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Semi-automated fluorescence microscopy and automated image analysis as a tool to study neuronal survival

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List of Abbreviations

Bcl-xL	B-cell lymphoma-extra large
CAG	CMV early enhancer/chicken beta-actin
CAMKII 1.3	Calcium/calmodulin-dependent kinase II 1.3
CNS	Central nervous system
CMV	Cytomegalovirus
CTRL	Control group
DAPI	Diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle medium
d2eGFP	Destabilized enhanced green fluorescent protein
EB	Electroporation buffer
EF1α	Elongation factor 1α
eGFP	Enhanced green fluorescent protein
FCS	Fetal calf serum
FER	Ferritin
FOV	Fields of view
GD	Glucose deprivation
GFP	Green fluorescent protein
GOI	Gene of interest
HIS	Histidine
hSYN1	Human synapsin 1
HKII	Hexokinase II
LCIS	Live cell imaging solution
MoG	Mixture of Gaussian
mOrange	Orange fluorescent protein
NBM-A	Neurobasal Medium A
NC	Negative control
OD	Oxygen deprivation
OGD	Oxygen-glucose-deprivation
PBS	Phosphate-buffered saline
PC	Positive control
PDL	Poly-D-lysine
RNAi	RNA interference
RT	Room temperature

SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TBS	Tris-buffer saline
UBI	Ubiquitin

Abstract

Fluorescence microscopy is an essential tool in biology and medicine. At present, almost any gene of interest (GOI) can be fluorescently labeled within a living cell, enabling research on protein localization, dynamics, and interactions or on biochemical reactions. Furthermore, by using semi-automated or automated fluorescence microscopes, hundreds of images can be acquired with little human intervention. However, the quantification of image-based data obtained through (semi-)automated imaging systems is an elaborate task and requires careful consideration. Therefore, the aim of this PhD thesis was to develop a tool to study the effects of GOIs on neuronal survival after metabolic stress. For this purpose, an overexpression neuronal survival assay was combined with semi-automated fluorescence microscopy and automated image analysis. The development process included modulating the neuronal cell culture protocol, changing the localization of the fluorescent marker protein to enable a transition from a whole-cell fluorescence signal to fluorescent neuronal nuclei, facilitating semi-automated image acquisition using the Leica Matrix screener application, and designing a pipeline for the automatic quantification of neuronal nuclei using the CellProfiler software. Finally, using this assay, neuronal survival quantification can be rapidly performed at different time points while the cells express a detrimental or beneficial GOI. Furthermore, this assay can be easily adapted to various cell types and different stimuli and therefore represents a powerful tool for the broader scientific community.

Additionally, as gene overexpression constituted a major component of the neuronal survival assay, different promoters and their performance were tested. As a result, the ubiquitin (UBI) promoter performance was regarded as the most appropriate for the neuronal survival assay. Furthermore, I found evidence for an intrinsic hypoxia inducibility of the cytomegalovirus (CMV) promoter. Destabilized enhanced green fluorescent protein (d2eGFP) expression was decreased around days four through seven in neuronal cultures when d2eGFP was expressed under the control of the CMV promoter, whereas constant d2eGFP expression was detected under UBI promoter control. Remarkably, non-fluorescent cells became bright green fluorescent cells following oxygen-glucose-deprivation (OGD). Likewise, d2eGFP protein levels were readily detectable for all time points when expressed under the control of the UBI promoter, whereas d2eGFP protein levels were measurable only until 24 h after hypoxia when the CMV promoter was employed for d2eGFP overexpression. Consequently, we excluded the CMV promoter as a candidate promoter for the neuronal survival assay because

the results indicate that the CMV promoter is hypoxia sensitive and can be reactivated after a short period of hypoxia.

Zusammenfassung

Die Fluoreszenzmikroskopie ist eine wesentliche Methode im Bereich der biologischen und medizinischen Forschung. Gegenwärtig kann fast jedes Gen von Interesse (GOI) in einer lebenden Zelle fluoreszenzmarkiert werden, welches die Erforschung von Proteinlokalisation, -dynamik und -interaktionen oder sogar von biochemischen Reaktionen ermöglicht. Darüber hinaus lassen sich durch den Einsatz halb- oder vollautomatisierter Fluoreszenzmikroskope Hunderte von Bildern ohne nennenswerte menschliche Intervention aufnehmen. Jedoch stellt die Quantifizierung der durch (halb-)automatisierte Bildgebungssysteme gewonnenen bildbasierten Daten, eine aufwendige und anspruchsvolle Aufgabe dar und erfordert eine eingehendere Betrachtung. Daher ist das Ziel dieser Doktorarbeit, ein Werkzeug zu entwickeln, mit dem die Effekte von Zielgenen auf das Neuronenüberleben nach metabolischem Stress untersucht werden können. Hierzu wurde ein Überexpressions-Neuronen-Assays weiterentwickelt und mit halbautomatisierter Fluoreszenzmikroskopie sowie automatisierter Bildanalyse kombiniert. Der Entwicklungsprozess umfasste Modulationen des neuronalen Zellkulturprotokolls, die Änderung der Lokalisation des Fluoreszenzmarker-Proteins von einem Ganzzell-Fluoreszenzsignal zu fluoreszierenden neuronalen Kernen, die Verwendung von halbautomatisierter Bildaufnahme mithilfe der Leica Matrix Screener-Anwendung sowie den Entwurf einer Pipeline für die automatische Quantifizierung von neuronalen Zellkernen durch die CellProfiler-Software. Letztlich kann durch die Verwendung des Assays, das Neuronenüberleben unter der Expression eines schädlichen oder nützlichen GOI zu verschiedenen Zeitpunkten quantifiziert werden. Darüber hinaus lässt sich dieser Assay leicht an verschiedene Zelltypen und Stress-Stimuli anpassen und stellt daher ein leistungsfähiges Werkzeug für eine breiter gefächerte Wissenschaftsgemeinde dar.

Da die Genüberexpression eine Hauptkomponente des neuronalen Überlebenstests darstellt, wurden verschiedene Promotoren bezüglich ihrer Leistung getestet. Infolgedessen wurde die Ubiquitin (UBI) Promotorleistung als die für den neuronalen Überlebensassay am besten geeignete Leistung angesehen. Darüber hinaus fand ich Hinweise auf eine intrinsische Hypoxie-Induzierbarkeit des CMV-Promotors. Die Expression eines destabilisierten, verstärkt grün fluoreszierenden Proteins (d2eGFP) war während der Tage vier bis sieben der neuronalen Kultur verringert, wenn d2eGFP unter der Kontrolle des CMV-Promotors exprimiert wurde, -

während eine konstante d2eGFP-Expression unter der Kontrolle des UBI-Promotors nachgewiesen wurde. Interessanterweise wurden aus nichtfluoreszierenden Zellen nach Sauerstoff-Glukose-Entzug, hellgrün fluoreszierende Zellen. Ebenso waren die d2eGFP-Proteinkonzentrationen zu allen Zeitpunkten leicht nachweisbar, wenn sie unter der Kontrolle des UBI-Promotors exprimiert wurden. Die d2eGFP-Proteinkonzentrationen waren dagegen nur bis 24 Stunden nach Hypoxie messbar, als der CMV-Promotor für die d2eGFP-Überexpression eingesetzt wurde. Folglich schlossen wir den CMV-Promotor als Promotor-Kandidat für den neuronalen Überlebens-Assay aus, da die Ergebnisse darauf hinweisen, dass der CMV-Promotor Hypoxie-sensitiv ist und nach einer kurzen Hypoxie-Periode reaktiviert werden kann.

