

PRAQA: Protein Relative Abundance Quantification Algorithm for 3D Fluorescent Images

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Abstract: In confocal fluorescent microscopy, the quality of the acquisition strongly depends on diverse factors including the microscope parameterization, the light exposure time, the type and concentration of the antibodies used, the thickness of the sample and the degradation of the biological tissue itself. All these factors critically influence the final result and render tissue protein quantification challenging due to intra- and inter-sample variability. Therefore, image processing techniques need to address the acquisitions variability to minimize the risk of bias coming from changes in signal intensity, noise and parameterization. Here, we introduce *Protein Relative Abundance Quantification Algorithm* (PRAQA), a 1-parameter based, fast and adaptive approach for quantifying protein abundance in 3D fluorescent-immunohistochemistry stained tissues that requires no image preprocessing. Our method is based on the assessment of the global pixel intensity neighborhood dispersion that allows to statistically infer whether each small region of an image can be considered as positive signal or background noise. We benchmark our method with alternative approaches from literature and validate its applicability and efficiency based on synthetic scenarios and a real-world application to post-mortem human brain samples of Alzheimer's Disease and Lewy Body Dementia patients. PRAQA is implemented in Matlab and freely available at <https://doi.org/10.17881/j20h-pa27>.

1 INTRODUCTION

Life relies on the proper function of its essential building blocks, the cells (Alberts, 2017). The functionality of cells is ensured by the orchestrated action of a plethora of molecules where in particular proteins encoded by genes are essential for structure, function and regulation. Impairment in protein functions caused by mutations in the encoding genes or by external perturbations often result in perturbed regulatory mechanisms and subsequent changes in protein abundance which in turn may trigger different diseases including cancer, diabetes and neurodegeneration. To investigate underlying regulatory disease

mechanisms, it is therefore essential to characterize protein abundance and localization also in the context of cellular heterogeneity (Komin and Skupin, 2017).

Immunostaining methods, including immunofluorescence, revolutionized the scientific world by exploiting antibody specificities to target and visualize antigens of interest in biological tissues by microscopy (Im et al., 2019). This technique allows for estimating the distribution and the quantification of targeted proteins at the microscopic scales, which are essential for assessing the roles of proteins or cellular and subcellular alterations in pathological conditions. A general challenge is thereby the post-acquisition analysis of microscopy images for which different methods have been developed as comprehensively discussed in (Varghese et al., 2014).

In fluorescent microscopy, a common drawback of these methods is the typical need of a preprocessing step to transform the image into an appropriate in-

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put format fulfilling requirements such as background uniformity or noise reduction, or the dependence of more than one tuning parameter in order to achieve the quantification. The wide range of options for parameterizing has the advantage that the quantification procedure can be adapted to different imaging conditions but irremediably increases the procedure complexity and subsequently leads to potential biases originated by an inaccurate choice of methods or parameters. While deep-learning methods are able to provide parameter-free analysis, the adaptability to a plethora of acquisition conditions requires huge amount of data to train the network (Belthangady and Royer, 2018). This requirement cannot always be satisfied, particularly for limited sample availability such as for human brain samples.

Furthermore, advances in high-resolution fluorescent microscopy now allow for processing of thicker samples in the range of 50 to several hundreds of micrometers. Larger 3D acquisitions have higher information content but, on the other hand, they are affected by larger signal intensity variability caused by the thickness of the section (Chung et al., 2013; Bouvier et al., 2016) that makes reliable and robust quantification challenging.

To address these challenges, we here introduce PRAQA (*Protein Relative Abundance Quantification Algorithm*), that is optimized for protein quantification in 3D fluorescent confocal tile scan microscope acquisitions. With only one parameter and no preprocessing required, PRAQA is easy to use, fast and extremely resilient to intensity changes as well as robust for noise compromised images. We benchmark PRAQA against alternative approaches in synthetic scenarios and test its efficiency by an application to paraformaldehyde fixed human brain samples in the context of neurodegeneration representing a challenging scenario due to extreme inter- and intra-sample variability.

The manuscript is organised as follows:

- Section 2 describes the algorithm and its implementation.
- Section 3 presents two synthetic scenarios and benchmarks PRAQA with alternative algorithms from literature.
- Section 4 presents an application on a real scenario.
- Section 5 discusses advantages and drawbacks of PRAQA and points to future work.

