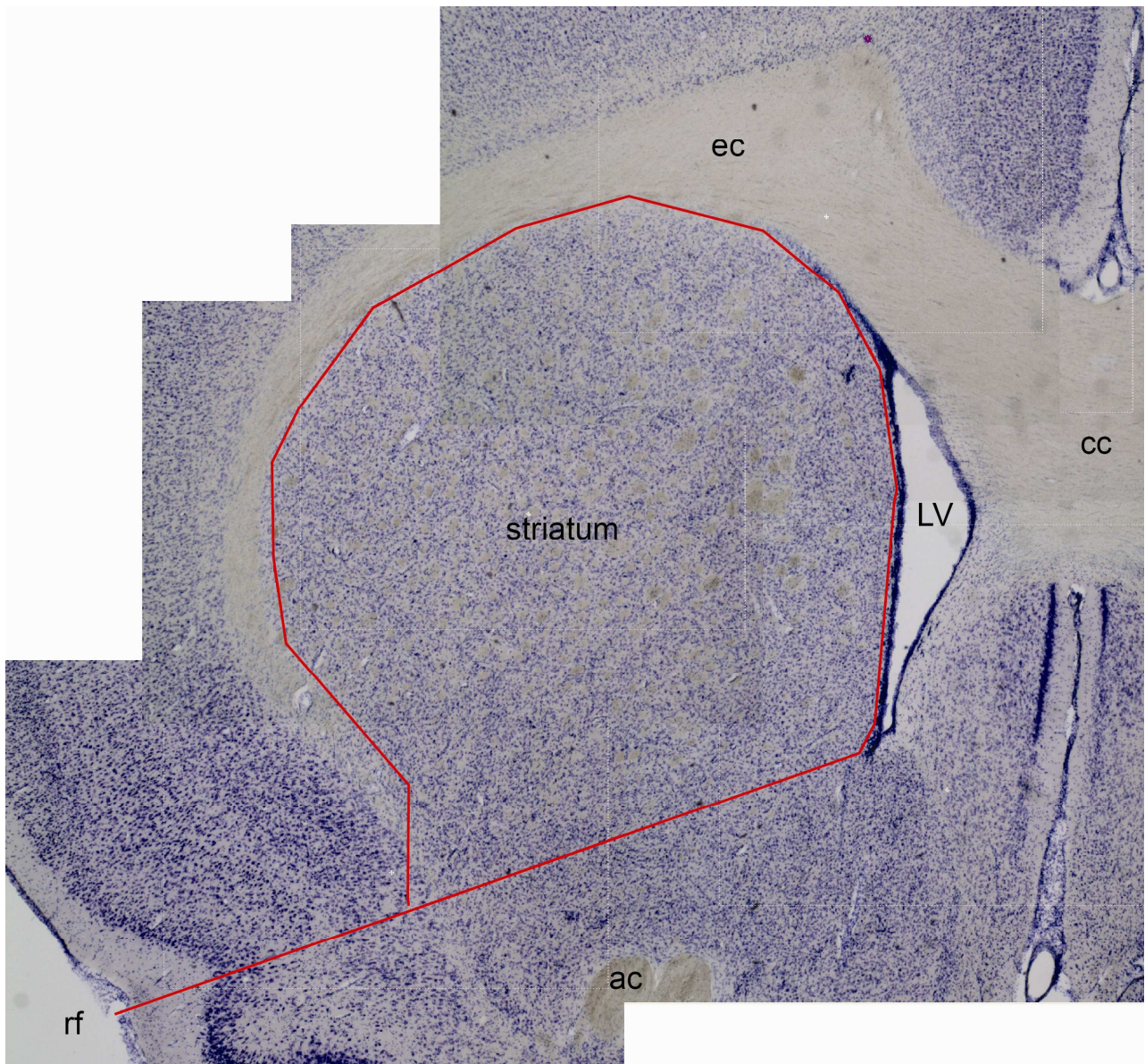


A stereological approach to measuring striatal cellular changes in transgenic Huntington's disease rats

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1. Abstract

Huntington's disease (HD) is an inheritable degenerative disease of the brain characterized by progressive neural dysfunction and degeneration in the striatum and cerebral cortex, resulting in abnormal motor- and cognitive function, emotional disturbances and premature death. While considerable knowledge has been gained about the pathophysiological mechanisms of HD, curative treatment is still not available. For further elucidation of the pathological processes and identification of potential targets for therapeutic interventions, experimental investigations in animal models are necessary. Transgenic rats in which a truncated HD gene has been inserted, develop progressive HD-like behavioural changes, and thus represent a promising model for experimental investigations of HD. It remains uncertain to what extent striatal neurodegeneration is present in these rats, and it is therefore important to evaluate this quantitatively. Quantitative assessment of cellular populations is typically obtained using a stereological method. In order to select an appropriate stereological study designs for such investigations, the objective of the present study has been to establish a procedure for quantifying striatal cell numbers in thionin stained sections in the rat brain. A non-systematic literature review, combined with snowball sampling, was conducted to retrieve original articles and review of interest and to delineate a suitable design and protocol. This protocol was tested on a limited material of thionin stained sections from a transgenic HD-rat and wildtype littermate control. The optical fractionator protocol was found to be the most suitable in the objective of population estimation, and a set of recommended sampling parameters were identified. We conclude that the proposed Optical Fractionator protocol is suitable to quantitatively test the hypothesis that striatal neurons degenerate in transgenic HD rats.

2. Introduction:

Huntington's disease (HD) is an inheritable degenerative disease of the brain characterized by progressive neural dysfunction and degeneration in the striatum (Vonsattel et al., 1985; Harper, 1996; Harper et al., 1997; Heinsen et al., 1994). HD patients have an unstable, polymorphic CAG-trinucleotide repeat (polyglutamin) expansion within the HD gene (Huntington's Disease Collaborative Research Group, 1993), leading to accumulation of mutant huntingtin protein in the brain. This eventually leads to regional degeneration of medium spiny projection neurons (MSP-neurons) in the striatum, as well as the cerebral cortex and other basal ganglia regions (Huntington's Disease Collaborative Research Group, 1993; Vonsattel et al., 1998; Heinsen et al., 1994; Hedreen et al., 1991; Sapp et al., 1999). Loss of basal ganglia function in turn causes symptoms of abnormal movement, emotional changes, motor deficits and premature death (Vonsattel et al., 1998; Folstein, 1989; Harper, 1996).

While considerable knowledge has been gained about the pathophysiological mechanisms of HD, curative treatment is still not available (Zuccato et al., 2010). For further elucidation of the pathological processes and identification of potential targets for therapeutic interventions, experimental investigations in animal models are important. Several different animal models have been generated in different rodent and primate species. This includes inducible (excitotoxin-induced, etc.) and genetically manipulated models (knock-in, knock-out, transgenic) (Mitchell et al., 1999).

