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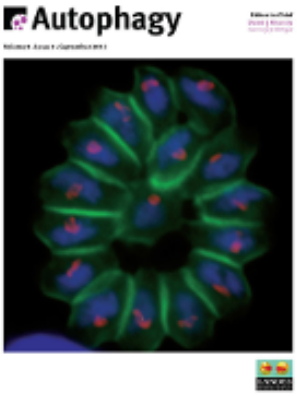

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Cutting a fine figure

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Cutting a fine figure

On the use of thin sections in electron microscopy to quantify autophagy

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Over the last few years, two guideline articles have been published with recommendations for assessing autophagy. These articles contained advice on quantification of autophagy by electron microscopy and proposed using thin slices for quantifying autophagic structures. Here, we expand on what *can* and *cannot* be quantified using single 2D slices and give some suggestions for efficient and minimally biased approaches for quantifying this fascinating and important process. We recommend that the journal *Autophagy* follow other journals in demanding stringent random sampling design and application of unbiased design-based quantification when reviewing submitted manuscripts.

Keywords: stereology, sampling, autophagy, electron microscopy, quantification, systematic uniform random

Abbreviations: 2D, two-dimensional; 3D, three-dimensional; EM, electron microscopy; SUR, systematic uniform random

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The Power of Electron Microscopy (EM)

EM is, without doubt, the gold standard for organelle characterization. It provides exquisite resolution that is orders of magnitude better than any conventional, or even super-resolution, light microscopy allowing display of membranes, cytoplasmic coat structures and lumenal content that are all relevant in autophagy studies.[1,2] A less well-known but nevertheless significant additional advantage is that, in addition to the organelle of interest, EM displays a wide array of structures within the cellular framework.[3] This visualization of the structural framework provides a huge array of possibilities for reliable identification, context analysis, connectivity and spatial analysis, all at high resolution.

The Strange World of Two-Dimensional (2D) Slices

With such detailed structural/molecular information, the possibilities for quantifying cellular structures and processes should be extensive. However, the display of structural details depends on slicing and, here, we describe how 2D information is "disconnected" from three-dimensional (3D) information. As a consequence, when it comes to quantifying from EM sections, extreme care and appropriate design is needed from the outset to get reliable and efficient results that really mean something about cellular quantities.

A first problem to solve with EM sections is that of organelle identification, because, in essence, one cannot measure that which one cannot "see"! Thus, for any quantitative analysis, organelles or subsets thereof, need to be reliably identified as they appear in the slices. It is therefore important to first draw up clear identification criteria, which allow reproducible assignment of any image to an organelle category of interest. It may even be necessary in some circumstances, to check by serial sectioning, that specific features utilized in the slices really do report on 3D structures, or to label using immuno-EM to characterize the organelle type. Autophagic structure identification is not the main focus of this article but examples of criteria used in autophagy studies are structural features such as the presence of a double membrane of early autophagic structures (discussed below) or the molecular markers identified using immunogold labeling such as LC3.[2]