Aberrant neuronal cell death constitutes one of the major causes for acute neurodegenerative diseases such as stroke or central nervous system (CNS) trauma as well as for chronic neurodegenerative diseases including Alzheimer's and Parkinson's (Fricker et al., 2018). During ischemic stroke, the lack of blood flow due to the blockage of a brain artery leads to necrosis, apoptosis and other forms of regulated cell death of neurons. The region that experiences severe hypoperfusion comprises the core of the infarct. Neurons within this region are lethally injured, whereas neurons surrounding this area remain metabolically active for a certain period (Dirnagl et al., 1999; Mergenthaler et al., 2004; Puig et al., 2018). Protecting these neurons from death can profoundly influence the outcome of a stroke. Therefore, it is important to identify potential neuroprotective key components with pro-survival functions - including genes, proteins, enzymes or small molecules - through research on neuronal survival by mimicking stroke *in vitro* through oxygen-glucose-deprivation (OGD) or by provoking metabolic stress, for example, by glucose deprivation (GD) (Carmichael, 2006; Mergenthaler et al., 2012b). Furthermore, the gained insights can help in developing new therapeutic strategies to alleviate symptoms of acute and chronic neurodegenerative diseases.

Hexokinase II (HKII) is a well-characterized enzyme with pro-survival functions. Hexokinase II facilitates the first step of glycolysis - the conversion of glucose into glucose-6-phosphate (Wilson, 1997) - and its positive effect on cell survival has been reported for various cell types (Ahmad et al., 2002; Bryson et al., 2002; Mergenthaler et al., 2012b; Pastorino et al., 2002; Roberts et al., 2013; Sun et al., 2008). However, few studies have examined the potential anti-apoptotic effect of HKII on cellular survival following metabolic stress (e.g., GD) in live neurons. This may be explained by the fact that culturing primary neurons is challenging. Because mature neurons do not proliferate, frequent neuronal preparations from fresh brain tissue (e.g., rat or mouse brain) are necessary. Furthermore, neurons derived in such a manner have a finite lifespan, which restricts the time course of experiments (Gordon et al., 2013). In contrast, using secondary neuronal cell cultures, such as the popular SH-SY5Y neuroblastoma cell line, enables obtaining an unlimited number of cells in a quick and easy manner (Kovalevich and Langford, 2013). However, most cell lines have been in culture for decades, owing to which these cells are highly adapted to their two-dimensional culture environment. Consequently, cell lines show genetic and phenotypic alterations such as atypical morphology (Pan et al., 2009). Therefore, the use of primary neuronal cells is favored over using secondary cell cultures. Moreover, the use of primary neuronal cells makes the assessment of *in vivo*

neuronal properties more feasible than the use of immortalized neuronal cell lines because the gene expression profiles of primary neurons more closely resemble the properties of neuronal cells *in vivo* (Daub et al., 2009). Therefore, when possible, primary neuronal cells should be used to study neuronal survival.

Currently, primary and secondary cell culture models and biochemical methods are often combined to study the elaborate network of genes, RNA molecules, and proteins that participate in neuronal survival (MacDougall et al., 2019; Murillo et al., 2017). However, important spatiotemporal information is lost when only genomic and proteomic methods are used for this type of research. In contrast, when candidate gene approaches are combined with fluorescence microscopy, researchers can address important "when and where" questions regarding gene regulation (Jain and Heutink, 2010). However, extracting quantitative information from the acquired images still represents a difficult task. To accomplish this task, researchers can use open-source image analysis programs such as ImageJ or CellProfiler (Carpenter et al., 2006; Collins, 2007; Schneider et al., 2012). CellProfiler relies on advanced image analysis algorithms, which are packed in modules. These modules can be placed in sequential order, thereby forming an image analysis workflow or pipeline. Using this pipeline, biological objects such as cells, cellular compartments or organisms can be quantified, and certain descriptive features of these biological objects, including size and fluorescence intensity, can also be measured. Ultimately, by combining cell culture models and assays with (semi-)automated fluorescence microscopy and automated image analysis, the quantification of cellular events within live cells becomes achievable.

Several fluorescent labeling techniques can be used to identify the relevant cell type or subcellular compartment for (semi)-automated fluorescence microscopy (Giepmans et al., 2006; Lavis, 2011). An approach widely used to fluorescently label cells is the fusion of a fluorescent protein such as green fluorescent protein (GFP) to a GOI and introducing it to a cell system via an expression vector. These vectors contain certain regulatory elements, such as enhancer and promoter regions, allowing for a constitutive or regulated expression of the inserted GOI. One of the most commonly used promoters in expression vectors is the cytomegalovirus (CMV) immediate-early enhancer promoter. However, its long-standing reputation of being able to yield a strong and constitutive expression of the transgene is currently being reevaluated. Remarkably, there is a growing body of evidence indicating that CMV promoter performance is susceptible to cellular stress stimuli such as hypoxia or to the application of chemical compounds (Bäck et al., 2019; Wendland et al., 2015).

An alternative method for labeling transgenes includes the introduction of polypeptide tags such as histidine (HIS) tag or FLAG-tag (DYKDDDK) (Einhauer and Jungbauer, 2001). Those tags are smaller than fluorescent proteins, which constitutes an advantage regarding protein folding and shuttling of the tagged protein to the intended cellular location because the short amino acid tag is less likely to interfere with these processes. However, the most common polypeptide tags are non-fluorescent and need to be labeled by immunofluorescence, which requires additional time. A primary antibody against the tag is applied on the cells, and a fluorescence signal is generated by a fluorescently labeled secondary antibody - a process called “immunodetection.” Another disadvantage is that this procedure requires cell fixation. Consequently, studying dynamic processes such as responses to certain stress stimuli is only possible using different cell populations. In contrast, imaging of a fluorescently labeled protein introduced into a primary neuronal cell culture using an expression vector enables the rapid examination of dynamic cellular processes - such as neuronal cell survival after stress - in the same transfected neuronal cell population at different time points.