The first rat model of Huntington's disease was introduced in 2003 (von Hörsten et al., 2003), with an insertion of truncated Huntingtin cDNA-fragment of 51 glutamine-repeats within the original rat Huntingtin promoter. The model exhibits the adult onset of HD phenotype, with emotional disturbances, cognitive decline and motor deficits (von Hörsten et al., 2003; Nguyen et al., 2006). This is combined with neuropathological hallmarks of nuclear inclusions, enlarged lateral ventricles, neuropil aggregation and loss of projection neurons in the striatum (von Hörsten et al., 2003; Nguyen et al., 2006; Kántor et al., 2006; Petrasch-Parwez et al., 2007)

While a qualitative comparisons between transgenic HD rats and wildtype control animals are sufficient to detect gross differences (presence or absence of protein markers, distinct abnormalities, etc.) (e.g. Reiner et al., 1988), are quantitative measurements necessary to detect more subtle differences by the use of statistical methods. Furthermore, since potential therapeutic interventions have a better likelihood of being effective at early stages of disease, is it also important to establish sensitive, quantitative measures of pathology.

Stereology applies the grounding principle of statistics, topology and geometry, (Miyamoto, 1994) in order to retrieve unbiased quantitative information from three-dimensional tissue based on measurements from two-dimensional histological sections (Lucocq, 2007; Schmitz et al., 2005). Cells loss can therefore be quantified in histological tissues by the use of stereological methods (Miyamoto, 1994; Lucocq, 2007; Schmitz et al., 2005). A range of study designs have been developed for the purpose of quantifying different cellular parameters in histological material.

Reduced striatal volumes have been described in transgenic HD rats (Nguyen et al., 2006), but so far no quantitative assessment of striatal cell loss has been made. Having access to a collection of histological sections from transgenic HD rats and wildtype controls, we have the ambition to test the hypothesis that cell numbers are reduced in old HD rats. However, to quantify cell loss in a rodent model of a neurodegenerative disease we first need to select an appropriate study design and to establish a workflow for conducting such measurements in the available histological material.

The aim of the present study is thus to review stereological approaches to quantify cellular elements in histological tissues, and to select and apply such a method in a pilot investigation of HD rats. Following a literature review of stereological methods, a cellular quantification procedure is presented, and tested in a limited pilot study.

3. Method:

1. Literature review:

A literature study was performed to review key articles on morphology, stereological methods and region of interest, with the intention of identifying approaches suitable for quantifying cellular elements in the available histological material. An initial non-systematic approach was performed using PubMed (<http://www.ncbi.nlm.nih.gov/pubmed>), followed by a so-called snowball sampling method¹, to identify review articles and original papers. The accumulated information was used to delineate a procedure for quantifying cell numbers in the rat basal ganglia, using thionin stained histological material. Specifically, the results of the literature queries were used to 1) select an appropriate stereological design and counting parameters, 2) delineate the anatomical boundaries of the dorsal and ventral striatum, and 3) define morphological criteria for differentiating striatal cell types.

2. Pilot study:

To test the stereology procedure and validate the chosen parameters, a pilot study was conducted in histological material derived from two 16.5-month-old male rats, kindly provided by Professor Olaf Riess and Dr. Hoa Phuc Nguyen (Department of Medical Genetics, University of Tübingen, Germany). One rat (H29.04, +/+) was transgenic for Huntington's disease (carrying a truncated HD gene with 51 CAG-repeats under control by the native rat HD promoter), and the other rat (H29.04, -/-), was a wildtype littermate control. The rats were deeply anaesthetized by sodium pentobarbital (50 mg / kg) and perfused with 50 ml PBS followed by 200 ml 4 % paraformaldehyde. The brains were extracted and postfixed overnight in 4 % paraformaldehyde, and then transferred to phosphate-buffered saline (PBS). Prior to sectioning, the brains were cryoprotected by immersion overnight in 30 % phosphate buffered sucrose. Coronal sections were cut at 50 micrometer using a freezing microtome, mounted and stained using a standard thionin protocol (see box 1). Sections were dried and coverslipped using Eukitt (Kindler, Freiburg, Germany).

From each rat, four sections were chosen for this analysis in a systematic and random manner, spaced at an interval of 200 μm from each other within the striatum. Anatomically corresponding levels were identified by estimating the distance from the genu of the corpus collosum by section serial number and section thickness. Due to the possibility of bias in the process of recognition, a blinding of the investigator was proposed by an anonymization of the slides, after generation of a "random number" identification for each one of the sections, using the stereological software. Analyses were only conducted in the left side of the brain. Live images of the specimen were acquired through a Zeiss Axioskop II, EC-Plan Neofluar 63x/09 dry objective, captured with a MicroFire-digital camera mounted on the microscope. As detailed below, a fractionator procedure was performed

¹ A snowball sampling method, also referred as chain-referral sampling, is a sampling method, utilized to track down subgroups within a vast population (Goodman, 1961). Several new objects are retrieved from the referral from a primary object of interest (Goodman, 1961). Applied in the interest of literature review, it would mean to search for new articles referred by a primary article/review.

using the software StereoInvestigator 8.0 (MicroBrightField Bioscience Inc, Williston, VT, USA), with a counting frame size set to 65x65 μm^2 , and 27-35 counting frames per sections. The frames were automatically positioned in a systematic random fashion by the software. For further details, see the procedure given in the Results section below.

Box: 1 Standard Thionin Staining Protocol

Dry sections were defatted with alcohol of different concentration, in the order and time given below.

Order:		Substance:		Minutes:	
Substance:	Minutes:	Substance:	Minutes:	Substance:	Minutes:
1 Water	2	11 Water x 4	0,5		
2 70% ETOH	2	12 70% ETOH x 3	3		
3 96% ETOH	2	13 100% ETOH x 2	1		
4 100% ETOH	2	14 Xylene	1		
5 Xylene	10	15 Xylene**	1-1,5		
6 100% ETOH	2	16 Eukitt***			
7 96% ETOH	2				
8 70% ETOH	2				
9 Water x 2	1				
10 Thionin*	2				

* 0.05% thionin in 0.1 M acetate buffer (3 week old), pH 4

** Stored while cover slipping

*** Coverslipping done manually

4. Results:

The purpose of the present study was to review stereological methods in order to select an appropriate design to count cellular elements in histological material from a transgenic rat model of HD. The literature review was conducted to elucidate the following questions:

- 1) Which stereology designs are suitable for measurement of altered cell numbers in thionin stained histological material from the rat striatum?
- 2) Which cell types is the rat striatum composed of, and how can these cells be morphologically differentiated?

Based on the retrieved review and articles, a practical procedure is suggested, and then tested in a limited pilot study.

4.1. Review of stereology: Which design is appropriate?

4.1.1 Principles of stereology

Stereology is based on the concepts of; “unbiased” and “efficiency”, defined as “...without the systematic deviation from the true value” and “with a low variability after spending a moderate amount of time”, in that order (Gundersen et al, 1988). “Unbiased” is crucial in obtaining information free of systematic errors, which often depends upon random sample site, and sometimes, specific orientations (Lucocq, 2007). Efficiency, is associated to reproducibility, cost etc., and has improved drastically, with the advancement of computer-based stereology (Glaser et al., 2000). To perform measurements, a geometrical entity,

combined with a set of counting rules is applied, named a stereological probe (Glaser et al., 2000; Howard et al., 1998). Various kinds of geometrical entities and counting rules exist (Glaser et al., 2000; Gundersen et al., 1988), and different combinations of these two, yields different stereological probes (will be elaborated under Choice of design, below). Since stereology is superimposed upon statistical probabilities, the result based on the sampling will only be estimates, thus furthering the need to evaluate the variability of these (Schmitz et al., 2005). This evaluation is defined as “coefficient of error” (CE) (Schmitz et al., 2000; Schmitz et al., 2005). Because of this, there is a clear realization between sample size and accuracy of the estimate. A higher number of sample size, number of counting frames per sections and number of sections, results, as follows, in a more precise estimation, and thereby a lower CE (Schmitz et al., 2005).

