Article

Distribution of Particles in the Z-axis of Tissue Sections: Relevance for Counting Methods

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ABSTRACT

The distribution of particles in the z-axis of thick tissue sections has gained considerable attention, primarily because of implications for the accuracy of modern stereological counting methods. Three major types of artifacts can affect these sections: loss of particles from the surfaces of tissue sections (lost caps), homogeneous collapse in the z-axis, and differential deformation in the z-axis. Initially it was assumed that thick sections were not compromised by differential shrinkage or compression (differential uniform deformation). Studies in the last decade showed that such artifacts are common and that they depend on embedding media and sectioning devices. Paraffin, glycolmethacrylate and vibratome sections are affected by this artifact, but not celloidin sections or cryostat-derived cryosections. Differential distribution of particles in the z-axis is likely due to compression of the surface areas (margins) during sectioning, resulting in differential particle densities in the core and margin of tissue sections. This deformation of tissue sections can be rapidly assessed by measuring the position of particles in the z-axis. The analysis is complicated by potential secondary effects on section surfaces through loss of particles, the so-called "lost caps" phenomenon. Secondary effects necessitate the use of guard spaces, while their use in case of primary effects (compression due to sectioning) would enhance the artifact's impact on bias. Symmetric versus asymmetric patterns of z-axis distortion can give clues to distinguish primary and secondary effects. Studies that use the optical disector need to take these parameters into account to minimize biases.

Key Words: Stereology, morphology, histology, particle counting, bias, optical disector, sampling, cell count, lost caps

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Introduction

Quantification of morphological parameters in tissues is important in the biological sciences to understand changes that occur during development, aging, and with altered conditions such as pathological disease states or experimental manipulations. Tissues need to be cut into sections to visualize cellular and subcellular particles of interest. The relevance and significance of accurate counting of particles is evidenced by the large numbers of citations of key methods papers (**Table 1**). A major fraction of those citations are from studies in the area of neuroscience.

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Theoretically, counting of particles with design-based approaches is straightforward, but unfortunately, the real world intrudes with artifacts when tissue sections are generated, processed and analyzed. This review focuses on three major types of artifacts that can affect the distribution of particles in the z-axis of thick tissue section: loss of particles from the surface of tissue sections, shrinkage or collapse of the z-axis in cryo- and vibratome sections, and deformation in the z-axis that can occur during and after sectioning (**Fig. 1A-C**). The first and long-known type of artifact is the loss of particles from section surfaces, the so-called "lost caps" phenomenon (Hedreen, 1998a), also called "truncation" (Gundersen, 1986). The second, z-axis shrinkage or collapse, became relevant when thick cryo- and vibratome sections were generated to implement the increasingly popular optical disector counting method for quantitative

analyses. The third, more recently discovered artifact is a differential deformation of tissue sections in the z-axis (Hatton and von Bartheld, 1999; Dorph-Petersen *et al.*, 2001; Gardella *et al.*, 2003; Baryshnikova *et al.*, 2006).