2 METHODOLOGY

The different sources of variability mentioned above render a robust correction challenging and practically impossible. Hence, image processing aims at solving the parallel problem of an image binarization obeying invariance with respect to the overall signal power. PRAQA is approaching this challenge by a statistical test based on **Median Absolute Deviation** (MAD) to reliably distinguish background noise from fluorescent signal among images with different contrast.

The advantage of MAD compared to other dispersion or outlier detection methods is its robustness with respect to extreme values (Leys et al., 2013) and is therefore an appropriate choice for binarization. PRAQA classifies pixels as positive when the neighborhood average intensity exceeds θ scaled MAD where the scaled MAD of a vector x is determined by

$$MAD_S(x) = \frac{|x_i - med(x)|}{med(|x_i - med(x)|)} \quad (1)$$

with $med(x)$ being the median of the vector x .

In Section 3.3, we empirically evaluate the robustness of θ with respect to different signal strengths.

2.1 PRAQA Pseudocode

The implementation of PRAQA is described in the following by pseudocode and corresponding explanations. For a given 3D image I and a threshold θ , PRAQA calls the *binarizeMAD* function:

```

1 function binarizeMAD(I,theta)
2   I = I./max(I);
3   SE_List = getSE();
4   for z = 1 : numberOfSlices(I)
5     for i = 1 to numberOfSE
6       Feats(:,i) = getFeat(I(z),SE_List(i));
7       I_MAD(:,i) = MADS(Feats(:,i));
8       Positive_P(:,i) = I_MAD(:,i) > theta;
9     end
10  end
11  for i = 1 to numberOfSE
12    I_Positive = sum(Positive_P(:,i)) > 1;
13  end
14  return I_Positive;
15 end

```

In line 2, the image I is first normalized to a $[0, 1]$ range. In line 3, a predefined list of **Structuring Elements** (SE) is loaded into the memory which are used to define the local neighborhood. These structuring elements can be considered as an additional parameter of the algorithm but here we kept specifically 3 circle SEs with radius 4, 6, and 8 throughout the whole study.

From line 4 to line 10, we start iterating through each slice of the 3D image by moving in the z direction. The nested loop (line 5 to 9) iterates through all the SEs and retrieves the pixel features by the function *getFeat* (line 6). This function returns a vector which contains the value of the average neighborhood intensity defined by the SEs for each pixel (see below). Next, the scaled MAD (Eq. 1) is calculated for each feature (line 7) and all pixels for which the threshold θ is exceeded are marked as positive and stored together with the scaled MADs of each SE in the variable *PositiveP* (line 8).

Hence, this loop (line 4 to 10) generates *PositiveP* as a 3D matrix of size $N \times M$ where N is number of pixels and M is the number of SEs. For final pixel classification, we next loop through the matrix *PositiveP* and check whether a pixel has been labeled as an outlier in more than one SE-based MAD (line 11 to 13). If this is the case, the corresponding pixel is classified as a positive signal pixel (line 12). The imposed requirement of at least two outlier classifications in the same pixel reduces the probability of false positive or false negative detection. Finally, the function returns the binarized image (line 14).

The aforementioned pixel features are calculated by the routine *getFeat* as

```

1 function getFeat(I, SE)
2   SE = SE./sum(SE);
3   feat = convolute(I, SE);
4   return feat;
5 end.
```

The feature is evaluated by convolving a moving average filter with the SE neighborhood (obtained in line 2) with the image. The returned variable *feat* is a $1 \times N$ vector containing the average neighborhood intensity for all N pixels.

2.2 Comment on Implementation

PRAQA is splitting the 3D images into 2D stacks and is iterating through these in a 2D-wise manner. The reasoning behind this is twofold. First, due to image acquisition and corresponding optical constraints, the z resolution is typically lower compared to x and y directions making the use of 3D SEs not suitable. The second reason is that signals from different slices will vary in intensity caused by different penetration of antibody staining and optical constraints. This effect is proportional to the sample thickness. Hence, the definition of one global threshold for the standard intensity-value is not a suitable approach. However, since our algorithm is invariant with respect to the signal intensity, we can still adopt a one-threshold policy for the whole image with no prior slice-wise normal-

ization required.

Furthermore, we chose here circular shaped SEs since most of the protein localization do not occur in sharp edge shapes but rather as smooth edgeless objects. However, this choice may not be optimal when dealing in a poor 2D resolution scenario with proteins that come in a filamentous or ramified arrangement.