Altogether, studying neuronal survival after applying a certain stress stimulus such as GD while inducing the overexpression of, for example, putative neuroprotective transgenes represents a valuable live cell approach to gain important insights into cellular survival mechanisms. However, revealing a quantifiable readout for such an assay is a rather complicated task. Therefore, the main objective of this PhD research was to develop a tool to investigate neuronal survival after metabolic stress in live neurons in the context of HKII neuroprotection. By using this tool, pro-survival or detrimental effects of a GOI in live neurons can be determined with automatic quantification of the image-based data. This was achieved by combining an overexpression neuronal cell approach to semi-automated fluorescence microscopy and automated image analysis. The overexpression approach starts with the co-transfection of a GOI and GFP in order to label the transfected live neurons and constitutes a major part of the developed neuronal survival assay described in this thesis. Consequently, addressing overexpression was an important component of my PhD research. The overexpression of a protein of interest can have detrimental effects on a cell (Prelich, 2012). Cellular defects caused by overexpression can largely be attributed to two mechanisms: resource overload and stoichiometric imbalances (protein complexes) (Moriya, 2015). Resource overload means that owing to the strong overexpression of a protein, important protein processing steps such as protein folding, localization, and degradation are overstretched and that important cellular resources are depleted and/or monopolized, ultimately leading to cellular defects. Additionally, a stoichiometric imbalance of the subunits of a protein complex

due to the overexpression of only one component of the complex (subunit) is believed to be another primary mechanism responsible for the development of cellular defects upon protein overexpression (Papp et al., 2003). Thus, near-physiological GOI expression is favorable and deserves close attention. Therefore, while developing and improving the optimized neuronal survival assay, different promoters were tested to investigate their main expression properties (e.g., time course of expression and sensitivity to experimental conditions). The results are summarized in *Publication 2* (Wendland et al., 2015).

The basic version of the neuronal survival assay was developed and employed by Mergenthaler et al. (2012) to investigate the pro-survival effect of HKII after neuronal damage induced by OGD, GD, and oxygen deprivation (OD) (Mergenthaler et al., 2012a). Its central feature was a co-transfection/co-cultivation approach in which one population of neurons was transfected with GFP, and another population was transfected with an orange fluorescent protein (mOrange). Following transfection, both populations were mixed at a 1:1 ratio and maintained in culture (co-cultivation). To investigate the effect of a GOI on neuronal survival, the transgene was co-transfected with GFP. Owing to its widely recognized anti-apoptotic function, B-cell lymphoma-extra large (Bcl-xL) was used as the positive control (Kale et al., 2018; Youle and Strasser, 2008), and single transfections with mOrange were used as the negative control. Finally, the ratio of the number of green fluorescent cells to the number of orange fluorescent cells was used as a measure of neuronal survival. A ratio of one indicated that the GOI had no effect on neuronal survival, a lower ratio demonstrated a damaging effect, and a higher ratio indicated a protective effect. The advantage of this assay is its high adaptability to different laboratory conditions and equipment; it can be applied to diverse types of damage models and different cell types. Because manually counting fluorescent neurons is an error-prone and tedious task, establishment of a semi-automated image acquisition approach with subsequent automatic quantification of fluorescent neuronal nuclei were major steps used to improve the basic neuronal survival assay. *Publication 1* (Mergenthaler et al., 2014) provides a general overview of the basic neuronal survival assay, and *Publication 3* (Wendland et al., 2018) establishes profound improvements to the approach and demonstrates the applicability of the optimized neuronal survival assay.

This section is limited to a brief description of the key methods used in each publication. All details can be found in the three publications listed in the appendix.

Publication 1: A versatile tool for the analysis of neuronal survival

Cell culture and transfection. The preparation of the neurons was carried out on the basis of the guidelines established by Brewer et al. (1993) (Brewer et al., 1993). E15 mice and E17 rats were used (gestational age). The parent animals were allowed to mate only for a few hours to enable the calculation of the exact gestational age. Cortical and subcortical regions were separated to ensure homogenous neuronal preparations; this means that the hippocampus, striatum, olfactory bulb, and other subcortical regions were removed (Meberg and Miller, 2003). Cortical neurons were electroporated immediately after preparation to incorporate plasmid DNA using a Nucleofector device (Nucleofector-I/-II, Amaxa, Lonza). Each transfection experiment was performed using 3×10^6 cells and 5 µg plasmid DNA. Nucleofector device program O-003 was used for rat cortical neurons and O-005 for mouse cortical neurons. A self-made electroporation buffer (EB) (192 mM NaCl, 9.6 mM KCl, 15 mM MgCl2) was used with standard electroporation cuvettes with long electrodes, and neurons were incubated for no longer than 10 min in the EB. Immediately after electroporation, the neurons were resuspended in Dulbecco's modified Eagle medium (DMEM; e.g., Biochrom) containing 3.7 g/l $NaHCO_3$, 4.5 g/l D-glucose, 10% fetal calf serum (FCS), 1% penicillin/streptomycin, and 1% L-glutamine; they were plated at a final density of 3.1×10^5 cells/cm^2. After four to six hours in culture, the medium was replaced with a 1:2 mixture of fresh and conditioned DMEM, to which glutamate was added to achieve a final concentration of 25 µM. After 16 - 20 h in culture, this medium was replaced with a 1:2 mixture of fresh and conditioned NBM-A (LifeTechnologies; containing B27, 1% penicillin/streptomycin, 0.5 mM L-glutamine, and 25 µM glutamate). After five days, approximately one-third of the total culture volume was partially replaced with fresh, glutamate-free NBM-A.

Co-transfection/co-cultivation and damaging events (OGD, OD, and GD). One population of neurons was transfected with GFP and another population with an orange fluorescent protein (mOrange). By mixing these two differently labeled populations, neurons were co-cultivated. In order to investigate the potential effect of a GOI on neuronal survival, neurons were co-transfected with a GOI and GFP. Bcl-xL was used as a positive control because of its known

anti-apoptotic effects (Kale et al., 2018, p. 2), and single transfections with mOrange served as the negative control. Fluorescent cells were counted manually immediately before a damaging event (hypoxia or aglycemia) and 24 hours after the event, focusing on the same area of a well. At least 500 - 700 cells were counted per condition per experiment. The ratio of the number of green fluorescent cells to the number of orange fluorescent cells was considered a measure of cell viability. A ratio of one indicates that the GOI had no effect on neuronal survival. Ratios greater than one or less than one indicate a protective or detrimental effect, respectively, of the GOI on neuronal survival. Calculating these ratios before and after a damaging event also provides an easy normalization method for subsequent statistical analysis. For all experiments OGD, OD, or GD was used as a damaging stimulus (Mergenthaler et al., 2012b). To perform OGD and GD, cultures were washed twice with phosphate-buffered saline (PBS), and BSS0 (116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1 mM NaH_2PO_4, 26.2 mM $NaHCO_3$, 10 µM glycine, 1.8 mM $CaCl_2$, and 1 mM HEPES) was added in the case of OGD. For OD, BSS1 (BSS0 + 1 mM glucose) was added. Oxygen-glucose deprivation was performed at 0.3% O_2, 5% CO_2, and 37°C for three hours in an InvivO2 400 hypoxia chamber (Ruskinn), whereas OD was performed at 0% O_2 for six hours in a Concept 400 anaerobic chamber (Ruskinn). Glucose deprivation was conducted at 21% O_2, 5% CO_2, and 37°C for six hours.