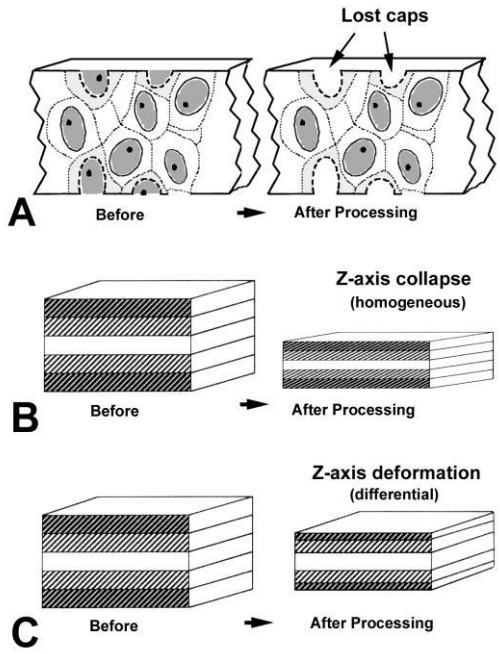

Figure 1. Illustration of three major types of artifacts that affect the distribution of particles in the z-axis of tissue sections. A. Loss of particles from the section surface – so-called "lost caps". Modified from Andersen and Gundersen, 1999. B. Collapse (shrinkage) of tissue section in the z-axis. This shrinkage may be either homogenous (as indicated, typically in cryosections) or differential (see panel C). C. Differential deformation of the z-axis. Particles in zones closer to the surface shrink or are compressed more than particles in the core of the tissue section, resulting in lower densities of particles in the core than in the margins of tissue sections (modified from Gardella *et al.*, 2003).

All three types of artifacts have different implications for quantification methods (profile counting, optical disector). It is important to recognize that these different artifacts can occur in combination within the same tissue section (Baryshnikova *et al.*, 2006). Nevertheless, the three different types of artifacts will be considered in this review in sequence. We will discuss likely causes, diagnostic procedures, implications for counting methods, the history of discovery of major concepts, and strategies to cope with these artifacts.

Table 1. Citations of key method papers for quantification of particles in tissue sections, according to the Web of Science (Thomson Reuters).

Authors	Year	Number of citations as of Aug 11, 2008	Aug 11, 2011
Abercrombie	1946	3226	3525
Konigsmark	1970	710	725
Sterio	1984	1463	1675
Gundersen	1986	1070	1245
Gundersen *et al.*	1988a	1235	1448
Gundersen *et al.*	1988b	1561	1817
Williams and Rakic	1988	265	308
West	1999	320	402
Coggeshall and Lekan	1996	651	735
Howard and Reed	1998	341	477
Schmitz and Hof	2005	78	171
	Total	10,920	12,528

History and Consequences of "Lost Caps"

Lost caps are particles located at the section surface that have fully or partially broken away from the surface during sectioning or tissue processing, or that are such small fragments that they cannot be optically resolved and recognized (Hedreen, 1998a, **Fig. 2A**). Three different methods have been used to assess lost caps. Historically, evidence for lost caps was documented in thin (about 5 µm) sections that had been re-embedded and sectioned in a plane perpendicular to the original sectioning plane (Helander, 1983). This method was recently called into question, because the re-embedding and re-sectioning process itself can cause secondary artifacts, not initially present in the original sections (Baryshnikova *et al.*, 2006). A second method is the quantitative measurement of particles in the z-axis as first described by Hatton and von Bartheld (1999) and Andersen and Gundersen (1999). Loss of particles from one or both surface regions is considered the most likely cause of "lost caps" (**Fig. 2B-C**), although incomplete stain penetration needs to be ruled out when particles appear to be missing on the lower (slide-side) surface (**Fig. 2D**). Finally, a 3D serial section reconstruction can provide evidence for lost caps when particle profiles such as large nuclear or nucleolar profiles are visible in one section, but the corresponding fragment is missing in the adjacent section (Coggeshall and Chung, 1984; Hatton and von Bartheld, 1999; von Bartheld, 2002).

Lost caps were a major concern and source of bias in older, profile-based methods (Hedreen, 1998a), because with profile-counting in thin sections, information about the frequency of all profiles is needed in sampled sections to apply appropriate correction factors. Correction factors were introduced on numerous occasions tominimize

the effect of lost caps for numerical estimates (Abercrombie, 1946; Konigsmark, 1970; Clarke, 1992, 1993; Hedreen, 1998b; Mouton, 2002). Although lost caps tend to partially correct the overcount caused by multiple profiles, it is still difficult to precisely estimate their impact. Without the use of time-consuming calibration strategies ("empirical method," Coggeshall *et al.*, 1990; Pover and Coggeshall, 1991; Hedreen, 1998a, b), the appropriate correction factor cannot be accurately determined, and therefore the lost caps error remains an inevitable problem in the profile-based counting methods (Hedreen, 1998a, b).