The fundamental question in the objective of population estimation-study is to define the minimum number of cellular elements to sample, to yield a valid estimate. The rule of thumb seems to be to count about 100-200 cellular elements/animal, as long as the CE is <0.1 (Gundersen et al., 1987; Gundersen et al., 1988; West et al., 1991; West et al., 1993; Keuker et al., 2001; etc.), even though there is no official consensus on this. Schmitz et al. (2000), on the other hand, recommends counting of about 700-1000 cellular elements/animal. With a defined cellular element count per counting frame (will be elaborated below), the variation lies primarily in the number of sections, both in itself and by affecting counting frame per section. To systemize this, a single formula is given below (West et al., 1991):

$$\sum Q \times 1/ssf \times 1/asf \times 1/tsf = N^{\text{of cells}}$$

N=number, SSF=section sampling fraction, ASF=area sampling fraction, TSF=thickness sampling fraction

Gundersen et al., (1987) shows the sampling of 100-200 cellular elements/animal, distributed over 10-20 sections and 100-200 counting frames (also called disectors) to be adequate in most studies (Keuker et al., 2001).. This results into a counting frame enclosing 1-2 cellular elements per frame (Gundersen et al., 1987; Keuker et al., 2001). It is proposed by the literature that the counting frame should not be too big, resulting in a declining efficiency, nor too small, leading to empty counting frames (Keuker et al., 2001). Similar to other parameters, is there no standardization, thus, yielding an appropriate cellular elements/counting frame ranging between 1-6 (StereoInvestigator Users Guide, 2008). With the other parameters determined, can the counting frame size and the grid size (distance between the different counting frames within a section) be defined.

Consequently, the weakness of the protocol lies in the selection of parameters, thus, a faulty selection results in a low precision, etc. To prevent this, a basic understanding of CE (coefficient of error) is capital (Schmitz et al., 2005; Keuker et al., 2001). The CE is in general comprised by total variance, which duty to sum up the 1) variance due to noise (intra-sectional variance) and 2) variance of systematic random sampling (inter-sectional variance) (or any other variant of these, depending on the type of CE used) (StereoInvestigator Users Guide, 2008; Keuker et al., 2001). An abnormal high number of any of these should result in either increased sampling within a section or sampling of more sections, respectively (StereoInvestigator Users Guide, 2008). In an optimal situation are the inter- and intra-sectional variance of the same magnitude (StereoInvestigator Users Guide, 2008). An optimal CE is set to about 0.05, but most studies try to retrieve a CE of at least 0.1 (StereoInvestigator Users Guide, 2008; Keuker et al., 2001; Gundersen et al., 1987). A part from this, the biological variability also has to be taken into account (Keuker et al., 2001). If this happens to be unknown, the recommendation seems be to conduct a study with between 3-5 animals in both groups (Keuker et al., 2001; Cruz-Orive et al., 1990; West, 1994;

Lucocq, 2007). Since the aim of this study is to produce a viable protocol, and not conduct a precise sampling, has this not been implemented in this production.

The tissue is exposed to a heterogeneous shrinkage, combined with surface aberrations (lost caps etc.) from the production, both of them affecting the top and bottom of the sections more, because of the larger surface area (Andersen et al., 1999; Schmitz et al., 2005). Consequently, the need for a guard zone is due, since the optical fractionator relies on a representable fraction of the section. The function of this zone lies in the name; it guards the counting frame from the corrupted part of the section along the z-axis, positioned at the top and bottom of the section (Andersen et al., 1999; Schmitz et al., 2005). Similar to other parameters, there is no unity on the size of the guard zone, but seems to range between 2-5 μm (StereoInvestigator Users Guide, 2008; Keuker et al., 2001; Schumann et al., 2006).

The sampling follows a set of rules (Gundersen et al., 1977; Keuker et al., 2001), with the aim to serve objectivity to the counting process. Apart from the criteria set to recognize cellular elements of interest, are the counting criteria given as follow: 1) All cellular elements falling within the counting square are counted, when they are in focus. 2) All elements touching the inclusion (green) line are counted when they are in focus. 3) All elements outside the square or touching the exclusion line (red), are not counted.

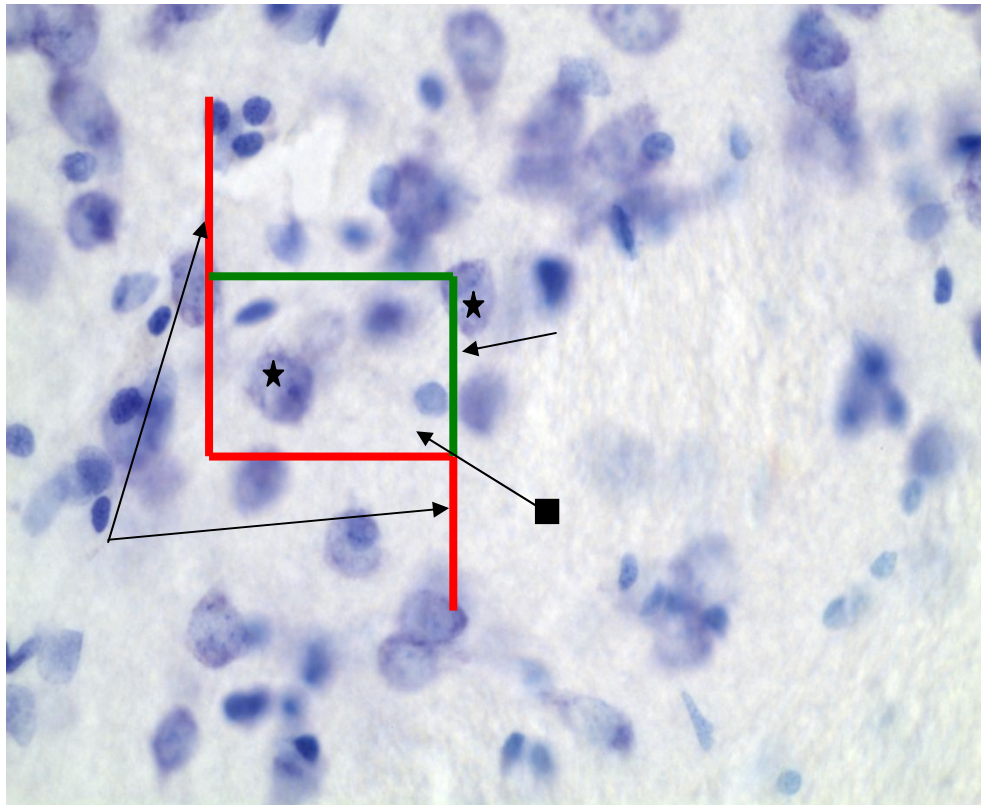


Figure 2: Counting frame, with in-and exclusion lines. The red lines are exclusion lines; the green lines are inclusion lines. All cellular elements in focus and 1) falling within the square, without touching the exclusion lines, or 2) touching the inclusion line, without being in touch with the exclusion lines, are marked with a star, and thereby counted. The counting square is marked by a square.