Publication 2: Intrinsic hypoxia sensitivity of the cytomegalovirus promoter

Imaging and image processing. Images were captured using a Leica DMI 6000B inverted fluorescence microscope equipped with a Leica DFC360 FX camera and an HCX PL APO 63×/1.3 glycerin-corrected objective, using eight-well µ-slides (Ibidi, Martiensried, Germany). Employing the Mark and Find function of the Leica software (Leica LAS AF, Wetzlar, Germany) made it possible to record images of the same position in each well, thereby capturing the changes in d2eGFP fluorescence intensity for all time points. The slight variations observed in the image positions can be attributed to µm-scale displacement of the eight-well µ-slides on the microscope stage. To prepare the figures, ImageJ (Fiji v. 1.47d, http://www.fiji.sc) was used to convert 16-bit TIF images to 8-bit TIF images with subsequent background subtraction, using a rolling ball algorithm and uniform contrast adjustment.

Western blotting. Baseline and post-OGD protein lysates (six-well plates) of TagRFP-mito or d2eGFP-transfected neurons were collected and processed on ice. After sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) using 12% Tris-Glycine gels (Thermo Fisher Scientific, USA), semi-dry western blotting and chemiluminescence detection were

performed using Thermo Scientific Pierce ECL Western Blotting Substrate. Primary antibodies for TagRFP-mito (Evrogen) were incubated overnight at 4°C, and primary antibodies for GFP (Santa Cruz, USA) were incubated for one hour at room temperature (RT). Anti-rabbit (GE Healthcare, USA) or anti-mouse (Santa Cruz, USA) horseradish peroxidase-linked secondary antibodies were used. Stripping was performed for ten minutes at 55°C. Membranes were then washed in tris-buffer saline (TBS) and blocked for 30 minutes with 5% milk in TBS at RT. This was followed by beta-actin (Cell Signaling, USA) primary antibody incubation at 4°C overnight.

Publication 3: Investigating gene function for neuronal survival after metabolic stress using semi-automated fluorescence microscopy and automated image analysis

Preparation, transfection, and cell culture of primary cortical neurons. Neuronal preparations and transfection were conducted as previously described in the Materials and Methods section of Publication 1 of this work, with modifications described below. Following transfection, neurons were resuspended in 500 ml DMEM (Biochrom) supplemented with 3.7 g/l $NaHCO_3$, 4.5 g/l D-glucose, and 10% FCS, and 4×10^4 neurons per well (2.6×10^5 cells/cm^2) were plated in poly-D-lysine (PDL; final concentration 20 mg/ml; Sigma) and collagen pre-coated half area 96-well imaging plates (Greiner mClear, #675090). To remove dead cells and debris generated during electroporation, the medium was replaced four hours after transfection. Twenty-four hours after transfection, cultures were gently washed with 1× PBS and NBM-A (Thermo Fisher Scientific, 2 mM L-glutamine) supplemented with B27 (Thermo Fisher Scientific). One week after transfection, 50 µl of culture medium were removed and replaced with fresh NBM-A supplemented with B27. The duration of cell cultivation was 10 days. Note that the ideal cell number depends on the growth area provided by the imaging slide or plate in use and the number of dead cells accumulated during neuronal culture preparation. Furthermore, electroporation itself is a detrimental event for neuronal cells. Twelve hours after electroporation, 50 - 70% of prepared neurons die, but the survivors grow well in culture. The main aim of this procedure was the co-transfection of a GOI and a fluorescent protein. The co-transfection rate achieved using electroporation is approximately 90% (previously established). As a major modification, a fusion protein consisting of GFP fused to a nuclear marker sequence (H2B, histone) was used to label the transfected population of neurons. Labeling only the nucleus of the neurons rather than the whole cell as in the basic neuronal survival assay made it possible to conduct automated image analysis using the CellProfiler software.

Semi-automated fluorescence microscopy. In principle, any automated research-grade inverted fluorescence microscope could be used for this type of assay provided that the controller software allows the user to save the X and Y coordinates for the field of view (FOV) to ensure consistency at subsequent time points. We used a fully automated Leica DMI 6000B microscope equipped with a Leica HCX PL FL L 20_/0.4 objective and a Leica DFC360 camera. The microscope was controlled using Leica LAS AF v. 2.7 software with HCS A Matrix screener extension software. On day nine following transfection, imaging before a damaging event was performed. The medium of the cultured cells was collected in 96-well plates, and primary neuronal cultures were washed once with 1× PBS; subsequent imaging was done using the Live Cell Imaging Solution (LCIS, Thermo Fisher Scientific) containing 140 mM NaCL, 2.5 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 20 mM HEPES (pH 7.4). The Matrix screener application enables the user to design imaging templates that differ in FOV size and number. I used a 96-well template and collected 16 images per well from 10 wells per transfection, adding up to a total number of 160 images per condition. On day 10, that is, 24 hours after the damaging event, culture medium was removed, and neuronal cultures were washed with 1× PBS, followed by subsequent imaging of the same FOVs.

Automated image analysis using CellProfiler. I designed a template using CellProfiler 2.2.0 software (www.cellprofiler.org) to quantify fluorescent neuronal nuclei. The image-processing pipeline consisted of an image loading step directly followed by a primary object identification module; the primary objects in question were the fluorescent nuclei. As the signal-to-noise ratio of the images collected was sufficiently high, the morphing and illumination correction modules were redundant, as determined in my preliminary experiments. The fluorescent nuclei were counted based on two defined parameters: pixel diameter and thresholding. The pixel diameter for various live and dead neuronal nuclei was measured using the “measure length” tool of the CellProfiler software to empirically determine the proper settings. Based on the results of the measurements, a pixel diameter of 23–40 pixels was used to distinguish dead cells from live cells. Nuclei touching the border are automatically excluded from the image analysis. Thresholding classifies any given pixel either as foreground or background, with higher threshold values favoring brighter regions and lower threshold values including dim regions of an image. Threshold values, which range from 0 to 1, can be manually selected in CellProfiler or automatically calculated using different thresholding methods such as Otsu, mixture of Gaussian (MoG), background, or robust background approaches. I used the MoG algorithm to automatically calculate the threshold value, which classifies each pixel as either foreground or background. Its usage is recommended when less than 50% of the image is defined as

foreground, and this criterion was fulfilled by the images obtained in the neuronal survival assay. The last module in the pipeline saved the images of outlined nuclei for quality control and exported a CSV file with all parameters measured, including identified objects and the fluorescence intensity values for each nucleus. A more detailed description of the CellProfiler pipeline is provided in Supplementary Note One of Publication 3 in the appendix.