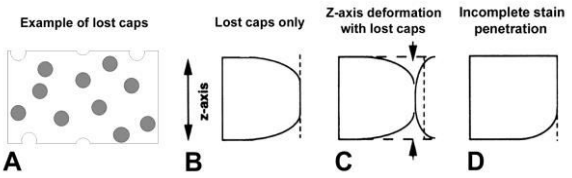

Figure 2. Schematic illustration of lost caps and their quantification by z-axis analysis. A. Example of lost caps at both section surfaces. B. Graphical illustration how lost caps can appear in z-axis analyses when this is the only artifact. C. Z-axis analysis when lost caps are superimposed upon differential z-axis deformation. D. Z-axis analyses indicating lost caps must be distinguished from data indicating incomplete stain penetration. Panels B-D are modified from Gardella *et al.*, 2003.

In the first decade of the introduction of the optical disector, lost caps were widely believed to be irrelevant for this counting method (Gundersen, 1986; Williams and Rakic, 1988; Clarke, 1993; Hedreen, 1998a). This belief was based on the notion of theoretical cancellation of lost caps mistakes at the top and the bottom of the counting chamber (Hedreen, 1998a). However, this design required the use of guard spaces. Unfortunately, the use of guard spaces makes the assumption that, if the section is deformed, only homogenous, but not differential deformation occurred in the z-axis, the topic of a later section in this review (see below). Thus, along with the use of guard spaces, the lost-caps problem needs to be re-evaluated for its impact on the optical disector.

The prevalence of lost caps in thick sections is controversial. One contributing factor to this uncertainty is that this phenomenon is usually not assessed with sufficient diligence and hardly ever documented (Guillery, 2002; von Bartheld, 2002; Geuna, 2005). Different tissue processing protocols used by different investigators seem to yield amounts of lost caps that vary to a surprising degree between studies. The frequency and severity of lost caps is currently controversial and seems to be highly dependent on the histological protocols used. Some histologists find minimal evidence and impact of lost caps in serial reconstructions or z-axis analyses (Hatton and von Bartheld, 1999; Gardella *et al.*, 2003; Baryshnikova *et al.*, 2006), while others report minor effects based on calibration studies (Pover and Coggeshall, 1991), or even major effects after analysis of particle distribution in the z-axis (Andersen and Gundersen, 1999; Dorph-Petersen, personal communication). Thus, the actual prevalence, magnitude, and impact of lost caps is not precisely known, and requires further study.

Z-axis Collapse and Modern Stereology

Shrinkage of tissue sections or z-axis collapse with dehydration, drying and coverslipping has been a familiar phenomenon among histologists, but it did not have any major implications for processing and analysis of the relatively thin (5-15 µm) sections that are typically used for traditional profile counting (Clarke and Oppenheim, 1995). With the advent of design-based stereology and especially the optical disector methodology, thicker (20-40 µm) sections were needed. Many accomplished and aspiring stereologists became frustrated when they cut 30- or 40 µm sections on cryostats or vibratomes, only to find that these sections shrink in the z-axis during section processing by 60-80%, to a final thickness of about 10-15 µm. Such sections were too thin to be used for optical disector analyses. It should be noted that celloidin and paraffin sections do not shrink nearly as much as cryo- and vibratome sections (Fig. 3A, Ward *et al.*, 2008). When tissue samples are embedded in paraffin, the whole specimen shrinks prior to sectioning, but the paraffin sections tend to retain their final thickness close to the nominal section thickness. This can be easily measured by comparing the nominal section thickness (verified by measuring the block advance) with the final z-axis thickness after coverslipping (measured by focusing and read-out on a microcator (Williams and Rakic, 1988; Dorph-Petersen *et al.*, 2001; Guillery, 2002; Williams *et al.*, 2003).

