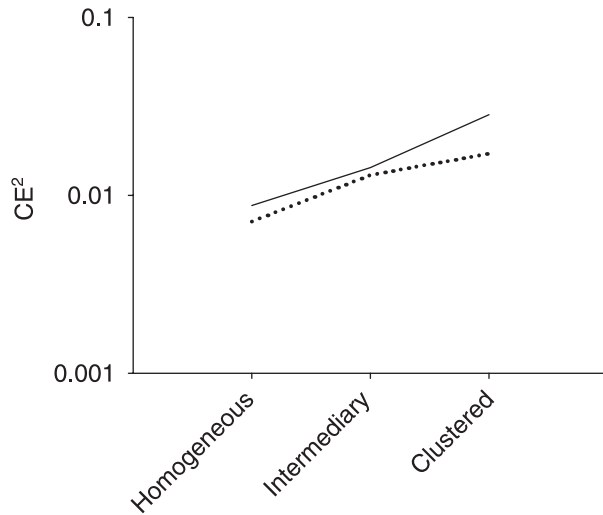
- 1 Create a synthesized image of cells, knowing the exact number, position and size of all cells.
- 2 Randomly tessellate the synthesized image region into a complete and uniform tessellation of fields of view.



**Fig. 9.** Comparison of simple random sampling (full line), systematic uniformly random sampling (dotted line) and smooth sampling (dashed line) with regards to cell counts in homogeneous (A), intermediary (B) and clustered (C) cell distributions. In all cases, the region size and shape was  $0.9375 \text{ mm}^2$  with 2500 cells (approximately  $11 \mu\text{m}^2$ ), the field of view size was  $2700 \mu\text{m}^2$ , and the counting frame size was 52% of the field of view. No noise was present. CE, coefficient of error.



**Fig. 10.** Comparison of simple random sampling (full line), systematic uniformly random sampling (dotted line) and smooth sampling (dashed line) with regards to noise in homogeneous (A), intermediary (B) and clustered (C) cell distributions. Noise was homogeneously distributed. In all cases, the region size and shape was  $0.9375 \text{ mm}^2$  with 2500 'normal' cells (approximately  $11 \mu\text{m}^2$ ), the field of view size was  $2700 \mu\text{m}^2$ , the counting frame size was 52% of the field of view, and the total cell count was fixed at approximately 100. The noise was a percentage of the 2500 'normal' cells placed, i.e. 50% noise was 1250 'ghost' cells, a total of 3750 cells. CE, coefficient of error.

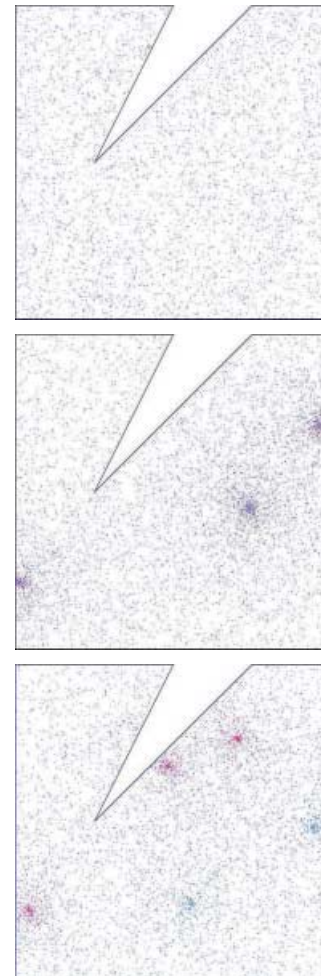


**Fig. 11.** Smooth sampling with counting frame in the centre of the field of view (full line) or counting frame in the lower left (dotted line). In all cases, the region size and shape was  $0.9375 \text{ mm}^2$  with 2500 cells (approximately  $11 \mu\text{m}^2$ ), the field of view size was  $2700 \mu\text{m}^2$ , the counting frame size was 52% of the field of view, and the total cell count was fixed at approximately 100. CE, coefficient of error.

- 3 Sample a subset of the fields of view according to the selected sampling paradigm and parameters.
- 4 Count the cells in the selected fields of view from Step 3 based on the known position and size from Step 1 (a task that is usually done by a human user when performed outside the simulation scope).
- 5 Estimate the total number of cells based on the sampling paradigm, design and parameters from Step 3 and the actual cell count from Step 4.
- 6 Compare the estimated number of cells provided in Step 5 to the expected cell number in Step 1.