Results

Publication 1 describes the concept of the basic neuronal survival assay. The assay combines an overexpression approach using primary cortical neurons and fluorescence microscopy to analyze neuronal survival after nutrient deprivation. Primary neuronal cells were prepared from mouse or rat embryos. In order to investigate the function of a GOI on neuronal survival, the GOI was co-transfected with GFP. Moreover, an additional cell population was transfected with an orange fluorescent protein (mOrange). The two differently transfected neuronal cell populations were then co-cultivated at a ratio of 1:1. Green fluorescent cells also expressing the GOI and orange fluorescent cells were cultivated for nine days under identical conditions. Next, the number of transfected neurons was quantified by manually counting green and orange fluorescent cells before and 24 hours after nutrient deprivation (e.g., hypoxia or aglycymia) using live-cell imaging. In total, the basic assay protocol requires 10 days. In all experiments, 500 - 700 cells per condition per experiment were counted. By comparing the calculated ratios (number of green fluorescent cells divided by the number of orange fluorescent cells) before and after a damaging event as well as among experimental groups, the effect of a certain transgene on neuronal survival can be easily and rapidly investigated. Notably, positive and negative controls need to be employed in the experimental paradigm, and the GOI expression results should be compared to these controls. Furthermore, an "endogenous" control was implemented in every condition of this assay, that is, one population of transfected neurons (orange fluorescent neurons) served as the control for another transfected neuronal cell population (neurons co-transfected with GFP and GOI).

The basic assay was used to characterize the neuroprotective function of HKII. Mergenthaler et al. (2012) reported a neuroprotective function of HKII following OGD in primary cortical neurons; however, under GD conditions, HKII promoted cell death. Cells were manually counted while performing live cell imaging in order to calculate the ratios of green and orange fluorescent cells. Figure 2 (A) displays the results of the cell counting in the control group (CTRL), a ratio of approximately 1. This result indicates that the transfection of the

fluorescent protein (mOrange) has no measurable effect on neuronal survival. In the positive control group overexpressing Bcl-xL and GFP, more green fluorescent cells survived the damaging event, resulting in a higher survival ratio following OGD. Moreover, Figure 2 (B) indicates the protective function of HKII after OGD, and its detrimental effect following GD is illustrated in Figure 2 (C). Taken together, these results demonstrate the applicability of the basic neuronal survival assay to analyze the protective or detrimental effect of a GOI on neuronal survival.

Publication 3 describes the applicability of the further developed version of the basic neuronal survival assay. In contrast to the basic assay described in Publication 1, the analysis process changed from manual cell counting while performing live-cell imaging to semi-automated image acquisition with subsequent automatic image analysis using the CellProfiler image analysis software. In order to analyze neuronal survival using the CellProfiler software, the localization of the fluorescent protein needed to be altered. The detection of a more distinct fluorescence signal was required - in contrast to a whole-cell fluorescence signal produced by a non-targeted fluorescent protein as was used in the basic neuronal survival assay - because the separation of a fluorescent neuronal network into single neuronal entities would be a very error-prone task for an image analysis program. Therefore, the nuclear marker H2B (histone) was fused to GFP, and the overexpression of this fusion protein led to bright green fluorescent neuronal nuclei. Owing to their defined shape and bright fluorescence, the nuclei served as a perfect quantification marker for the optimized neuronal survival assay. Consequently, primary embryonic rodent cortical neurons were co-transfected with H2B-GFP and Bcl-xL as the positive control (PC). As the negative control (NC), H2B-GFP and an empty vector were co-transfected. Likewise, to analyze the pro-survival or detrimental effect of a GOI on neuronal survival, the GOI was co-transfected with GFP. Next, semi-automated image acquisition was conducted using a fully automated Leica DMI 6000 microscope equipped with the HCS A Matrix screener extension software. With the Matrix screener software, different screening templates can be generated and adjusted. A 96-well template was adapted to collect 16 images per well per transfection, adding up to a total of 160 images per condition. Using this template, images displaying the same FOVs were taken before and 24 h after nutrient deprivation. An FOV describes the maximum area visible through the microscope camera or eyepiece (eyepiece FOV). In contrast to the results of the basic neuronal survival assay, the outcome of the optimized neuronal survival assay was stored as images and was automatically quantified using the CellProfiler software. CellProfiler enables the generation of image processing pipelines to

automatically measure and quantify biological phenotypes. In order to quantify fluorescent neuronal nuclei, the image analysis software must distinguish between foreground signal and background signal of each image and identify live neuronal nuclei depending on their size. This was achieved by defining two parameters: by using pixel diameter and by thresholding. The pixel diameter of viable fluorescent neuronal nuclei was empirically determined by using the "Measure length" tool provided by CellProfiler. Especially when analyzing fluorescent nuclei after nutrient deprivation, live and dead fluorescent nuclei must be separated by the image analysis software. Thus, to exclude dead nuclei from the analysis, the pixel diameter was set to 23 - 40 pixels. Notably, Figure 2 (A) illustrates size differences between live and dead fluorescent neuronal nuclei. Thresholding defines whether a certain pixel is classified as foreground or background. Higher thresholding values favor brighter regions of an image, whereas lower threshold values include dimmer regions of an image. Based on the image characteristics, I used the thresholding method mixture of Gaussian (MoG) included in the software. The MoG algorithm is recommended if less than 50% of an image is foreground, which was the case for the images obtained through the neuronal survival assay. Thus, by implementing semi-automated fluorescence microscopy and automatic image analysis, the development of a tool for neuronal fate quantification through the observation of survival or death of individual fluorescent neuronal nuclei was accomplished.

Further modifications of the basic neuronal survival assay, such as adapting the primary cell culture protocol, were necessary to improve image acquisition and image-based data analysis. I found that introducing a washing step on day one after transfection promoted the health and survival of the neuronal cell culture. A possible reason for this beneficial effect was that less dead cells and cellular debris remained in the culture. Dead cells and debris resulted from the introduction of plasmid DNA into the cells via electroporation. This process employs an electrical pulse, which generates temporary pores in the phospholipid bilayer of the cell membrane, which allows molecules to enter (Gärtner et al., 2006; Rubinsky, 2007; Sukharev et al., 1992). Under suitable biological conditions, these pores close rapidly, restoring the cell to its original state while retaining the newly introduced plasmid DNA. However, depending on the cell type, the pore-closing process may be prolonged or impaired, ultimately resulting in cell death (Grys et al., 2017). Furthermore, the excess accumulation of dead cells (still fluorescent) in a neuronal cell culture negatively influences the overall health of the culture and makes it difficult to achieve imaging with precise cellular resolution.