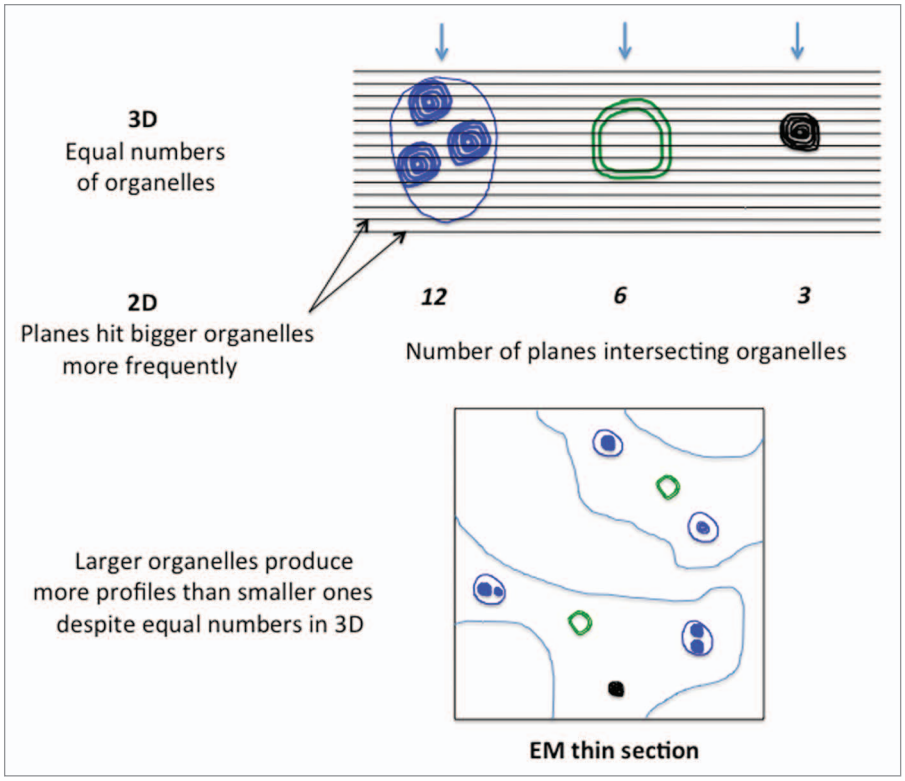

Figure 1. The size of an organelle determines its chance of occurring in a section plane. Different types of autophagic structures (left and center) and a late endosome/lysosomal organelle (far right) are illustrated and are cut by a series of 2D section planes (black horizontal lines). The number of section planes that hit these organelles is determined by the relative heights orthogonal to the direction of sectioning (this direction is indicated by arrows). Even though the numbers of each organelle in 3D are equal, the bias caused by sectioning would produce four times as many profiles when the section planes intersect the largest organelle (in this case 12 section planes) compared with smallest organelle (4 section planes). As an illustration of this effect, the thin section in the lower part of the figure displays profiles of these different organelles with frequencies that reflect their relative sizes and not their equal number in 3D.

A second problem to solve, when ultrathin sections are used, is how to quantify, and this is dealt with in detail here. Slicing is a widespread approach for visualizing the inner fabric and composition of biological structures. Taking the example of the human body, it is obvious that the shape and size of an individual organ (such as the liver) cannot be directly represented on a single slice and this is a good reason for reassembling the 3D structure of organs in silico for better clinical diagnosis. At the cellular level it is clear that something more needs to be done to extract volume, surface, length and number in 3D from the 2D information displayed on an ultrathin section examined in electron microscopy.

Number is not Accessible from a Single EM Section

This "disconnect" between the 2D profiles and the 3D objects is well illustrated when we consider how the "dimensions" of size and number are actually represented on a slice.[4,5] For example, an object with volume in 3D is actually represented by an area on the plane of a thin slice, while a surface in 3D produces a line in 2D, and a linear feature interacts with the slice-plane to produce a point. Importantly, all this shows that a plane section represents each 3D parameter at a dimension reduced by one in each case—a fact that provides us with some insight into whether we might be able to count items such as autophagic structures. As number is zero dimensional, it cannot be represented by any dimensional representation on a slice-plane. And if this is so, then what does a count of autophagic structure profiles represent? The answer has everything to do with the probability that a section plane intersects with the 3D objects in question. Intuitively, one might appreciate that the chance of a section plane transecting an object is related to its height in the direction of sectioning (see refs. 5 and 6; **Fig. 1**). A simple illustration would be a cell within which an organelle only stretches across half of the cell. Clearly, of all the possible randomly placed section planes, only one half of those falling in the cell will transect the organelle. Therefore, by counting profiles there would then appear to be only half as many organelles as there are cells, even though there is exactly one of each in 3D! This means that the frequency of organelle profiles (e.g., autophagic structures) is determined not just by their number in 3D but also by their 'height' in the direction of sectioning. In fact, this 'height' is sensitive to factors such as size, shape and orientation. In the case of autophagic structure quantification, any change in the number of profiles may be linked to changes in height or number or both. Any counts of profiles expressed as a density or relative proportion between vacuoles of certain types should therefore be interpreted with extreme care.

So how might numbers be counted using slicing procedures? The solution is to use a volume-based probe and this can only be generated at the EM level by combining more than one section or by using electron tomography. This is rarely done, but if the extra work is invested, then there can be rich rewards.[7-9]

The above considerations emphasize that autophagic structures cannot be counted reliably using single EM sections and must be counted by combining information obtained from sets of parallel slices. An estimate of profile number per unit area, or the ratio of profiles of one type to another, is sensitive to both organelle number and other organelle parameters such as size. When comparing, say, control and experimental cells, the only way a profile count in a 2D plane can reliably reflect relative numbers in 3D is when particles have identical size (height) distributions in both groups—and this is mostly unknown and would take a significant amount of work to check. So, however compelling and intuitive it seems, to start counting structures or measuring

them on the slices, it is a simple fact that the information present in the slice needs to be carefully considered before valid data can be derived from it.