#### *Simulation tool functionality*

- Synthesize a lifelike image of cellular spatial distributions.
- Run the different sampling paradigms on the (same or different) synthesized cell images.
- Repeat (requested number of times) the sampling method on the same or different synthesized cell image. Repeating the sampling simulation a number of times will provide more estimates. This will reduce the simulation standard error of the mean (SEM of the estimates). By having the simulation SEM low, the noise from the simulation itself is essentially irrelevant in any graphs, values or trends on which the results are based on.
- Noise is introduced in the simulation as ghost cells. Noise cells contribute to the weight as the colour of real cells. However, noise cells do not have a counting unit and therefore cannot contribute to the count (see Fig. 12).



**Fig. 12.** Synthesized images from the simulation camera. Upper image – homogeneous distribution of both cells and noise. Middle image – nonhomogeneous distribution, three origins, with noise clustered around the same origins. Lower image – nonhomogeneous distribution of both cells and noise, three origins for cells, three other origins for noise.

- Automatically perform the counting of the cells on any part of the synthesized image, using a counting frame.
- Intensive reporting and analysis for the sampling paradigms, design, parameters, sampled fields of view, noise, counted cells and results.

#### *CAST as a simulation platform*

The advantages of using a commercial software package are:

- Easier access to third party cameras and stages for using the sampling paradigms outside the simulation framework.
- Reducing time to market when research is completed.

#### *External plugins*

The following external components will change when used outside the simulation scope:

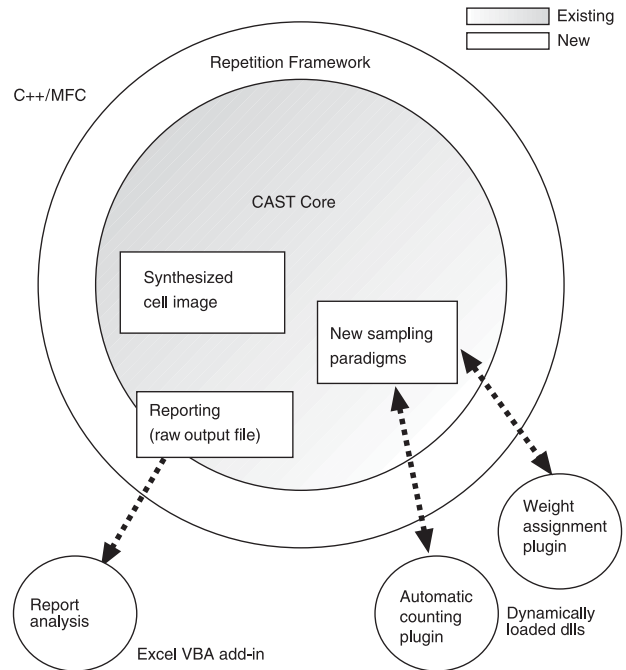


- *Weight assignment plugin.* Assigns a number to an image according to the presence of requested visual features. The more requested visual features found, the higher the result. In case of the simulation's synthesized image of cells, this number is the percentage of the pink or blue areas in the image, out of the maximum possible value in an image of the same size.
- *Automatic counting plugin.* A counting frame probe is used for counting the cells on the sampled field of view (Sterio, 1984). Usually, a human user is the one doing the counting task. During simulation, the counting can be done automatically because the system knows the cell's exact position and size. When used outside the simulation scope, this plugin has the potential to count automatically or suggest a count value to the user.

*Technical details*

See Fig. 13 for a technical illustration. The core changes in CAST are all C++/MFC. The external plugins are dynamically loaded dlls with a clear interface. The simulation output is one or more text files. The analysis of these (potentially huge) text files is via an Excel VBA add-in that extracts the requested information and displays a summary in an Excel spreadsheet.

This technical and logical component separation ensures encapsulation, minimum (unavoidable) changes of existing CAST code, easier debugging, and independent analysis of the simulation output.



**Fig. 13.** Simulation tool platform – based on computer-aided stereology tool (CAST) software. The repetition framework is built around existing CAST functionality and the synthesized images and the new sampling paradigms are built inside. The arrows show the flow of information.