4.1.2 Choice of design

There are different stereological approaches to estimate population density/population number, all with different strengths and weaknesses, (Lucocq, 2007; Schmitz et al., 2005, Gundersen, 1986; Mayhew et al., 1996.). So-called method-based probes are restricted to sampling from uniform tissues, while design-based probes also can cope with inhomogeneous tissue (West, 2001; StereoInvestigator Users Guide, 2008). A minimum of four varieties of design-based probes exist, including the Optical Fractionator, Physical Disector, Optical Disector, and Fractionator probes. The main difference between optical and physical probes relates to different preferences for sections thickness; while the physical-based probes are suitable for thin, parallel sections, are the optical-based probes more suitable for thick sections (Andersen et al., 1999; Schmitz et al., 2005). Disector (Sterio, 1984; Gundersen, 1986) is a probe with several variations (physical-, double- and optical version), and common for them all is the application of the counting frame (also called a disector) mentioned above (Lucocq, 2007). Fractionator (Gundersen, 1986; West et al., 1991), on the other hand, is a probe that measure the population within a fraction of the thickness, in a fraction of the areal of the whole section, and in a fraction of all the sections (see the formula given above) (West et al., 1991). The Optical fractionator is a combination of optical disector and fractionator (West et al., 1991), yielding the strength of both probes, thus a natural selection in this production, providing a powerful tool for population size- and density estimation, and used applied in many studies (Keuker et al., 2001; Schumann et al., 2006; etc.)

The strength of optical fractionator relates to its independency from size, orientation and spatial shape of the cells/tissue, as well as changes in reference volume (Schmitz et al., 2005; Lucocq, 2007; West et al., 1991). This provides a probe that is independent change in the volume (shrinkage/compression) of the region of interest (in contrast to the Physical/Optical Disector) (Lucocq, 2007). Since this is an optical probe, it is recommended that the tissue sections have a minimum thickness of 15 μm (after shrinkage due to preparation), and optimal over 20 μm (StereoInvestigator Users Guide, 2008). The weakness of the probes lies in the dependency on fixed height of the counting frame (along the z-axis) (West et al., 1991, Andersen et al., 1999). The insensitive nature of design-based probes, and optical fractionator in particular, towards the uniformity of the tissue, resides on the principle of systematic, random sampling (Andersen et al., 1999; West et al., 1991; Lucocq, 2007; Schmitz et al., 2005; StereoInvestigator Users Guide, 2008). A systematic, random sampling of the whole region of interest, grants every cellular element within this reference volume the chance of beings sampled (Lucocq, 2007; StereoInvestigator Users Guide, 2008) (will be elaborated under Sampling parameters). Optical fractionator has also been found to be more efficient than many of the other methods (Lucocq, 2007).

Thus, to quantify cell populations in 50 μm thick thionin stained sections from the rat striatum, we conclude that an optical fractionator approach is most suitable.

4.2 Review of cellular morphology in the striatum

The striatum contains different types of projections neurons, interneurons and glial cells. About 95 percent of all neurons are medium spiny projection neurons (MSP-neuron) (Kemp et al., 1971; Mitchell et al., 1999; Dray, 1979).

The neuron population can be categorized in three different groups based on size: neurons with a soma diameter smaller than 9 μm , a medium group with soma diameters between 10-20 μm (consisting of four different types of neurons), and a group neurons with soma diameters between 22 – 30 μm (consisting of one type of neuron) (Mensah et al., 1974). There seems to be some minor variability in the population estimation and limitation

of the different groups of neurons between studies (Kemp et al., 1971; Dimova et al., 1980; Dray, 1979)., and it is unknown whether this is due to variation between different animals models applied or because of other variances. Regardless, Mensah et al., (1974) and Dimova et al., (1980), both based on rat-models, confirm high concordance with the result of Kemp et al., (1971), which is based on a cat-model. The variation between different species, could therefore be of minor importance, based on the similarities of several studies, from a wide range of animals (Kemp et al., 1971; Mensah et al., 1974; Dimova et al., 1980; Cajal, 1911; Leontovich, 1954). Thus, it seems to be possible to extrapolate the morphology described in cat by Kemp et al., (1971) over to a rat-model.

The most numerous group of neurons, the MSP-neurons, are defined by an average diameter between 12-14 μm , but with a variation between 9-18 μm (Kemp et al., 1971; Dimova et al., 1980) and with a scant/medium and pale cytoplasm. These cells have a large, round and pale nucleus, combined with a round/polygonal body (Kemp et al., 1971; Dimova et al., 1980). The group of small-sized neurons can sometimes be hard to differentiate from glial cells based on size, but the neurons have more oval/round nuclei, pale and with distinctive nucleoli, and a dark cytoplasm with few Nissl-bodies (Kemp et al., 1971; Dimova et al., 1980). By contrast, glial cells have dark and irregular nuclei with clumped chromatin, which makes it hard to distinguish the nucleoli (Kemp et al., 1971). Table 1 (modified from Kemp et al., 1971) gives a summary of the distinctive morphological features of the different striatal cells.

Table 1: Listing of the major different groups of neurons in the striatum and glial cell	
Cells:	Morphology:
Small:	Round/oval nucleus, pale prominent nucleoli. Dark cytoplasm. Few Nissl-bodies.
Medium spiny projection neuron:	Round and pale nucleus. Scant/moderate amount of cytoplasm. Round/polygonal soma.
Medium long axon:	} Like MSPN, but the differentiation from the former lies in numbers of dendrites, axon length, spines, and collaterals.*
Medium smooth:	
Varicose dendrite:	
Large:	Resemble MSPN, but have more indentations and Nissl-bodies. Differentiate also in the aspect dendrites, etc.
Glial cells:	Dark and irregular nucleus, with crumbled chromatin.

* For further details, see Kemp et al., (1971); Mensah et al., (1974) and Dimova et al., (1980).

4.3. Protocol

The objective of the study is to perform a quantitative comparison of cellular elements in transgenic HD rats and a wildtype control. To establish this design to the material at hand, we need to define a) a reference volume, b) sampling parameters for the optical fractionator method, and c) morphological parameters to differentiate striatal cells.

4.3.1 Defining the region of interest (ROI)

To calculate a reference volume, the region of interest must be delineated in both groups of animals using explicit anatomical criteria. In the present study, the primary region of interest is the dorsal striatum. No formal consensus seems to exist in the literature about the anatomical delineation of the striatum, and sometime a combination of neurochemical and anatomical criteria are employed (Voorn et al., 2004; Kántor et al., 2006; Ingham et al., 1998; Van de Berg et al., 2000). In histological material, the dorsal and lateral boundary of the striatum is readily defined by the overlying white matter (external capsule). Anteriorly, the genu of the corpus callosum provides a distinct boundary, and posteriorly the fornix joining the diencephalon forms an important landmark. The medial boundary follows the lateral ventricles. The ventral boundary of the dorsal striatum is difficult to delineate cytoarchitectonically (Voorn et al., 2004) and we have therefore employed an arbitrary definition (Ingham et al., 1998; Van de Berg et al., 2000), by drawing an imaginary line between the inferior tip of the lateral ventricle and the rhinal fissure (Fig. 1). In contrast to studies: Kántor et al., (2006), Ingham et al., (1998), Van de Berg et al., (2000), and even though this study is basically enclosed to the striatum, is there no exclusion of globus pallidus in this study, due to the indifference in the objective of both the protocol and the pilot study.