3 BENCHMARKING WITH SYNTHETIC SCENARIOS

To evaluate the performance of PRAQA, we benchmark and compare our method with alternative image processing pipelines in terms of accuracy, robustness and runtime. For this purpose, we synthetically generated 3D images that can resemble an ideal acquisition without noise or intensity changes, and keep those images as ground truth to benchmark the algorithms efficiency on the identical images with added noise and intensity changes. We generated these images by randomly placing two kinds of shapes, either spheres or spirals, of different sizes throughout the whole 3D image. The two different object shapes were chosen to have a simpler scenario with well defined contours (spheres) and a harder scenario that contains irregular thinner shapes (spirals). Small binary spheres are approximated and do not look perfectly round.

In analogy to real image data, the synthetic image dimension was set to 2048 by 2048 by 30 pixels. Table 1 summarizes the object sizes and quantities used to fill the images.

Table 1: Description of objects used in the synthetic images.

Sphere		Spiral	
Radius	Quantity	Size (x y z)	Quantity
10	100	25 25 6	100
15	50	45 45 8	50
20	30	70 70 10	15

Additional 3 slices were added at the top and the bottom of the image stack before randomly placing the objects to avoid having less positive pixels on the boundaries. Those additional slices were subsequently removed leading to some partly cut objects in analogy to experimental image acquisition.

Noise and intensity alteration were introduced at different strength to evaluate the resilience of the pipelines tested. We used Gaussian white noise ($\mu = 0, \sigma = 1$) multiplied by a scaling factor η and added to each pixel. Figure 1 and 2 show a zoom in of 110 by 110 pixels on the two scenarios for different values of η . We also simulated the **luminescence loss**

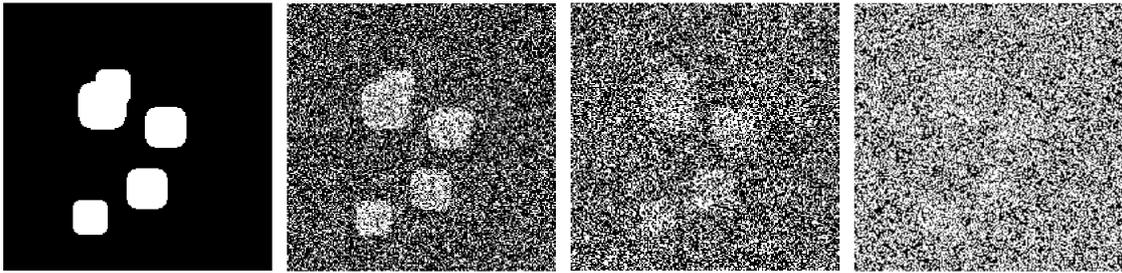


Figure 1: Zoom in for the sphere noise scenario with noise intensities $\eta = [0, 1, 2, 3]$ from left to right, respectively.

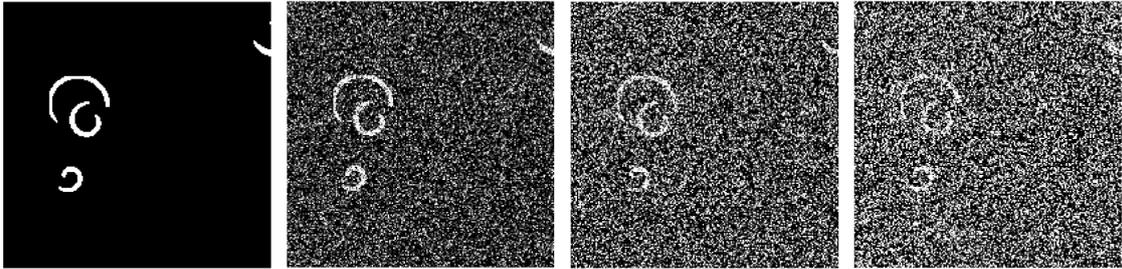


Figure 2: Zoom in for the spiral noise scenario with noise intensities $\eta = [0, 0.5, 0.75, 1]$ from left to right, respectively.

(LL) and **luminescence variability** (LV) throughout the z dimension by additionally reducing the intensity of each slice away from the middle of a factor t . In dependence to the experimental setting this is one likely scenario but in different settings the LV can have also an inverse character where the middle average intensity is lower than at the borders. Since PRAQA works in a 2D slice-specific mode, the direction of LL and LV does not affect the results.