As gene overexpression was a major component of the further developed neuronal survival assay, the most suitable promoter for transgenes and for the nuclear marker protein expression

was determined. Thus, properties of popular promoters were studied (e.g., time course of expression and sensitivity to experimental conditions). The main results are summarized in ***Publication 2.*** This investigation consisted of transfecting primary rat cortical neurons with a plasmid encoding for a destabilized eGFP (d2eGFP) under the control of either the CMV or UBI promoter (Li et al., 1998). Next, neurons were imaged directly before OGD and two, three, and 24 h post OGD. Using the “Mark and Find” function of a software program that enabled the storage of X and Y coordinates (positions) to define different FOVs of the μ-slide made it possible to visualize the changes in the d2eGFP expression patterns in the same cell at all measured time points. Remarkably, I found that the green fluorescence intensity levels diminished around days four through seven in culture when d2eGFP was expressed under the control of the CMV promoter; however, constant d2eGFP expression was found under UBI promoter control. Furthermore, our data suggest that the CMV promoter is intrinsically sensitive to hypoxia because non-fluorescent cells became bright green fluorescent cells following OGD. This phenomenon is illustrated for one cell, depicted with an open arrowhead, in Figure 1 a-i of Publication 2. In contrast, cells overexpressing d2eGFP under UBI promoter control exhibited a constant weak-to-moderate fluorescence intensity throughout the experiment, which is illustrated for one cell, depicted with a closed arrow, in Figure 1 a-ii of Publication 2. Next, protein-level changes were studied by means of western blotting of d2eGFP under the control of the CMV and UBI promoter, respectively. The d2eGFP protein levels were readily detectable at all time points when expressed under the control of the UBI promoter, whereas protein quantities were measurable only until 24 h after hypoxia under the control of the CMV promoter. Taken together, these findings indicate that the CMV promoter is hypoxia-sensitive and can be reactivated after a short period of hypoxia. Based on these findings, we excluded the CMV promoter as a candidate promoter for the optimized neuronal cell survival assay.

To investigate promoter performance, expression plasmids were generated by subcloning TagRFP-mito into vector backbones containing different promoter sequences, namely CMV, UBI, calcium/calmodulin-dependent kinase II (1.3) (CamKII 1.3), CMV early enhancer/chicken beta actin (CAG), and ferritin (FER). The highest protein levels were detected under CamKII 1.3 promoter control, followed by that under UBI promoter control. Moderate d2eGFP protein levels were detected when d2eGFP was expressed under the control of the CAG promoter, and very low levels of the d2eGFP protein were detected under CMV and FER promoter control. Mitochondrial targeted TagRFP-mito was almost undetectable until 24 h after OGD when expressed under CMV promoter control. Based on the findings described above,

we selected the UBI promoter as the most appropriate promoter for the neuronal survival assay. The UBI promoter has several advantages: (1) expression of the fluorescent marker protein under UBI promoter control produces a sufficiently bright fluorescence signal by using short exposure times, which benefits neuronal health; (2) protein quantities produced under UBI promoter control are easily measurable by means of western blotting using chemiluminescent substrates that require short incubation times; and (3) absolute construct/vector size considerations favor the UBI promoter because its relatively small size positively influences transfection efficiency (data not included). One possible explanation for this observation is that there is a potential relationship between plasmid size and nuclear uptake of plasmid DNA after electroporation (Ribeiro et al., 2012). Furthermore, our observations are corroborated by the findings of Lesueur et al. (2016), who demonstrated that decreased cell viability and transfection efficiency are directly linked to the physical size of the plasmid molecule (Lesueur et al., 2016).

Discussion

In this work, a versatile tool to study the effects of detrimental or beneficial GOIs on neuronal survival after metabolic stress was developed by combining a neuronal cell approach with semi-automated fluorescence microscopy and automated image analysis. The major advantage of this assay is its easy applicability to different cell types and stress stimuli and that even novice users of an image analysis program can easily adapt the provided image analysis pipeline to their own experimental needs. Finally, owing to its easy adaptability, the survival assay can be used beyond the biological fields of neuroprotection and neurodegeneration.

In addition, investigations of gene promoter performances provided evidence for an intrinsic hypoxia sensitivity of the CMV promoter. Therefore, it is not advisable to use the CMV promoter for gene overexpression when this type of stress stimulus is used in the experimental setup.

Establishing an image-based overexpression assay to study neuronal survival

By applying fluorescence microscopy, scientists can accurately study fundamental biological processes within an intact, healthy living cell (Pepperkok and Ellenberg, 2006). Furthermore, using (semi-)automated microscopy combined with subsequent automatic image analysis enables the conversion of phenotypic data stored as images into measurable biological parameters. In my PhD research, I have developed a tool to study neuronal survival that implements the above-mentioned approaches. The optimized tool enables investigations of a detrimental or beneficial effect of a GOI on neuronal survival in a rapid and straightforward manner. Most importantly, the measured effect can be automatically quantified. Extensive modifications were needed to optimize the assay, including adapting the neuronal cell culture protocol, changing the localization of the fluorescent marker protein to enable a transition from a whole-cell fluorescence signal to a nuclear fluorescence signal, facilitating semi-automated image acquisition using the Leica Matrix screener, and establishing automated image analysis using the CellProfiler software.

When cell-based imaging assays are performed, one of the main challenges is determining the ideal combination of the cell culture plate type, magnification, and desired image quality. Therefore, close attention was paid in choosing every component of this assay. Finally, the ideal cell culture and seeding procedures and semi-automated image acquisition requirements were determined in order to enable proper automatic quantification of neuronal nuclei using the

CellProfiler software. Another advantage of the optimized neuronal survival assay was that the number of replicates per imaging plate was increased because it used 96-well microtiter plates in contrast to the 48-well plates used in the basic assay.

A key limitation of the basic assay used by Mergenthaler et al. (2012) was the requirement for manual cell counting under the microscope, which is a tedious, time-consuming, and error-prone task. In contrast, semi-automatic microscopy, as performed here, enables the collection of hundreds of images in a short timeframe with little manual intervention (Wendland et al., 2018). The image acquisition process required 25 - 35 min for one 96-well plate, in contrast to two to three hours required for manual cell counting per 48-well plate in the basic neuronal survival assay. Thus, by introducing semi-automatic image acquisition, I was able to reduce the time spent on the microscope; more importantly, the sensitive neuronal cultures were exposed to phototoxic fluorescent light for a shorter time, which is beneficial for general neuronal viability. Indeed, the optimal viability of cell cultures is a high priority when performing live cell imaging. For example, the cellular response to light-induced photodamage may be observed as experimental artifacts (Icha et al., 2017; Magidson and Khodjakov, 2013). Consequently, the most challenging part of live cell fluorescence microscopy data collection is minimizing photodamage while retaining a useful signal-to-noise ratio of the obtained images (Ettinger and Wittmann, 2014). I addressed this challenge by identifying the most appropriate promoter facilitating the expression of the fluorescent protein and the GOI and by adapting the neuronal cell culture protocol. As a result, an appropriate cell number forming a neuronal monolayer with a low level of cellular debris and the use of a promoter that enabled the expression of a fluorescent protein in a detectable quantity by using short excitation times of fluorophores produced an appropriate signal-to-noise ratio for imaging neuronal nuclei using semi-automated image acquisition.