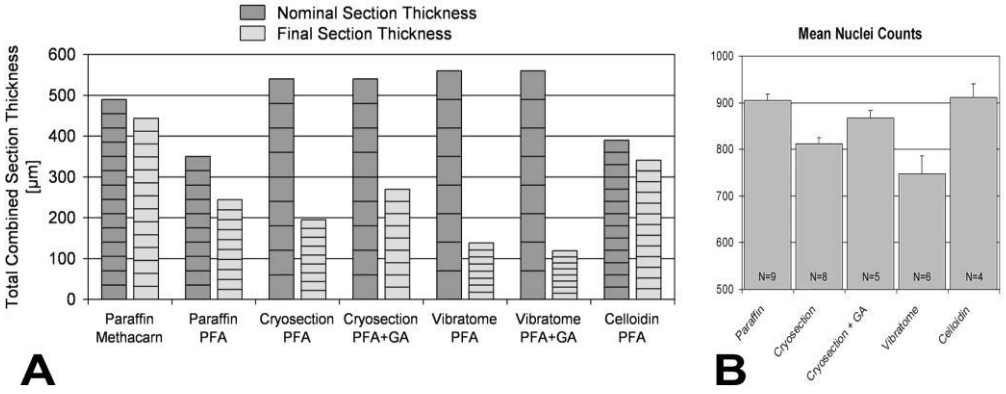

Figure 3. The extent of z-axis collapse with different embedding and sectioning protocols, and the empirical quantification of their effect on recognition of particles, using large motor neuron nuclei as particles. GA, glutaraldehyde; PFA, paraformaldehyde. A. Extent of z-axis collapse with different fixation protocols. B. Quantification of the effect of higher stacking density on particle recognition, and the predicted effect (bias) on number estimation with optical disector technology. Adopted with permission from Ward *et al.*, 2008.

Two different solutions for the problem of z-axis collapse were proposed: One study recommended omitting dehydration and clearing, and to coverslip cryosections in a hydrophilic mounting medium (Bonthius *et al.*, 2004). However, combining a water-based mounting medium that has a lower refractive index (1.42), with a higher refractive index (about 1.5) for the cover glass and oil immersion, is not advised for proper microscopy when reliable and precise measurements are needed (Williams and Rakic, 1988; Dorph-Petersen *et al.*, 2001; Guillery, 2002; Guillery and August, 2002). While the advocates of the water-based approach argued that the substantial error due to the Holmes effect would cancel out, no theoretical or empirical evidence for this notion was provided (Bonthius *et al.*, 2004). An additional major disadvantage of the proposed water-based mounting is the short durability of the sections, which makes it impossible to repeat, verify or check the initial counts.

Another solution to deal with z-axis collapse that is more generally used and established is to cut considerably thicker sections, 60-80 µm, and then to analyze the sections after they have shrunk to a final z-axis thickness of 20-30 µm (Ward *et al.*, 2008). One wrinkle in this strategy is that now the particles become stacked more densely on top of each other in the z-axis. This can lead to enhanced difficulty in recognizing or distinguishing small particles (Clarke, 1993; Ward *et al.*, 2008). When quantified, this indeed resulted in an empirically-derived bias (underestimate) of 10-25% (Ward *et al.*, 2008) (see **Fig. 3B**). The optical resolution of particles appears to be influenced by the type of fixative used. Addition of glutaraldehyde, even in very low concentrations, is surprisingly effective in improving recognition of particles in cryosections (Ward *et al.*, 2008).

The problem of z-axis collapse is largely restricted to cryo- and vibratome sections, but does not occur with celloidin, glycolmethacrylate or paraffin sections. When the project and study design allows different choices among embedding media and sectioning devices, the investigator should use the most suitable tissue processing and quantification protocol (von Bartheld, 2002).

Differential Deformation of Tissue Sections in the Z-axis

The third, and most recently discovered artifact is possibly the most significant, as it has consequences for the placement of counting boxes and guard spaces. Neglect of differential deformation in the z-axis can substantially bias optical disector counting, the most popular of the design-based counting methods.