Volume and other Sizes are Accessible on Single EM Sections

While it is clear that, with the dimensional reduction outlined above, number cannot be estimated on a single slice, thankfully other aggregate parameters of size such as volume and surface are accessible. In this article, we first consider the simplest and most straightforward parameter, which is volume. A principle described over a century ago by Delesse[10] established that the fraction of cytoplasm profile area that is occupied by organelle profiles on a plane section, the so-called "fractional area," is an estimate of "fractional volume" of the cytoplasm occupied by the organelle. In stereological parlance, the cytoplasm is called the reference space and the autophagic structures the component phase. One way to obtain an estimate of fractional volume is to employ a regular array of points, which can be used to interact systematically with the cytoplasmic space and count the fraction of points that land on the autophagic structures (see lower part of **Figs. 2 and 3**). Importantly, in order to ensure unbiased values, the point array must be placed at random on randomly-sampled sections. Note that in the case of autophagic structures it is reasonable to select the cytoplasm as the reference space, rather than the whole cell, since up to now autophagic structures have not been described in the nucleoplasm!

Finally it is worth reemphasizing the important issue of compartment identification. The earliest autophagic structures, also known as phagophores, are assembled as cisternal structures that eventually enclose a region of cytoplasm within an autophagic vacuole. Later on, the "ends" of the phagophore cisternal membranes appear to undergo fusion to form a closed vesicle surrounded by a double limiting membrane and these structures are then known as autophagosomes. Unless the open ends of the phagophores appear in the plane of section, these two structures may not therefore be differentiated. So while it is possible to carry out point counting on sections to quantify the total space enclosed by phagophores and autophagosomes together (both are surrounded by the double membrane), it may not be possible to differentiate how much of the space belongs to each. To determine the contribution from each, additional information may be needed from serial sectioning or by identifying a molecular/structural marker for phagophores or autophagosomes. In the case that phagophores present structures with extremely large openings it may be useful to quantify their membranes rather than the enclosed contents (see below).

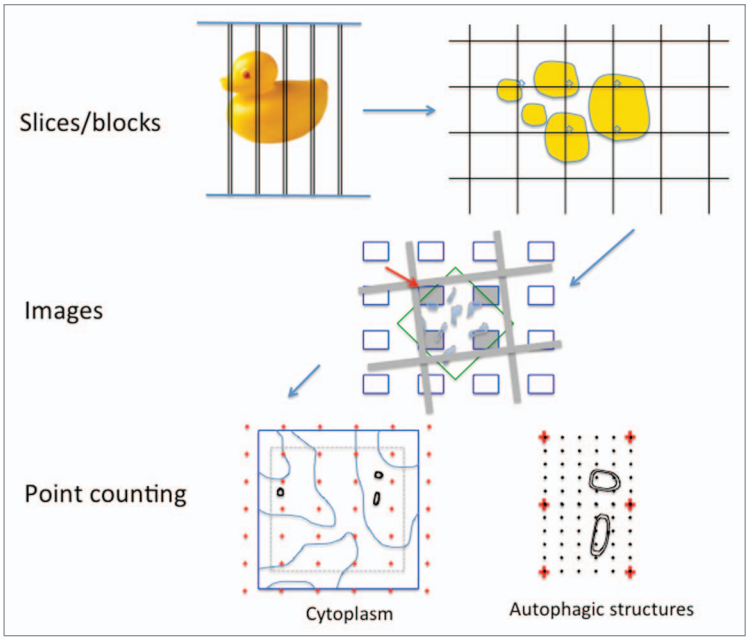

Figure 2. Sampling scheme for quantitative estimation of autophagic structure volume fraction in the cytoplasm. Systematic uniform random (SUR) sampling is the strategy of choice because of its efficiency and lack of bias. The tissue/organ/cell pellet can be sectioned into a randomly placed and evenly spaced set of slices. Systematic random locations mark the sampling positions of the blocks that will be processed and embedded for EM. First, sections taken from these blocks will be positioned by SUR inside the tissue/pellet. Appropriately stained sections are mounted on EM grid supports (gray lines). Micrographs are taken at low magnification in an SUR array, covering a whole section/pellet profile (typically, these number 10–20). The magnification is selected to contain maximal areas of the cytoplasm but allow clear identification of autophagic structure profiles for subsequent image recording at high power. Point counting proceeds on each of the micrographs as detailed in **Figure 3**. (Note that if necessary for volume fraction analysis, orientations of the cells can be preserved prior to the preparation of random sections, thereby preserving key spatial information about the location of autophagic processes relative to cell structure, as might occur in polarized cells).