We compare our algorithm with BM3D (Dabov et al., 2007), median filtering (Lim, 1990), non-local means filtering (Buades et al., 2005) and wavelet denoising (Donoho, 1995). We parameterized BM3D with $\sigma = 60$ without reporting benchmarks with lower values since this configuration always performed better than others. By contrast, we parameterized median filtering with different neighborhoods (5, 10 and 15 pixels) because in dependence on the input the different parameterization exhibited different performances. Since our algorithm directly outputs a binary image, we then used either a global threshold, Otsu method (Otsu, 1979) or Niblack thresholding (Niblack, 1985), for binarizing the results of the benchmark algorithms. All algorithms implementations are based on MATLAB and we used the native MATLAB implementations for all the competing algorithms except for BM3D for which the implementation of the original paper was used (Dabov et al., 2007).

In the following sections, we will refer to BM3D ($\sigma = 60$) as **B60**, to median filtering with the different neighborhood settings as **M5**, **M10** and **M15**, to non-

local means filtering as **NLF**, to wavelet denoising as **WAV** and to our algorithm as **PRA**.

3.1 Accuracy Benchmark

In this section we evaluate the algorithms performance by means of **Receiver Operating Characteristic** (ROC) curve and **Precision-Recall** (PR) curve (Saito and Rehmsmeier, 2015). In these first tests, we did not apply intensity changes ($t = 0$) and evaluated the average performance on slices in different noise conditions with $\eta = (1, 2, 3)$ for the sphere scenario and $\eta = (0.5, 0.75, 1)$ for the spiral scenario, respectively.

For the alternative approaches, the performance curves were obtained by changing the global intensity threshold after filtering. Since PRAQA directly returns a binarized image, the performance curve was obtained by changing θ . To avoid the use of a trained classifier which could unevenly bias the result, we kept only one feature of the 3 features obtained by circular SEs. In particular, we chose the largest SE for the sphere scenario and the smallest SE for the spiral scenario, respectively.

Table 3 and 2 report the **Area Under Curve** (AUC) for both the sphere and spherical scenario, respectively. Without intensity changes along the z dimension, BM3D performs best and in particular better than PRAQA. Still, PRAQA performs typically better than all the other algorithms and yields acceptable results in all cases except in the noisiest spiral scenario. The great performance of BM3D is based on

Table 2: Spiral scenario accuracy benchmark.

	$\eta = 1$		$\eta = 2$		$\eta = 3$	
	ROC	PR	ROC	PR	ROC	PR
PRA	.99	.99	.99	.94	.98	.77
B60	.99	.99	.99	.96	.99	.88
M5	.99	.88	.90	.28	.80	.10
M10	.99	.98	.98	.79	.93	.45
M15	.99	.98	.99	.90	.97	.71
NLF	.99	.98	.98	.78	.94	.44
WAV	.99	.97	.99	.84	.97	.61

Table 3: Sphere scenario accuracy benchmark.

	$\eta = 0.5$		$\eta = 0.75$		$\eta = 1$	
	ROC	PR	ROC	PR	ROC	PR
PRA	.99	.87	.99	.70	.97	.45
B60	.99	.91	.99	.80	.99	.68
M5	.99	.81	.97	.53	.94	.24
M10	.98	.55	.97	.43	.95	.28
M15	.98	.35	.97	.29	.94	.20
NLF	.99	.91	.98	.60	.95	.25
WAV	.97	.63	.93	.33	.88	.14

its specific focus on white Gaussian noise and its performance still remains a gold standard nowadays.

3.2 Performance with LV Benchmark

Next, we investigated the performance of the algorithms in a scenario with both noise and intensity changes. We fixed $\tau = 0.03$ and $\eta = 0.75$ in the spiral scenario and measured the performance with respect to **true positive rate (TPR)** and **true negative rate (TNR)**. The MAD_S threshold parameter θ was set to 3. Since using just one global threshold for the binarization for the competing algorithms would be unfair, we picked automatically a threshold for each slice by the Otsu method and Niblack thresholding. In Figure 3, the variability of TPR among slices for the different algorithms is shown. We empirically assess the robustness of PRAQA with respect to intensity changes among slices emphasized by the small TPR variance compared to the other methods. This result further demonstrates the great potential of PRAQA for images with high LV that is typically observed in tissue staining.