I. Calibration analyses and the discovery of z-axis distortion. In the last two decades, two conflicting schools of thought existed regarding calibrations of the new stereological disector methodology. One school, represented by Gundersen, West, Cruz-Orive, Mayhew, Pakkenberg, Mouton, and Howard believed that calibrations were not

only unnecessary, but also actually irrelevant or counterproductive, because the new methodology was theoretically unbiased and therefore was deemed to be inherently accurate (Cruz-Orive, 1994; Mayhew and Gundersen, 1996; Howard and Reed, 1998; Mouton, 2002). Another school of thought (represented by the works of Coggeshall, Farel, Guillery, Saper, Geuna, Kaplan, von Bartheld, Williams, and Rosen) held that calibration was a useful and necessary tool to validate new methods and to detect, correct and minimize biases (Coggeshall *et al.*, 1990; Pover and Coggeshall, 1991; Coggeshall, 1992; Hatton and von Bartheld, 1999; Saper, 1999; von Bartheld, 1999, 2001, 2002; Farel, 2002; Guillery, 2002; Williams *et al.*, 2003; Geuna, 2005; Baryshnikova *et al.*, 2006). Calibration analyses of the disector methodology were conspicuously absent in the 1980s and emerged only in the 1990s, first for the physical disector (Pover and Coggeshall, 1991; Popken and Farel, 1996; Basgen *et al.*, 2006), and subsequently for the optical disector (Hatton and von Bartheld, 1999). This delay was likely related to the fact that the most influential advocates of the new methods considered calibrations unnecessary, and also because calibration analyses are cumbersome and tedious, with most model systems being too large or complex for easy comparison with complete serial section reconstructions.

The first calibration analysis of the optical disector was performed by serial section reconstruction for a small (trochlear) motor nucleus with about 900 neurons (Hatton and von Bartheld, 1999). The authors noticed significant (15-25%) deviations from true numbers when compared with the "gold standard." The resulting discrepancy prompted Hatton and von Bartheld to further analyze and question the use of guard spaces. To address the problem, von Bartheld and colleagues developed a simple, but highly effective and quantitative method to assess the z-axis distribution of particles. This led to the discovery of z-axis deformation as the apparent cause of the considerable numerical discrepancy (15-25%). In 1999, two groups independently published a similar design for scoring the z-axis position of particles to determine shrinkage and/or compression: Within months, first Hatton and von Bartheld (June, 1999) and subsequently Andersen and Gundersen (December, 1999) reported the z-axis distributions of particles in tissue sections. Curiously, somewhat different results were obtained: The vibratome sections in Andersen and Gundersen's study showed considerable lost caps, while there were minimal lost caps in Hatton and von Bartheld's paraffin, cryo- and plastic sections. Later studies with vibratome sections showed that the discrepancy could not be explained by differences between the type of embedding and sectioning devices alone (discussed in detail in Gardella *et al.*, 2003; Baryshnikova *et al.*, 2006). The discrepancy is still not fully resolved, even after more systematic z-axis analyses were extended to all types of tissue sections, including paraffin, vibratome, methacrylate, celloidin and cryosections (Gardella *et al.*, 2003; Baryshnikova *et al.*, 2006) (Fig. 4A-E). Interestingly, a recent study of cryosections that were not obtained in a cryostat chamber but rather on a sledge microtome (where the specimen is frozen, but not the knife blade) revealed that those sections presented with z-axis deformation (Carlo and Stevens, 2011), unlike cryostat-derived cryosections (Gardella *et al.*, 2003). This suggests that the interactive mechanics and physical thermal properties of a frozen vs. thawed blade are crucial for z-axis deformation.

II. Modern stereology and the introduction of guard spaces. To understand the relevance of z-axis deformation, it is useful to briefly review the concept of guard zones in the optical disector design. When the optical disector methodology was developed, it was realized that potential artifacts at tissue surfaces – lost caps – had to be avoided. To deal with this potential artifact, Gundersen (Gundersen, 1986; Gundersen *et al.*, 1988a) introduced guard spaces – zones that were specifically excluded from sampling (Fig. 5), so that one would avoid sampling of particles in surface areas that were potentially affected by lost caps. However, there was a price that had to be paid for this solution. This was clearly recognized and stated by Gundersen and coworkers in their 2001 publication as "specific requirements for optical design" – "no differential deformation in the direction of the z-axis may occur" (Dorph-Petersen *et al.*, 2001). When one uses guard spaces, one makes the implicit assumption that there is no differential deformation of the tissue sections. In other