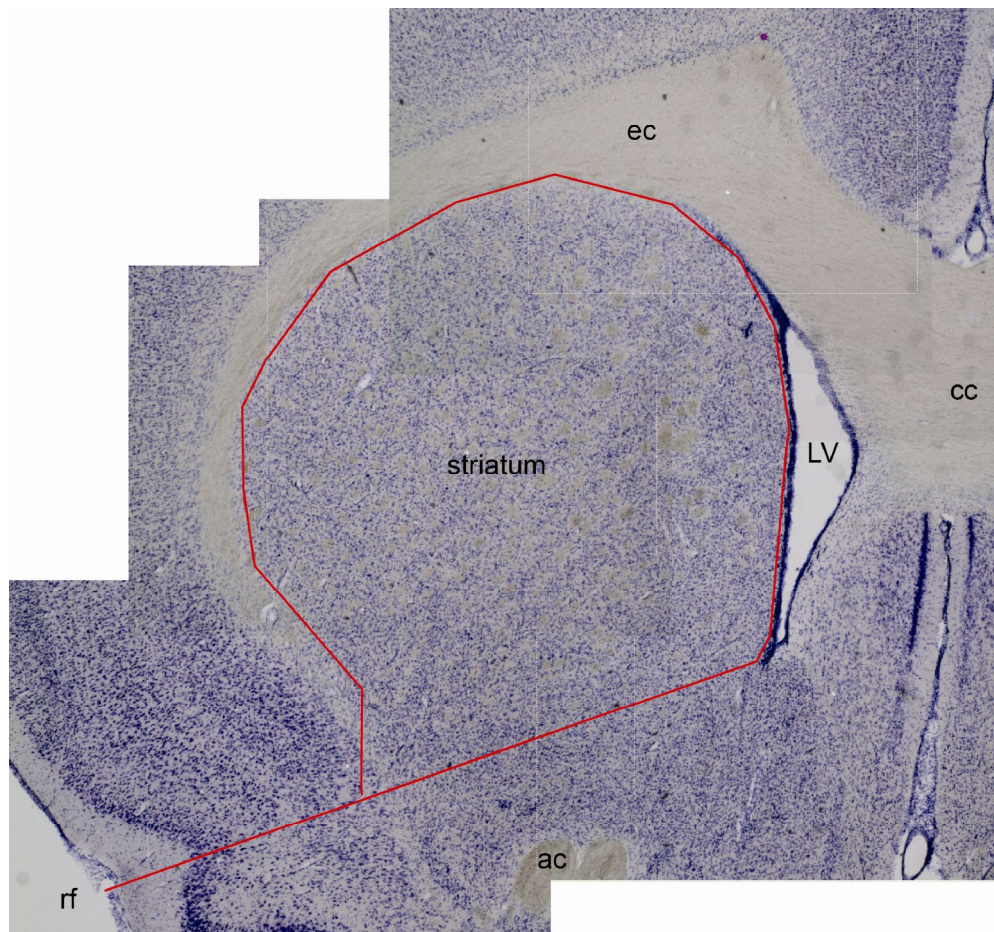


Figure 1: montage of five different photomicrographs showing the dorsal striatum. The boundaries employed in the present study are marked with a red contour. ac: anterior commissure, cc: corpus callosum, lv: lateral ventricle, rf: rhinal fissure

4.3.2 Sampling parameters

After the recognition of the appropriate specimen, defining ROI and some of the different parameters, a determination of the number of sections needed should be done. Several factors influence the decision, ranging from the tissue at hand, ROI to timeline. The numbers of sections must succeed each other, thereby generating a sections-series. In this production, every fourth section was counted, with an interval of 200 μm , resulting in a succeeding section-series of total four sections from one single animal. Gundersen et al., (1987) recommends 10-20 sections per animal.

The independent nature of the optical fractionator protocol towards size, orientation and spatial shape of the cells/tissue (Schmitz et al., 2005; Lucocq, 2007; West et al., 1991), is embedded in the concept of systematic, random sampling (Andersen et al., 1999; West et al., 1991; Lucocq, 2007; StereoInvestigator Users Guide, 2008). A two-step systematic, random sampling is applied (West et al., 1991; StereoInvestigator Users Guide, 2008), with first a random selection of the start-point of the section-series, and second, a random set point for the counting frame (both generated by the same software) (StereoInvestigator Users Guide, 2008). The latter yields a randomly placed mesh of counting frames within the section, with every counting frame being spaced in a standardized distance from each other.

Due to the possibility of bias in the recognition process, a blinding of the investigator is proposed by an anonymization of the slides. It is done by generation of a “random number” identification for each one of the sections, using the stereological software.

In the study of Gundersen et al., (1987), 100-200 cellular elements were counted, with the application of about 100-200 counting frames, in 10-20 sections per animal. The counting frame was sized to sample 1-2 cellular elements each. In the objective of a pilot study, an oversampling is advised, due to an unknown CE, etc. (StereoInvestigator Users Guide, 2008). A count between 500-1000 cellular elements/animal is, thus, set as the aim of the study and protocol. A feasible count of cellular element/counting frame is therefore set to minimum 5 elements/counting frame for this study. This should in general range between 1-6 (StereoInvestigator Users Guide, 2008). In the thionin stained striatum of rats, is a reasonable size of the counting frame therefore 65 μm x 65 μm . With only four sections being counted per animal, does this account for about 25-35 counting frame/section, and set the grid size within the section to 600-650 μm x 600-650 μm , calculated automatically by the stereological software in this production. The number of counting frame per section defines the grid size. A part from this, the biological variation is an important factor, and a study with 3-5 animals in each group should be conducted, if it happens to be unknown (Keuker et al., 2001; Cruz-Orive et al., 1990; West, 1994; Lucocq, 2007). The guard zone and counter frame height seems to be the parameter which are hardest to define for this protocol. There is seemingly no consensus about these, but several articles have defined them between 2-5 μm (StereoInvestigator Users Guide, 2008; Keuker et al., 2001; Schumann et al., 2006) and about 10 μm (West et al., 1991), accordingly. One approach is to define the upper guard zone in the range of 2-5 μm , the counting frame height at 10 μm (West et al., 1991), and let the lower guard zone vary coherently with the tissue thickness (Keuker et al., 2001). Regardless of this; no specific size on the guard zones were used in this production, but they were defined by the distance between the section top/bottom and first/last cellular element fulfilling the requirements during a scroll-down from the top to bottom, respectively, thus yielding a more unsecure, but, a more efficient protocol. Counter frame height was set between the two guard zones. This strategy was seemingly allowed by the comparative nature of the study, and would

otherwise be defined in accordance to studies like Keuker et al., (2001) and Schumann et al., (2006).