In Figures 4 and 5, we next investigated the performance of the algorithms by averaging TPR and TNR slice-wise for the different threshold approaches, respectively. These plots indicate that PRAQA robustly binarizes images with high accuracy since it exhibits high TPR and TNR while the alternative algorithms typically are more targeted for one criterion. Thus,

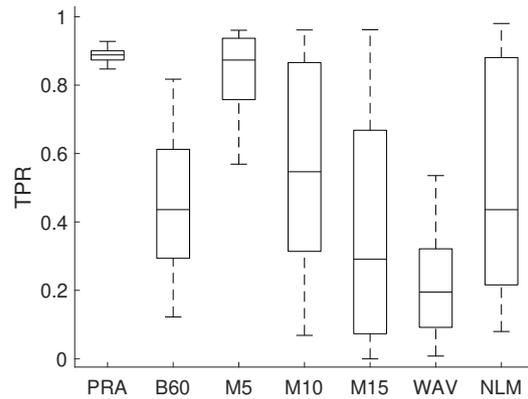


Figure 3: TPR Boxplot slicewise.

WAV and B60 exhibited a slightly higher TNR compared to PRAQA but performed significantly worse with respect to TPR using Otsu thresholding (Fig. 4). For Niblack thresholding, WAV resulted in a higher TPR than PRAQA but had the worst TNR performance of all algorithms (Fig. 5). Hence, the targeted approach of PRAQA represents a good compromise for binarization that significantly outperforms the other algorithms.

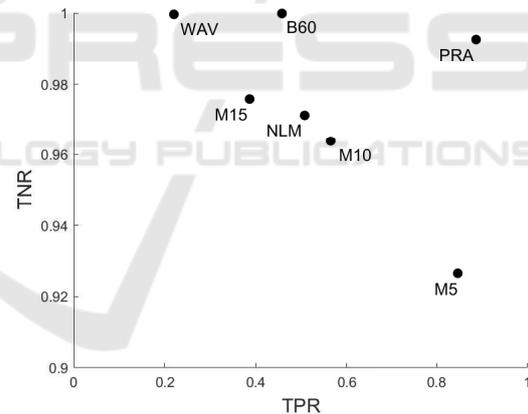


Figure 4: Performance Scatter Plot with Otsu thresholding.

3.3 Robustness against Positive Pixel Content

To investigate the performance of PRAQA in dependence on signal (i.e. object) density, we fixed the threshold parameter θ in the *binarizedMAD* routine (Section 2.1) and considered the spiral scenario with $\eta = [0.5, 0.75]$ and $\tau = 0.03$. We first investigated the dependency of MAD (Eq. 1) with respect to the number of objects and subsequently confirmed the results in the synthetic scenario. For this purpose, we increased the number of objects by adding a multiplying constant to the number of objects listed in Table 1.

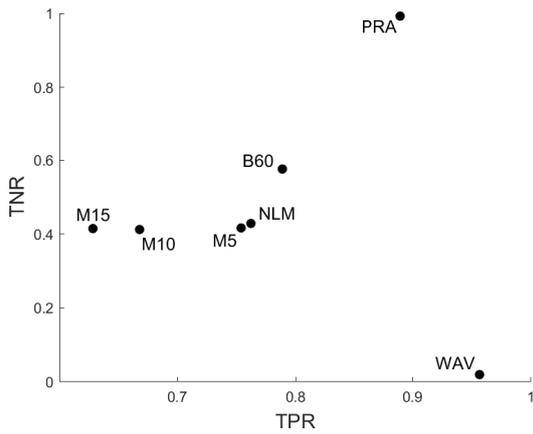


Figure 5: Performance Scatter Plot with Niblack thresholding.

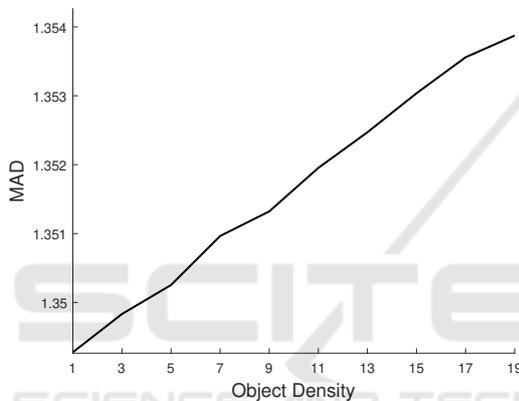


Figure 6: Variation of MAD with respect to object density.