words, one assumes that the guard spaces (spaces that are not sampled) contain or used to contain at a previous time point the same density of particles as the core of the tissue section. If this assumption was not true, meaning that if such deformation occurred and resulted in differential particle densities, then the guard spaces would violate the "holy grail" of design-based counting, the key rule that "a priori all particles have the same chance of being sampled" (Gundersen *et al.*, 1988a). In this case, differential deformation would result in bias, because guard spaces are included in the total reference space, but the density in the guard spaces differed from the density in the core areas that were sampled.

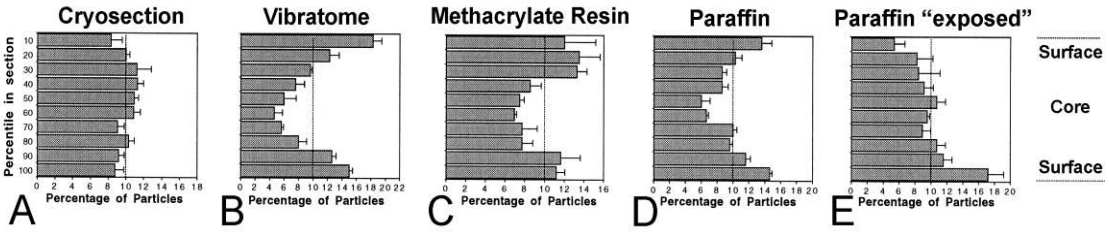

Figure 4. Examples of distribution profiles (z-axis analyses) of different types of tissue sections. The percentage of particles (theoretically = 10% in each of 10 bins = 100%) is plotted against the percentile in the section. The top bin is at the top (upper surface), the bottom bin (against the glass slide) is at the bottom of each section. **A**. Cryosection generated in a cryostat. **B**. Vibratome section. **C**. Methacrylate (plastic) section. **D**. Paraffin section. **E**. Paraffin section that was cut with a knife angle tilt that makes the exposed surface more vulnerable to particle loss during section processing (floating, staining, dehydration, Baryshnikova *et al*., 2006). Note the secondary loss of particles from the bin at the upper surface. All error bars = SEM. Approximately 400-600 particles were scored for each tissue section. Data are compiled from Hatton and von Bartheld, 1999; Gardella *et al*., 2003; and Baryshnikova *et al*., 2006.

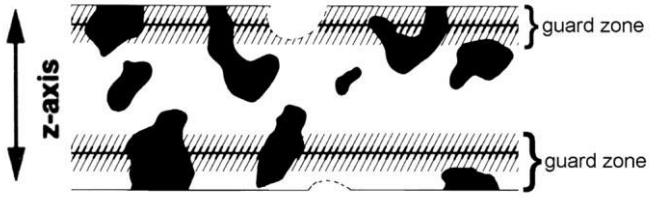

Figure 5. Example of the placement of guard spaces (guard zones, exclusion zones). Modified from Gundersen, 1986, Mouton, 2002, and Baryshnikova *et al*., 2006.