Quantitative analysis of images obtained using fluorescence microscopy has long been a tedious and time-consuming undertaking, with few possibilities to conduct large-scale investigations of biological mechanisms. Indeed, manual image analysis restricted this type of research to low-throughput experiments. However, the introduction of robotics for sample preparation and the further automation of imaging systems present new opportunities for high-throughput imaging (Pepperkok and Ellenberg, 2006). In fact, high-throughput microscopy systems facilitate image-based screening to measure diverse phenotypes of cells and whole organisms (Conrad and Gerlich, 2010). For example, large libraries of small molecules have been screened using

cell-based assays and automated microscopy to identify novel drug candidates (Carpenter, 2007). Additionally, this approach has been used to assess gene function after introducing genetic perturbations or RNA interference (RNAi) (Boutros and Ahringer, 2008). Notably, a straightforward readout of most image-based cellular assays is detection of a fluorescence signal produced by fluorescently labeled proteins or by antigen immunostainings. For example, these techniques have been used to assess lipoprotein uptake to identify genes that regulate cellular cholesterol homeostasis and to investigate mitochondrial Ca^{2+} transport, thereby revealing the mitochondrial Ca^{2+}/H^{+} antiporter function of the human Letm1 protein (Bartz et al., 2009; Jiang et al., 2009). However, these approaches have a disadvantage in that immunodetection requires that cells be fixed, prohibiting the investigation of dynamic cellular processes such as cellular reactions to stress stimuli of the same cell population. Furthermore, evidence suggests that cell fixation introduces artifacts in cell structure (Li et al., 2017). Moreover, most image-based research is conducted using cell lines, whereas the usage of primary cells, especially neurons, is relatively rare. One example of the usage of mouse primary neuronal cultures is the study by Nieland et al. (2014), who investigated excitatory and inhibitory synapse development using RNAi to evaluate novel regulators of synapse formation (Nieland et al., 2014). Ultimately, the optimized neuronal survival assay enables the investigation of responses of live primary cells to various stress stimuli. Importantly, this assay allows researchers to measure the behavior of the exact same live cell population at different time points. This constitutes an advantage over performing immunostaining after cell fixation because investigation of dynamic processes is only feasible when different cell populations are stained.

Since its introduction in 2005, CellProfiler has enabled scientists to create and share modular image analysis pipelines for the quantification of various phenotypes of different cell types (Bray et al., 2015; Gasparini et al., 2017; González et al., 2012; Kamentsky et al., 2011). Since then, automated microscopes have further influenced modern research. In current research, well-developed automated microscopes enable the collection of thousands of images that demonstrate even subtle phenotypic changes caused by genetic perturbations or chemical compounds (Boutros et al., 2015). Extensive data sets are collected, and computer algorithms need to be able to accurately identify single cells, cell compartments, or organisms. Additionally, important descriptive features, such as the compartment size (e.g., nucleus) or measured fluorescence intensity of an object, need to be extracted for each identified object. To keep pace with these requirements, CellProfiler 3.0 was launched in 2017 (McQuin et al., 2018).

Analysis of 3D images is now supported in many pipeline modules, and several new image-processing modules have been introduced. It now even supports the installation of deep learning frameworks such as TensorFlow and Caffe, which are used to model deep neuronal networks (Abadi et al., 2016; Jia et al., 2014). This development suggests that there is a scientific demand for more advanced image analysis programs, which leads to the question of why it is beneficial to use image analysis programs in the first place. First, using image analysis programs reduces human selectivity and bias. Different sets of images obtained, for example, before and after a stress stimulus can be analyzed, and biological objects can be quantified based on the exact same image analysis pipeline. Furthermore, performance differences are avoided. If different staff members were to count, for example, fluorescent nuclei of neurons, the obtained results would likely differ (Carpenter et al., 2006). Possible causes are differences in education regarding the context (size and structure of viable neuronal nuclei), ability to focus on a task, and motivation to perform the task. Second, by using image analysis programs, scientists save time because manual cell counting is highly time-consuming. Third, the reproducibility is very high because hundreds, thousands, or even millions of images can be analyzed to quantify biological objects. Furthermore, because automated image analysis programs facilitate analyzing large image sets, even subtle phenotypic changes become measurable; such subtle changes in phenotypes would not necessarily be detectable by the human eye. Fourth, using image analysis programs provides more options than only counting and quantifying biological objects. Important object information, including size, shape, fluorescence intensity, and texture of a biological object, can be determined and saved for subsequent analysis.

By developing a tool to automatically analyze neuronal survival after stress, I demonstrated that with a common research-grade fluorescence microscope capable of storing and locating X and Y coordinates, near high-throughput imaging is possible through a combination of semi-automated fluorescence microscopy and automated image analysis. The major advantage of this optimized neuronal survival assay is its straightforward adaptability to different cellular models and stress conditions. This assay relieves an important bottleneck because even novice and untrained users of image analysis programs can utilize the described pipeline parameters as a starting point for their own image-based data analysis. One way of adapting the described pipeline could be to use the "Measure Length" tool provided by the CellProfiler software to determine the pixel diameter of cellular compartments other than nuclei such as peroxisomes or liposomes and quantify changes in organelle number due to a given stress stimulus. Thus,

this assay can be used and adapted to help answer various research questions asked by a broad scientific community and therefore constitutes an excellent cell research tool.

Gene promoter characterization

Comprehensive research on the function and components of promoters enables gene expression modifications, and therefore gene promoters represent powerful molecular biology tools (Alberts et al., 2002). However, promoter performance differs among promoters. These differences include variations in target protein levels and the time course of transgene expression (Moritz et al., 2015; Raup et al., 2016). However, comparative studies that provide profound insights regarding promoter properties are rare. For this reason, and to select the most appropriate promoter for the optimized neuronal survival assay, we tested different promoters to investigate their effect on target protein expression. We provide evidence that the target protein quantities and presence are mediated by promoter performance. We illustrate that, under the control of the CMV promoter, d2eGFP or TagRFP-mito protein content was diminished to almost undetectable levels on day 10 in culture. Interestingly, the d2eGFP fluorescence signal was restored after exposing the neurons to OGD, indicating a hypoxia sensitivity and inducibility of the CMV promoter. Therefore, it is challenging to use the CMV promoter to express a protein of interest or to incorporate stress stimuli such as OGD into the experimental paradigm. Consequently, the CMV promoter was excluded as a candidate promoter for the optimized neuronal survival assay.