As proposed, must the different parameters be calibrated to the goal of the study, tissue at hand etc., so a pilot study is always recommended by the literature (StereoInvestigator Users Guide, 2008; Schmitz et al., 2005; Keuker et al., 2001). A CE should always be calculated, also (Keuker et al., 2001; StereoInvestigator Users Guide, 2008).

4.3.3 Morphological criteria

Given the morphological characteristics of striatal cells (Table 1), it is possible to differentiate them in thionin stained sections, at least in the objective of neurons and glial cells. Glial cells have a more irregular and dark shape of the nucleus with no clear nucleoli (Kemp et al., 1971), thus, facilitating the differentiation from pale and oval/round formed nucleus of the neuron, with distinctive nucleoli and granules (Figure 3D). This differentiation can be done without difficulties in older animals (Fentress et al., 1981). The criteria defines that only cellular elements with oval or circular nuclei (Fig. 3A), as well as visible granules and nucleoli (Fig. 3B), will be counted, thus, there is no distinction between different neuron groups. To avoid multiple sampling of the same cellular elements, only elements in focus are sampled. Somewhat similar approach has been used in other population estimation-studies (e.g. Schumann et al., 2006; Bjaalie et al., 1997).

In summary, the following inclusion criteria are employed to ensure that our quantification is restricted to striatal neurons. Sampled cellular elements must:

1. be in focus
2. have a visible oval or circle shaped nucleus
3. have visibly labelled granules or nucleoli

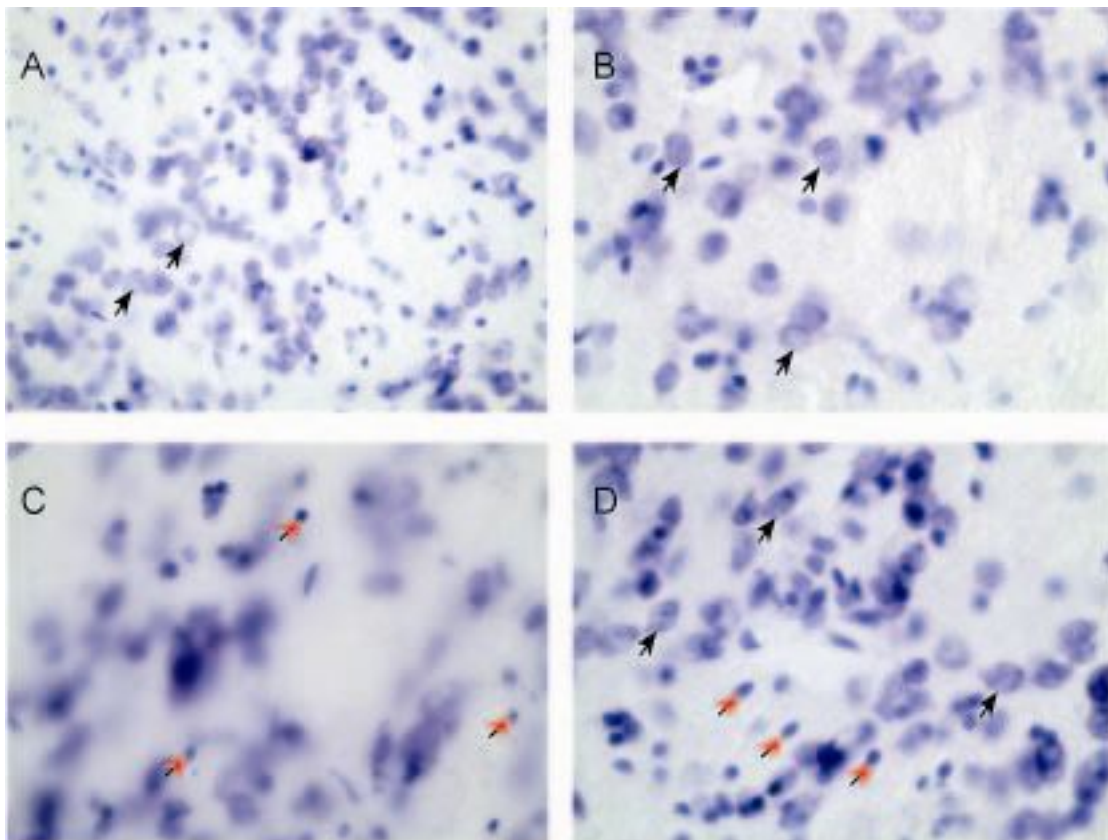


Figure 3: High-power photographs of a coronal thionin stained section through the striatum showing detailed cellular morphologies. Panels A and B show neuronal nuclei (black arrows), with prominent nucleoli and “granules”. Panels C and D show glial cells (red arrows), with dark and clumpy nuclei.

4.3.4 Summary

Using the methodological parameters described above, is the following procedure suggested to perform a quantification of cell numbers and densities in thionin stained sections from the rat striatum. Only a short summary of the procedure will be given here (see the Appendix for further details). The basis of the pilot study is to determine appropriate sampling parameters and counting criteria. After a calibration of the parameters, a more profound study can be initiated, implementing the given criteria.

1. Define the goal of the study, along with the specimen of interest, etc.
2. Define the number of animal needed. The biological variation can be delineated from a pilot study with 3-5 animals in each group (Keuker et al., 2001; Cruz-Orive et al., 1990; West, 1994; Lucocq, 2007).
3. Define the number of sections needed. Gundersen et al., (1987) recommends between 10-20 sections.
4. Define the block advance (the succession within the sections-series). This is based on several factors, like availability of sections, and can be calibrated with the use of “variance of systematic random sampling“ (intersectional variance). A high number of intersectional variance in itself or compared to “variance due to noise” (intra-sectional variance), should yield a larger section-series within an animal (StereoInvestigator Users Guide, 2008). Define the counting frame size. It should enclose 1-6 cellular elements per frame (StereoInvestigator Users Guide, 2008).
5. There is no consensus about this, but several articles have defined the guard zone between 2-5 μm (Keuker et al., 2001; Schumann et al., 2006), and the counter frame height about 10 μm (West et al., 1991, etc). A method described in the literature (Keuker et al., 2001), is to place a standardized guard zone in the top, set the counting frame height about 10 μm , and let the bottom guard zone vary in accordance with the sections thickness.
6. A blinding of the investigator is proposed by an anonymization of the slides, after generation of a “random number” identification for each one of the sections, using the stereological software. With all of this done, start sampling with the given morphological criteria

4.4 Pilot study

To test the delineated optical fractionator procedure on a realistic material, we performed a limited investigation using a total of eight thionin-stained sections from two rats, one transgenic HD rat and one wildtype control. The protocol was implemented with the block advantages set at 200 μm , counter frame size of 65 μm x 65 μm and the grid size to 600-650 μm x 600-650 μm (see section: Stereological parameters, above). Due to the variation of sectional area, was the number of systematically, but random positioned counting frames, put between 27-35 frames per section. A 63x-objective was used to facilitate the detection of cellular morphology (Figs. 2 and 3). Details and result are shown in Table 2. A simple density estimation, based on the total number of cellular elements in all counter frames, divided on the total number of counter frames, points towards a lower density rate in the advantage of tgHD (9.23 compared to 9.36 cellular elements/counting frame). This seems to correspond with population estimation of tgHD-rat in other studies (e.g. Kántor et al., 2006), and, thus, supporting the theory of striatal atrophy in Huntington’s disease (Huntington’s Disease Collaborative Research Group, 1993; Vonsattel et al., 1998; Heinsen et al., 1994; Hedreen et al., 1991; Sapp et al., 1999). However, no conclusions can be drawn from this

pilot study due to limited number of sections and animals investigated, etc., and the differentiation could be due to i.e. biological variance. Presently, we have no measurements of the biological variation, so a larger study is recommended, with a group size of 3-5 animals (Keuker et al., 2001; Cruz-Orive et al., 1990; West, 1994; Lucocq, 2007).