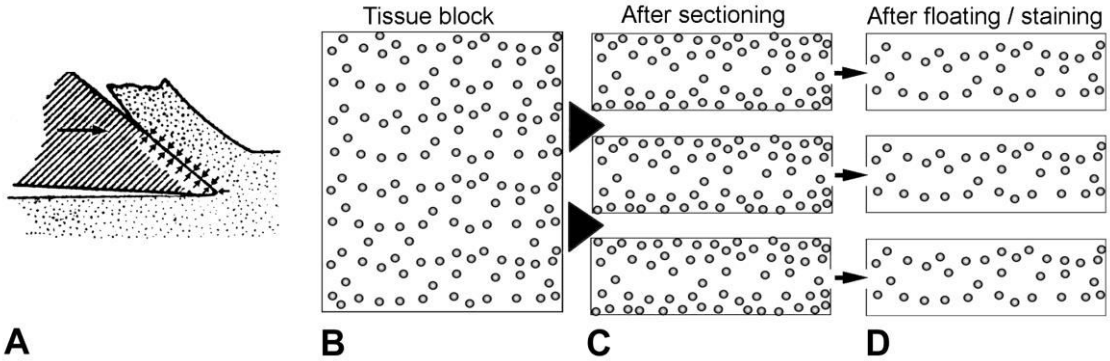

Figure 6. The schematic illustrates the proposed 2-step mechanism of z-axis deformation. **A**. Forces exerted upon the tissue by the knife during sectioning. Modified from Dempster, 1942. **B**. Particle distribution in the tissue block prior to sectioning. **C**. Sectioning results in compression of margins with higher particle densities at the surfaces. D. Loss of particles from the section surfaces during tissue processing. Panels **B-D** are modified from Baryshnikova *et al*., 2006.

The theoretical assumption of lack of differential deformation (no differences between core and surface regions) was recognized and was still believed to be largely met in the work of Dorph-Petersen *et al.* (2001), but ultimately this assumption turned out to be incorrect in many cases – in fact tissue deformation is quite frequent in several, but not all, types of embedding media, as a series of studies showed (Hatton and von Bartheld, 1999; von Bartheld, 1999; von Bartheld, 2002; Gardella *et al.*, 2003; Williams *et al.*, 2003; Baryshnikova *et al.*, 2006). Thus, the introduction of guard spaces (Gundersen, 1986) ironically violated the key principle that the new methods had emphasized as being superior to the older profile-based (and often so-called assumption-based) methods (von Bartheld, 1999; von Bartheld and Hatton, 1999; von Bartheld, 2001; 2002; Gardella *et al.*, 2003).

The initial response by stereologists to the publication of the guard space problem was mixed. Some experts immediately recognized the merits and importance of the new findings and embraced them as an opportunity to improve the optical disector concept (e.g., Williams and Rakic, 1988, see update at http://www.nervenet.org/papers/3DCounting.html; Guillery, 2002; Williams *et al.*, 2003; Geuna, 2005; Schmitz and Hof, 2005), while others chose to either ignore it or to regard the problem as a "staining artifact" (Dorph-Petersen *et al.*, 2001, 2004) or as a "non-reproducible lab-specific problem" (Evans and Nyengaard, 2004). To definitively address this issue, an international study was designed in which not only tissue sections generated in the senior author's lab were examined by (initially skeptical) experienced experts in other countries, but also new tissue sections were generated in two different labs located on two other continents (Europe and Asia) (Baryshnikova *et al.*, 2006). The results of this study confirmed and expanded the initial results of z-axis deformation, thus convincing many skeptics. Regarding the notion that the observed z-axis patterns may reflect staining artifacts, it was demonstrated conclusively that the observed effect could not be explained as a staining artifact, because (1) the pattern of distribution was symmetrical rather than asymmetrical as expected (and verified) for incomplete stain penetration. Furthermore, (2) it was shown that incomplete stain penetration generates a pattern that differs considerably from those observed, and (3) the quality of particle staining and the number of particles resolved in the different bins along the z-axis did not vary qualitatively or quantitatively (Gardella *et al.*, 2003; Baryshnikova *et al.*, 2006; Ward *et al.*, 2008).

III. Attempts to identify the mechanistic cause: the concept of a two-step process. The above-mentioned study, Baryshnikova *et al.* (2006), was the first to systematically address the likely mechanisms of differential shrinkage in the z-axis. Importantly, by closely examining the patterns of deformation under numerous experimental conditions (flipping of sections so that different surfaces were exposed to different sequences of conditions), the authors provided evidence for a two-step process. The section surface of "soft" sections first becomes compressed, resulting in higher densities of particles at bins close to the surfaces, but subsequent exposure to harsh solutions may lead to secondary loss of particles from the bin immediately adjacent to the exposed surface, resulting in lost caps in a secondary step and a reduction of particle densities at the bin(s) closest to the surface (**Fig. 6**).