Increasing Efficiency: Random Sampling with a Twist

Any estimate of the fractional volume will be valid for the part of the cytoplasm that lies in the immediate vicinity of the chosen section, but the challenge is to obtain estimates of fractional volume of autophagic structures that are valid for the *whole* cell pellet, tissue, organ, animal, experimental condition or cell culture. In EM, micrographs act like extremely small windows through which small portions of the section are visualized, and the sections are themselves samples of the whole specimen. To link the information within these small windows to the whole specimen, it is necessary to carry out a sampling protocol that ensures all possible sections and micrographs derived from the specimen are equally likely. This means that some form of random sampling is the method of choice.

However, because a random process has a tendency to cluster, it can lead to relatively large fluctuations in the estimates because of local "over-representation" and "under-representation" of biological

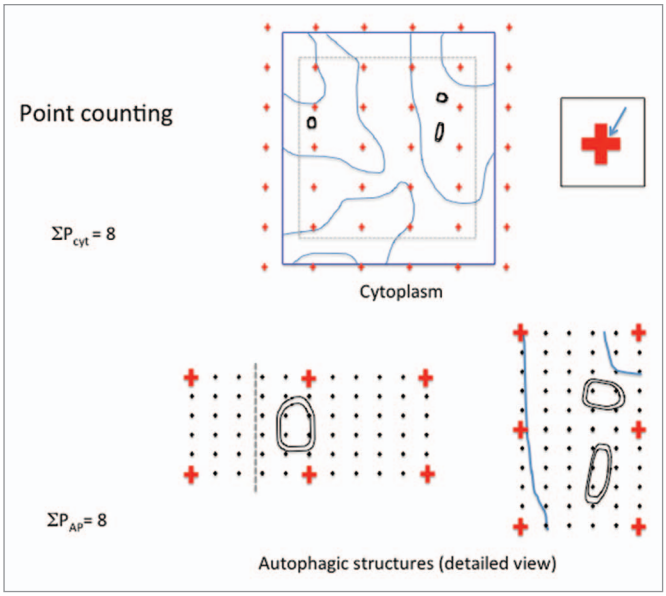

Figure 3. Principle of point counting to estimate fractional volumes of autophagic structures in the cytoplasm. A systematic grid lattice of widely spaced points (red crosses) is placed randomly over the micrograph (blue lines) containing an inset quadrat (gray dotted lines). The inset quadrat enables cell features such as autophagic structures within this rectangular area to be reliably identified even if they cross edges of the quadrat. Points (P) are defined by the corner at which the two lines of the cross meet (see inset top right). In the case illustrated here, points that interact with the cytoplasm (P_{cyt}) are 8 in number. Often, autophagic structures are infrequent and small in size and it may be preferable to carry out point counts on autophagic structure profiles (P_{AP}) found within the cytoplasm micrographs using a grid lattice with a higher density. An estimate of volume fraction = $\Sigma P_{AP} / \Sigma P_{cyt} . \rho$, where ρ is the number of points on the dense grid used for autophagic structures that represent each point on the grid used for the cytoplasm (in this case 25). So in this example with 8 point-hits over autophagic structures, an estimate of the volume fraction = 8/200. A key advantage of point counting is that the counts are decisions about whether a point is in or out of a structure and not traces or measurements.

structures, especially if they are inherently rather heterogeneous in their distribution. A better approach in most biological systems is to spread the samples through the whole specimen at regular intervals, avoiding bias by positioning the whole array with a random start. This twist on random sampling is termed systematic uniform random sampling or SUR sampling. For EM and other estimation purposes, SUR sampling is likely to be more efficient than simple random sampling and is of widespread utility in cell biology.[11,12] The only factor likely to compromise its efficiency is the presence in the specimen of a pattern, which happens to coincide with the frequency interval of SUR samples. However, this can be overcome easily by altering the sampling interval. SUR sampling is particularly useful whenever biological spaces/surfaces need to be sampled, as in the case of autophagic structures.