The stereological parameters and counting criteria chosen seems to correspond to other population estimation studies (i.e. Schumann et al., 2006; Bjaalie et al., 1997), even though they seemingly result in an oversampling. We conclude that the procedure is readily implemented, and yields realistic numbers, but has to be calibrated to decrease the chances of oversampling.

Table 2: Details and result from conducted pilot study on thionine-stained rat; tgHD and wildtype littermate control, cut thickness of 50 µm								
Parameters:								
Number of animals:	2	Staining:	Thionin	Counter frame:	65 µm x 65 µm			
- tgHD:	1	Orientation:	Coronal	Grid size:	600-650 µm x 600-650 µm			
- wildtype:	1	ROI:	left, striatum					
Section per animal:	4	Cut-thickness:	50 µm					
Block advantage:	200 µm	Elements/frame:	≥5					
Result:								
TgHD				Wildtype:				
Section No.:	C#:	Sect. Areal:	No. CF:	Section No.:	C#:	Sect. Areal:	No. CF:	
	1	239	10398600	27	1	275	9786410	29
	2	230	11152200	28	2	289	10442400	30
	3	334	12955100	33	3	253	12138700	33
	4	332	13523400	35	4	290	13208600	33
Total:	1135	48029300	123		1170	45576110	125	
Density:	9,23					9,36		
C#: Total cellular elements in all counting frames/section								
Sect. Areal: Total area of the section. Given in µm ²								
No. CF: Number of counting frame								
Density: C# /Total No. CF								

5. Discussion

The objective of the present study has been to establish a stereological procedure for quantifying cellular elements in thionin stained, striatal sections in the rat brain. A literature review was conducted to select an appropriate design, a practical procedure was suggested, and then tested in a limited pilot study.

The literature review reveals that there is limited consensus in the use of stereological methods and their parameters (Gardella et al., 2003), even though some recommendation exist (Gundersen et al., 1987; West et al., 1991; Keuker et al., 2001; Schmitz et al., 2000). The challenge has therefore been to extrapolate these recommendations to fit our needs. There are several protocols for quantification of cellular populations (Lucocq, 2007; Schmitz et al., 2005; Gundersen, 1986; Mayhew et al., 1996), each with many variable parameters that need to be adapted to the material investigated.

The optical fractionator probe is found to probably be the most popular probe to estimate population densities (Glaser et al., 2000), and even though it is quite efficient and precise (West et al., 1991), does it have its restrictions (Schmitz et al., 2005); some confined to the design-based nature, while other more specific for the optical fractionator. The more

general restrictions are primarily confined to inhomogeneous shrinkage and compression, accessibility to all the whole region of interest and to incomplete penetration of dye (Schmitz et al., 2000; Schmitz et al., 2005; Gardella et al., 2003; West et al., 1991). In contrast to many other probes, is the fractionator approach seemingly unaffected by the former (Lucocq, 2007). Some of the specific restrictions to the optical fractionator probe, are confined to its need for a standardized height of the counting frame (disector) and guard zones (West et al., 1991; Andersen et al., 1999). While the former is needed for the calculation of the fraction the virtual counting frames constitute of a reference volume (i.e. West et al., 1991), are the latter necessary to prevent aberration on the surface after production of the sections, like lost-caps, fragments etc., to exert bias (Schmitz et al., 2005, Andersen et al., 1999). In the pilot study, we used a simplified approach, defining the guard zones by the distance between the section top/bottom and first/last cellular element fulfilling the requirements during a scroll-down from the top to bottom, respectively, and the counter frame height was defined as the distance between these two. The literature (StereoInvestigator Users Guide, 2008; Keuker et al., 2001; Schumann et al., 2006; West et al., 1991; Andersen et al., 1999) as well as this protocol, recommends a counter frame height set at about 10 μm . The top guard zone should be between 2-5 μm , and the guard zone in the bottom, should vary in accordance with the section thickness, as done in the study by Keuker et al., (2001).

Other potential pitfalls with this study seem to be connected to the morphological criteria, primarily formulated into two distinctive problems. The first one is confined to the possibility of extrapolation of morphological data from one animal on to another, while the other is confined to potential of subjectivity, due to morphological criteria. As mentioned under section 4.2, above, is there seemingly high concordance of morphological features among different animal models, thus, making it possible to extrapolate the morphology described in cat by Kemp et al., (1971) over to a rat-model. In respect to the second problem, while the methods applied are quite objective, an element of subjectivity is introduced when the investigator selects which cellular elements to include. The use of explicit morphological inclusion criteria is therefore seemingly important, and should be in context with the difficulties in discrimination of the different cellular elements. If it is hard to differentiate the different cellular elements, should the criteria also be that precise. The discrimination between neuron and glial cells has been described as readily by the literature (Fentress et al., 1981), thus, lowering the chance for subjectivity. As a ramification of this, could it be interesting to see whether a modification of the protocol is able to differentiate the different groups of neurons. Regardless, a blinding of the investigator is proposed by an anonymization of the slides, after generation of a “random number” identification for each one of the sections, using the stereological software, to decrease any given possibility of subjectivity.

The primary alternative to the given protocol is the use of specific immuno-histochemical staining techniques to visualize specific cell types (see Pickel, 1981), thus providing a higher precision to the study and avoiding the possibility of subjectivity and morphology. A standard thionin-stain is, on the other hand, both cheaper and more time efficient, so a consideration must be done. In our case, the latter was found to be more important.

Our pilot investigation yielded population density counts in line with previous literature (Heinsen et al., 1994; Kántor et al., 2006). The average cell density was slightly lower in the transgenic HD rat compared to the wildtype control, which would fit with the a priori expectation. However, given the limited number of sections and animals investigated, these numbers cannot be used to draw any conclusions. The reciprocal value of the square root of total number counted, has been found to approximate the real CE of the fractionator

(Schmitz, 1998; Schmitz et al., 2000; Schmitz et al., 2005). With a count of minimum 1000 cellular elements/animal in the pilot study, should this yield a CE less than 0.05. Thus, the biggest variation would be due to biological variance (Keuker et al., 2001). With biological variance being unknown, is the recommendation (Keuker et al., 2001; Cruz-Orive et al., 1990; West, 1994; Lucocq, 2007), to conduct a study with 3-5 animals per group. The stereological parameters and morphological criteria chosen correspond to the recommendations and to other similar studies (i.e. Schumann et al., 2006; Bjaalie et al., 1997), even though they seemingly result in an oversampling. Consequently, this protocol should yield statistical valid result, even though a calibration through a larger study should be done.