The data obtained by measuring particle distribution in "flipped" or "double-flipped" sections was consistent with a 2-step process: first compression, deformation and shrinkage – then secondary loss of particles (lost caps) during staining or washing of tissue sections (Baryshnikova *et al.*, 2006). Relevant for diagnostic insights is that primary effects are typically symmetric (they affect both surfaces and margins of tissue sections, **Figs. 2A-C**, 6C), while secondary effects are typically asymmetric. They affect only or primarily the exposed side of the tissue section – if processed "on the slide" – but may be symmetric, if processed free-floating. Thus, the pattern of deformation in the z-axis, symmetric or asymmetric, gives important diagnostic clues about the mechanism how the deformation was initially generated. As mentioned above, artifacts of incomplete stain penetration typically affect only the "slide side" – the side that is protected from, and at the largest distance from the staining solution (Fig. 2D). Artifacts due to incomplete stain

penetration always need to be ruled out and need to be distinguished from true differences in z-axis distribution of particles.

Outlook

How to Cope with Artifacts and Achieve Minimal Bias When Counting Particles

The following are a few practical tips how to avoid artifacts in tissue sections, how to recognize them, and how to deal with them once they have been identified.

1. Prior to or during the design of a new study, take into account different vulnerabilities of embedding and sectioning protocols. Select those embedding and sectioning protocols that are best-suited for the purposes of the study and counting design (**Table 2**). Consider z-axis collapse and resulting biases (Ward *et al.*, 2008). For example, when particles of interest are small and densely packed, cryo- or vibratome sections should be avoided (Ward *et al.*, 2008). Consider adding glutaraldehyde fixatives to improve the particle recognition in cryosections. Major advantages and disadvantages for embedding media and sectioning techniques are briefly summarized in Table 2.

Table 2. Criteria for choosing embedding and sectioning techniques. * unless frozen sections are derived by a sledge microtome (Carlo and Stevens, 2011).

Technique	Advantages	Disadvantages
Cryosection	No z-axis deformation* Immunolabel Fast	Mediocre morphology Z-axis collapse Poor recognition of particles
Vibratome	immunolabel Fast	Mediocre morphology Z-axis collapse Z-axis deformation Lost caps (controversial)
Paraffin	Excellent morphology Minimal lost caps Immunolabel Fast	Z-axis deformation
Glycolmethacrylate	Good morphology	Z-axis deformation No immunolabel
Celloidin	Excellent morphology No z-axis deformation	no immunolabel Time-consuming embedding

2. Prior to large-scale tissue collection and processing, examine in a small, representative sample the distribution of particles in the z-axis. This will provide information about possible lost caps, z-axis collapse and z-axis deformation. If any of these parameters are severe, alternative protocols should be considered and tested (including different fixatives or different embedding and sectioning options). Examine sections for lost caps by analyzing how particles are distributed in the z-axis (von Bartheld, 2002).

3. Whenever potentially relevant changes are made to the protocol, such as use of a different fixative, different thickness of sections, different staining parameters, switching from processing "on the slide" to free-floating sections, then test the distribution of particles in the z-axis. Decide on the placement of guard spaces, if any are needed, only after completing a z-axis analysis to assess differential uniform deformation. This is particularly important for tissues embedded in paraffin or glycolmethacrylate, or when tissues are sectioned on a vibratome. When a new study is started or any significant variables are modified, it is advised to perform an exploratory minimal calibration (von Bartheld, 2002; Williams *et al.*, 2003), to make sure that no major biases have been overlooked or have crept into the analysis.

4. When the results of the study are published, include sufficient information about fixatives, staining protocols, section thickness, z-axis analyses, and placement of sample boxes and guard spaces, so that the results can be directly compared with other's work. This will also help to identify causes and mechanisms of z-axis artifacts. Collectively, such information will contribute to minimize and control biases in quantitative morphology.

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