The human CMV immediate-early promoter enables high-level expression of a transgene in various mammalian cell types. Thus, it is among the most commonly used promoters for commercial and customized expression vectors. The CMV promoter has even been used in gene therapy studies in the CNS because of its long-standing reputation for promoting stable and long-term transgene expression (Maguire et al., 2014; Piguet et al., 2017). However, there is a growing body of evidence that using the CMV promoter to drive transgene expression should be reevaluated. For example, when tumor suppressor protein p53 was overexpressed in HEK293T cells, the transcription rate of β-galactosidase expressed was drastically altered under the control of the CMV promoter but not under the control of two other promoters tested (Rodova et al., 2013). Remarkably, Bäck et al. (2019) found an increase in CMV-driven transgene expression induced by methamphetamine, glutamate, and kainic acid. In contrast, transgene expression under the human synapsin 1 (hSYN1) promoter or elongation factor 1α (EF1α) promoter did not alter transgene expression after exposure to the same neuronal stimulants (Bäck et al., 2019). Furthermore, the silencing of the CMV promoter by

DNA methylation and histone deacetylation is widely recognized (Brooks et al., 2004; Collas, 1998; Löser et al., 1998; Mehta et al., 2009; Prösch et al., 2009).

Finally, the results described in this work suggest that the assertion that the CMV promoter exhibits constitutive expression in neurons and other cell types should be reconsidered. Further research is required to test its applicability in cell-based overexpression approaches and in drug therapy research. In conclusion, the misinterpretation of cell biology data due to misconstrued promoter performance can be avoided by developing a thorough understanding of promoters.

In summary, every part of the advanced neuronal survival assay was established and tested with considerable effort and precision, resulting in the development of a powerful tool to study the effects of a gene of interest on neuronal survival after metabolic stress. Effects of various GOIs on cell survival can be rapidly investigated and automatically quantified. Even novice users of an image analysis software can use the provided pipeline as a starting point for their own experiments. Finally, the neuronal survival assay can be easily adapted to different cell types and stress stimuli and therefore constitutes a versatile tool for the broader scientific community.

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Appendix

Statutory Declaration

"I, Kristin Wendland, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic: "Semi-automated fluorescence microscopy and automated image analysis as a tool to study neuronal survival; Semi-automatisierte Fluoreszenzmikroskopie und automatische Bildanalyse als Werkzeug zur Untersuchung neuronalen Zellüberlebens", independently and without the support of third parties, and that I used no other sources and aids than those stated.
All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me."

Date Signature

Declaration of your own contribution to the publications

Kristin Wendland contributed the following to the publications listed below:

Publication 1: Mergenthaler, P., **Wendland, K.**, Meisel, A., **2014**. A versatile tool for the analysis of neuronal survival. Methods, Advanced Light Microscopy 66, 325–329.

Kristin Wendland was strongly involved in further developing the idea of the assay. Furthermore, she was involved in manuscript writing.

Publication 2: Wendland, K., Thielke, M., Meisel, A., Mergenthaler, P., **2015**. Intrinsic hypoxia sensitivity of the cytomegalovirus promoter. Cell Death Dis. 6, e1905.

Kristin Wendland conducted the experiments in the laboratory and played a decisive role in creating the experimental setup. This included designing and constructing the plasmids, maintaining the neuronal cell culture, performing the OGD experiments, fluorescence microscopy, and western blots including SDS-PAGE. Furthermore, she was responsible for the evaluation and interpretation of the results. Additionally, she was strongly involved in manuscript writing and figure preparation.

Publication 3: Wendland, K., Meisel, A., Mergenthaler, P., **2018**. Investigating gene function for neuronal survival after metabolic stress using semi-automated fluorescence microscopy and automated image analysis. Front. Mol. Neurosci. 11.

Kristin Wendland designed the neuronal survival experiments and conducted them in the laboratory. This included the assay design and implementation using molecular and biochemical methods (molecular cloning, western blot), neuronal cell culture, consumable testing and selection, and the establishment of semi-automated fluorescence microscopy (matrix screener). Furthermore, she independently developed the CellProfiler pipeline, including the testing process of all used program modules and the validation of the program features. She was also responsible for the interpretation of the collected data and the statistical analysis. Furthermore, she wrote the manuscript and prepared the figures. All figures included in this publication were produced on the basis of the experiments she designed and conducted.

Signature, date and stamp of first supervising university professor/lecturer

Signature of doctoral candidate

Publication 1: A versatile tool for the analysis of neuronal survival

Mergenthaler, P., **Wendland, K.**, Meisel, A., 2014. A versatile tool for the analysis of neuronal survival. Methods, Advanced Light Microscopy 66, 325–329.

Electronic Publication: Please follow this link:
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Impact factor:

Abbreviated Journal Title	ISSN	JCR Data						Eigenfactor Metrics	
		Total Cites	Journal Impact Factor	5 Year Impact Factor	Immediacy Index	Citable Items	Cited Half-Life	Eigenfactor Score	Article Influence Score
METHODS	1046-2023	14080	**3.645**	3.851	1.516	246	>10	0.01	1.516

Publication 2: Intrinsic hypoxia sensitivity of the cytomegalovirus promoter

Wendland, K., Thielke, M., Meisel, A., Mergenthaler, P., 2015. Intrinsic hypoxia sensitivity of the cytomegalovirus promoter. Cell Death Dis. 6, e1905.

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CELL DEATH DIS	2041-4889	8527	**5.378**	5.497	0.983	354	2.4	0.03659	1.588

Publication 3: Investigating Gene Function for Neuronal Survival After Metabolic Stress Using Semi-Automated Fluorescence Microscopy and Automated Image Analysis

Wendland, K., Meisel, A., Mergenthaler, P., 2018. Investigating Gene Function for Neuronal Survival After Metabolic Stress Using Semi-Automated Fluorescence Microscopy and Automated Image Analysis. Front. Mol. Neurosci. 11.

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Abbreviated Journal Title	ISSN	JCR Data						Eigenfactor Metrics	
		Total Cites	Journal Impact Factor	5 Year Impact Factor	Immediacy Index	Citable Items	Cited Half-Life	Eigenfactor Score	Article Influence Score
FRONT MOL NEUROSCI	1662-5099	4752	**3.720**	4.375	0.611	483	2.9	0.01422	1.346

Curriculum vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

List of publications

Mergenthaler, P., **Wendland, K.**, Meisel, A., **2014**. A versatile tool for the analysis of neuronal survival. Methods, Advanced Light Microscopy 66, 325–329.

Wendland, K., Thielke, M., Meisel, A., Mergenthaler, P., **2015**. Intrinsic hypoxia sensitivity of the cytomegalovirus promoter. Cell Death Dis. 6, e1905.

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