So how is SUR sampling applied to EM in practice (**Fig. 2**)? An SUR sample of tissues/organs can be produced by systematic selection from a complete array of slices sampled at intervals through the whole organ. The first slice of the series is selected at random to ensure the absence of bias. Each selected slice can be overlaid with a randomly placed systematic sampling framework and cut into blocks. This ensures SUR placement of sections produced from the embedded blocks and, again, micrographs can be positioned as an SUR array over the whole section profile with the first micrograph positioned randomly by alignment with the corner of a hole of the EM support grid square (**Fig. 2**).

Once micrographs have been recorded, the final step is to carry out estimation of the volume fraction using point counting with a systematic array of points (**Figs. 2 and 3**). Note that once the random or SUR sampling has been applied at all levels, including the point counting, the number of point counts needed to obtain reasonable precision is 100–200 for each element of the ratio (points on autophagic structures divided by points on reference space) in each experimental condition—this means the counts take minutes and are a minor outlay compared with the time required for processing, sectioning and imaging.

In the case of autophagy, the autophagic structures are rather infrequent and small compared with the cytoplasm, and so it is advisable, (1) to include as much cell space as possible by recording micrographs at a magnification that is the smallest at which the borders of the autophagic structures can be reliably identified, and (2) use two different densities of points in a so-called "coherent" point sampling grid which has a known ratio between the numbers of points at each density (**Fig. 3**). Another approach (not illustrated here) is to systematically scan all the available reference space (cytoplasm) that is displayed in the section and take pictures of *all* autophagic structures at high magnification and then take low magnification views for quantifying the area of the cytoplasm from which the autophagic structures were sampled. Point counting would proceed as already described (**Fig. 3**) except that in addition, account is now made for the different magnifications used when calculating the ratio of point counts that have been made over cytoplasm and autophagic structures, respectively.

As already suggested, there is a relationship between the boundary length of, say, membrane profiles and the packing density of surface within the cytoplasm. When early autophagic structure membranes are a particular focus, then this approach could be useful because it detects phagophores as well as other autophagic structures, irrespective of whether they have completely enclosed their contents. The principle is to apply an array of test lines and count intersections with the autophagic structure membranes (**Fig. 4**). By simultaneous point counting over the reference space it is then possible to estimate the line length applied to the reference space and a simple formula estimates the packing density of autophagic

structure membrane in the cytoplasm from the intersection counts. The basis for this relationship will not be discussed further here, but an unbiased estimate of packing density is obtained when randomization of specimen orientations is achieved in addition to the randomization of position described for volume fraction (**Fig. 4**; see ref. 14 for further details). Randomization of orientation for EM can be arranged easily by embedding the sample in a small ball of gelatin and rolling it prior to embedding in resin.[13]

The Reference Trap: Reporting Concentrations or Amounts?

The volume fraction is a ratio and is determined by two components: the volume of autophagic structures and the volume of the reference space (most often the cytoplasm). Similarly, surface density is determined by the surface of autophagic structure membrane in cytoplasmic volume. Herein lies the so-called reference trap and caution is required.[14] Why? Because while, on the one hand, an increase in say the volume fraction can reflect an increase in the volume of autophagic structures, on the other hand, it could also reflect a decrease in volume of the reference space. Imagine an autophagy stimulus, such as amino acid/growth factor deprivation, which also prevents cell growth (reducing increases in cell size) and causes an increase in autophagic structure fractional volume without any change in the autophagic structure pool. Of course, one could argue that, in the short-term, the chance that gross physiological causes of reference space change are unlikely to produce large changes in its volume. But a cautionary note comes from studies on human brain in which differential shrinkage of the reference led to the misguided impression that there was excessive depletion of neurons in older brains compared with young ones.[15]

The reference space volume can be determined in a number of ways. One is to estimate the volume from a set of slices using the principle of Cavalieri either by light microscopy (e.g., confocal) or by EM.[14] The principle is to take a randomly positioned systematic series of sections and to estimate the area of the reference volume displayed on these. The sum of the areas multiplied by the distance between the sections is an estimate of the volume. Other ways to estimate the cell volume are by analyzing live cells with cell content markers or by combining cell height measurements in EM with cell counts.[16]