6. Acknowledgments

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8. Appendix:

Name	Optical Fractionator
Date	28.09.2010
Software	Stereo-Investigator 8.0 (MircorBrightField, Williston, VT; USA).
Keywords estimation	Counting, optical fractionator, stereo investigator, population
Author	Saurabh Kumar Jain
Reference	MBF Bioscience (MicroBrightField, Inc.). (2008). Stereo Investigator 8 User's Guide - Document Version SI0108-08. MBF Bioscience (MicroBrightField, Inc.). Williston. ²

Optical Fractionator

Purpose: is to estimate the population of defined subject(s) within a specified area(s).

Prerequisites a “Preliminary Population Estimate” and/or “Optical Fractionator Workflow”- should be performed in advance to get the feeling for suitable parameters regarding the estimation. (You find both functions under “**Probes**”.)

Procedure

- 1. Tools->Random Number Generator.** Use this function to define which section you will work on.
- 2. Set a set point. TIPS:** Position the set point equivalently on every single section; i.e.: the top of the left corpus callosum.
- 3. Trace you region(s) of interest. TIPS:** It is easier to trace with the “**Rubber Line Tracing**”. It is also better to trace within the area of interest, rather than making the contour too vast, including too much unwanted area.
- 4. Change to magnitude of choice.** Remember to change the “**Change Lens**”- function in the program as well, to corresponding lens.
- 5. Hit the “Adjust Video Input”-button (Imaging->Adjust Camera Settings).** Use the auto-exposure to find the right exposure.
- 6. Probes->Define Counting Frame.** Define the counting frame size, as well as the position on the screen. **TIPS:** Place it within the auto-move box (the dotted-square right in the central of the screen).
- 7. Probes-> Preview SRS Layout. TIPS:** It is easier to define the “SRS Grid Size” based on “Desired Sampling Site”. Type in the desired number, and then hit: “Estimate Grid Size”. Round the size-number to nearest 50. Hit “OK”. If you are not satisfied, hit the “**Preview SRS Layout**” once more, and try again. Satisfied: hit anywhere on the screen to exit. An appropriate size is 600-650 μm x 600-650 μm , and should be kept equal for all sections.
- 8. Left side of the screen.** Choose a marker.

² This is a modified version of the protocol given in StereoInvestigator Users Guide version 8, (2008), made to fit this need of this study. Part of this protocol, can be quoted directly from the mentioned User Guide.

9. **Probes->Optical Fractionator.** Hit “NO” on the pop-up (if you are not using “**Serial Section Manager**”). Another pop will appear. Do not change anything, but hit “OK”. This will start the counting. Focus at the top of the section (debt wise). On the next pop-up, you could either find the bottom or just hit “Do Not Measure”. ‘

Counting:

The sampling follows a set of rules (Gundersen et al., 1977; Keuker et al., 2001), with the aim to serve objectivity to the counting process. A part from the criteria set to recognize cellular elements of interest, are the counting criteria given as follow: **1)** All cellular elements falling within the counting square are counted, when they are in focus. **2)** All elements touching the inclusion (green) line are counted when they are in focus. **3)** All elements outside the square or touching the exclusion line (red), are not counted.

TIPS: Remember to define standardized guard zones, and a standard height for the disector (Andersen et al., 1999). One approach is to define the upper guard zone in the range of 2-5 μm (StereoInvestigator Users Guide, 2008; Keuker et al., 2001; Schumann et al., 2006), the counting frame height at 10 μm (West et al., 1991), and let the lower guard zone vary in concordance with the tissue thickness (Keuker et al., 2001).

Click on the cell to mark them. Hit F2 to go to the next counting frame. Right mouse click provide other functions as well. **TIPS:** Have well-defined criteria before counting.

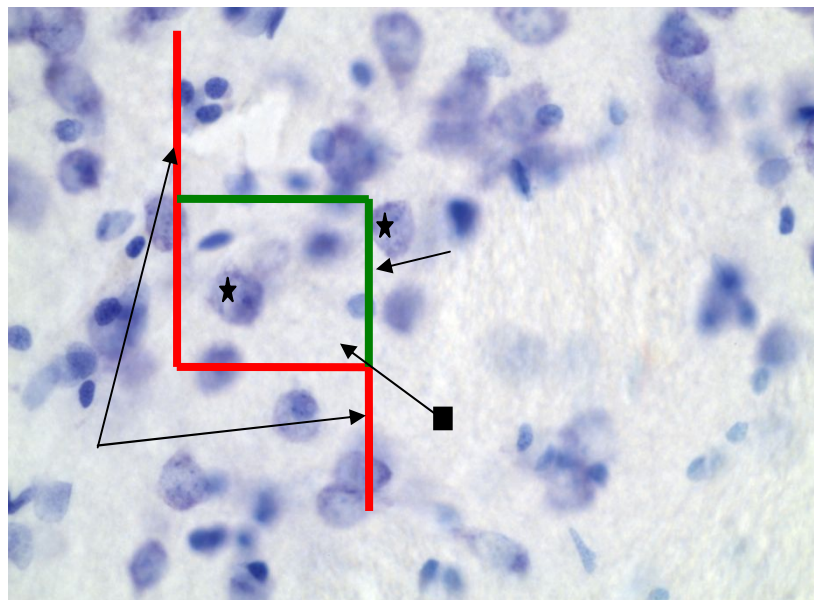


Figure 2: Counting frame, with in-and exclusion lines. The red lines are exclusion lines; the green lines are inclusion lines. All cellular elements in focus and 1) falling within the square, without touching the exclusion lines, or 2) touching the inclusion line, without being in touch with the exclusion lines, are marked with a star, and thereby counted. The counting square is marked by a square.

10. After finishing counting, go to **Probes->Display Probe Run List**. You can change the run list and repeat it, watch the results etc. For now, just watch the results. Then hit: **“Copy All Results to Clipboard”**. Paste the results into an excel-sheet, and save this.
11. If you want to save the tracing and marking as a photo-file, do as follows:
Toolbar: Find the button with a question mark and a box: **“Where is”**. Hit it, then: **Edit-> Copy to Clipboard (BMP)**, and finally hit **Edit->Paste from Clipboard**. **TIPS:** You should also acquire pictures within the contour of the section itself; **Image->Acquire picture**. Place yourself at several different positions within the contour to acquire picture of the whole section of interest. **Remember:** Joy-free/Joy-track must be turned off.
Then use **Edit->Copy to Clipboard (BMP)** and **“Paste from Clipboard”**
12. Save everything. **File->Save as**. Save the file either as .dat- or .asc-file. The acquired pictures should be saved as .tif.
13. Start with a new section. Repeat everything.

Optional:

Tool ->Start Serial Section Manager-> New Section

Block Advance: the thickness at which the tissue was cut. (Generally about 50 μm)

Mounted Thickness: Final mounted thickness (due to shrinkage). Generally about 12-20 μm . (You can change it later on.)

Evaluation Interval: Numbers of section that you skip over. (For instance: If you got total of 50 sections and you count 10, you type 5.)

Block advance: added (if you start counting from the rostral part)

Leave the rest as it is. After finishing this section, just add a new section the same way throughout the procedure.

+: It will calculate the result for every single/selected section compared to each other, so you do not have to do it manually.

-: The contours will be placed upon each other.