In Figure 6, we observe a linear dependency between the amount of positive signal given by the object density and resulting MAD values. Thus, the MAD scaling factor used to obtain MAD_S as defined by Eq. 1 leads to changes in the algorithm positive pixels detection. Figure 7 depicts the change of TPR in a spiral scenario with a fixed $\theta = 3$ for an increase of objects increases by 10 and 20 times and two different noise intensities. Interestingly, we observed only a minor loss in TPR of 0.008 for a 10 fold change in the amount of positive signal when noise is low ($\eta = 0.5$) and 0.025 for larger noise ($\eta = 0.75$). Even for the higher density with a scaling factor of 20, the reduction in TPR is relatively low following the linear dependency and further demonstrates the robustness of PRAQA.

3.4 Runtime Benchmark

To investigate the applicability of the different algorithms also with respect to real-world applications, we benchmarked the time required to process 30 slices

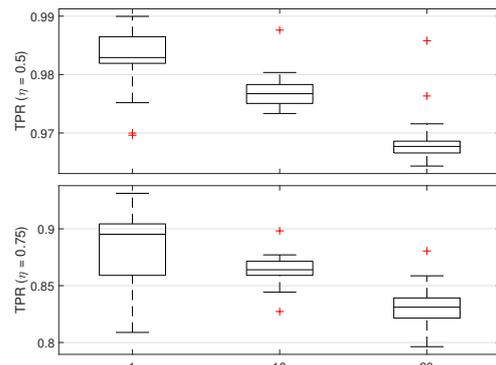


Figure 7: Boxplots of TPR performance with respect to object density ($\eta = 0.5$, bottom: $\eta = 0.75$).

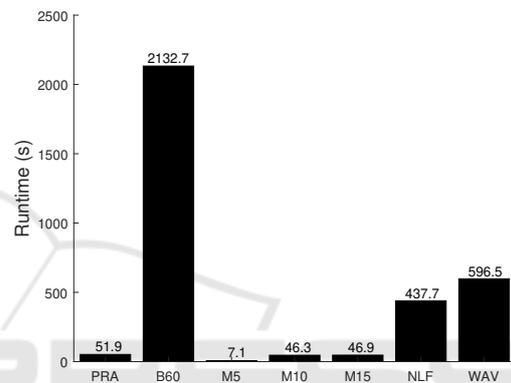


Figure 8: Runtime in seconds for each algorithm.

of size 2048 by 2048 pixels. The machine used for benchmarking was equipped with two Intel Xeon E5645 @2.40 Ghz and 96 GB RAM. Figure 8 depicts a bar plot for each tested algorithm demonstrating that PRAQA exhibits a great runtime performance. In particular, PRAQA performs significantly better compared with the second accurate algorithms WAV and BM3D (B60) with a 40 times faster performance than BM3D. The PRAQA algorithm execution time is independent with respect to the choice of θ but directly proportional to the number of SEs.

A GPU parallelized version of the algorithm has also been developed. However, most of the time needed for the processing is taken by the MAD evaluation, which is not a parallelizable operation when the exact MAD values are computed. A faster solution can be obtained by losing accuracy in the measurement via pixel intensity binning, which may make PRAQA be even suitable for real time applications. Overall, the extensive benchmarking of PRAQA has shown that its targeted approach for 3D fluorescent images with LV and LL outperforms alternative algorithms in terms of accuracy and runtime.

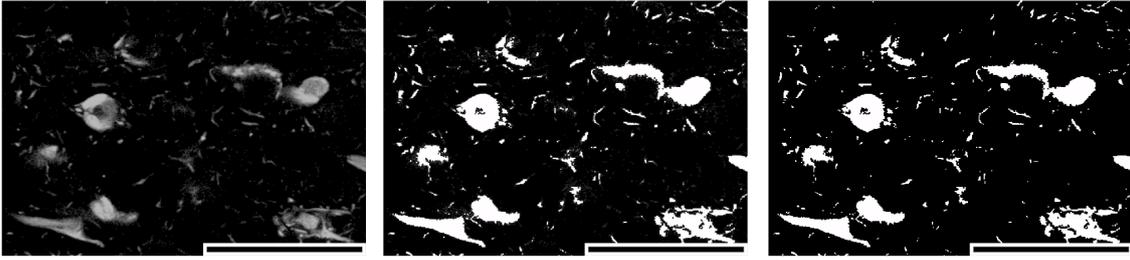


Figure 9: Hyperphosphorylated tau aggregation by AT8-staining acquisition in a region of interest. From left to right: original acquisition, original + PRAQA overlay, PRAQA. Scale bar: $60 \mu\text{m}$.