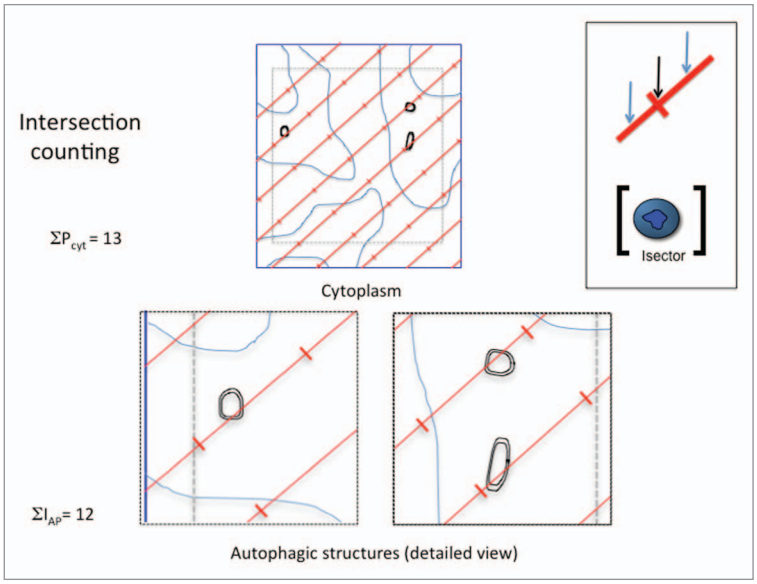

Figure 4. Principle of intersection counting to estimate surface density of autophagic structure membranes in the cytoplasm. A systematic grid of test lines (red) is placed randomly over the micrograph (blue lines) containing an inset quadrat (gray dotted lines) to aid identification of organelles. Intersections (I) are counted along one edge of the lines (see inset top right, blue arrows) and each line is adorned with points, positioned at regular intervals and identified as in **Figure 3** by the corners between crossed lines (black arrow). In the case illustrated here, points that interact with the cytoplasm (P_{cyt}) are 13 in number. Counts are made of intersections of the test lines with autophagic structure membranes (in this case each of two limiting membranes) and are 12 in number. An estimate of the surface density = $2\Sigma I_{AP}/ L_{cyt}$, where L_{cyt} is the length of test line applied to the cytoplasm (the reference space) and is determined by the number of points falling on the cytoplasm ΣP_{cyt} x the interpoint length. Thus, if the interpoint length is 5 microns, then the surface density estimate is 24/65 in μm^{-1}. A similar multistage sampling scheme can be used as for volume fraction, but orientations must be randomized in addition to the positions, using for example the isector (specimen encased in a ball of gelatin/agarose and rolled before resin embedding; bracketed insert).[13] Note that for surface density estimations on polarized or monolayer culture cells there is a specialized technique that allows the generation of randomly orientated line probes on a section with a chosen "vertical" direction. In this case the "isector "is not used and the random lines are represented by cycloids placed with reference to a vertical direction which can be chosen to preserve the polarized organization of the cell structures (see ref. 14 for details). For simplicity and clarity, the array of test lines illustrated in the figure are oriented parallel in one direction. Other arrangements include a square lattice or semicircles. Both of these arrangements can reduce the variance linked to any preferred orientations in the membranes of the target organelle.

Recommendations for Quantitative Assessment of Autophagy in Thin Section-EM

In summary, there are several dos and don'ts that show the way through to unbiased and efficient estimates of autophagic structures in thin sections. They stem from the strange relationship between the 2D slice-plane profiles and the quantitative 3D reality of the autophagic structures in the cell. A first step is to establish "cast-iron" criteria for assigning 2D profiles to 3D object sets. *Do not* count profiles, because autophagic structures vary in size and shape. Use fractional volume estimates as a preferred starting point. Make a sampling scheme that uses at least random and preferably SUR sampling at all sampling levels including the slices, blocks, sections and micrographs. The sampling is crucial to obtaining unbiased and also efficient estimates. Make sure that images of both the cytoplasm and the autophagic structures are recorded at magnifications that allow clear identification of compartments and

delineation of profile boundaries. Point counting stereology can then be employed and is a rapid and efficient way to estimate areas. Similar guidelines apply to surface density estimations but these also require randomization of either test line or specimen orientation in 3D. Importantly it is rarely necessary to count more than 200 points or intersections over each compartment of interest for reasonable precision and efficiency of estimation.

Finally we would urge the autophagy community and associated journals to adopt policies for manuscript submission that stipulate a requirement for the use of rigorous sampling techniques and unbiased design-based methods for quantifying autophagic structures.[17-19] One of us has recently emphasized the importance of doing this is in cell biology[12] and a number of journals are aligned with this view.

Acknowledgments

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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