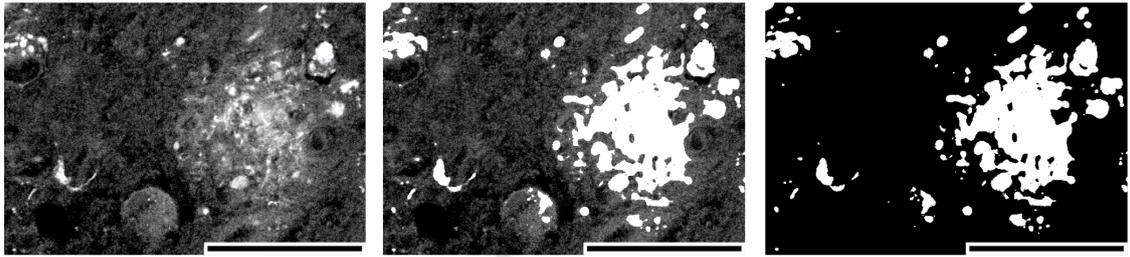


Figure 10: Amyloid-beta aggregation by 4G8-staining acquisition in a region of interest. From left to right: original acquisition, original + PRAQA overlay, PRAQA. Scale bar: $60 \mu\text{m}$.

4 REAL SCENARIO

Based on the successful benchmarking of PRAQA with the synthetic scenarios in Section 3, we next applied PRAQA to a real world application of neurodegeneration (Salamanca et al., 2019). A common feature of different neurodegenerative diseases such as Alzheimer’s or Parkinson’s disease is the accumulation of proteins either intracellular or extracellular (Ross and Poirier, 2004). While the function and impact of these aggregates is still not fully understood, they are essential for the diagnosis. Current biomedical research is focusing on the characteristics of these protein aggregates and their relation to different disease stages (Dugger and Dickson, 2017). For this purpose, a reliable quantification approach of protein aggregation in tissue is essential and PRAQA is exactly targeting this need.

Here, we demonstrate the potential of PRAQA in the context of **Alzheimer’s Disease (AD)** and **Dementia with Lewy Bodies (DLB)**. Both conditions share the accumulation of extracellular aggregation of amyloid-beta 1-42 and intracellular aggregation of hyperphosphorylated tau in the parenchyma but with a higher burden expected in AD samples. To characterize amyloid and tau aggregation in a cohort of human post-mortem brain samples (Tab. 4), we have stained our collection of samples with antibodies commonly used in neuropathology to visualize respective protein/peptide aggregation (Figs. 9 and 10),

acquired 3D stacks with a confocal microscope and performed relative protein abundance quantification with PRAQA (Figs. 11 and 12).

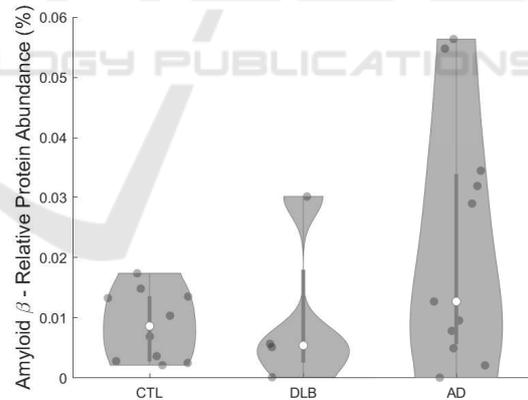


Figure 11: Amyloid peptide abundance per sample condition stained by 4G8.

4.1 Human Brain Sections Preparation and Data Acquisition

Anonymized human brain samples were provided by the Douglas-Bell Brain Bank (Douglas Mental Health University Institute, Montréal, QC, Canada) and all experiments were conducted in accordance with the guidelines approved by the Ethics Board of the Douglas-Bell Brain Bank and the Ethics Panel of

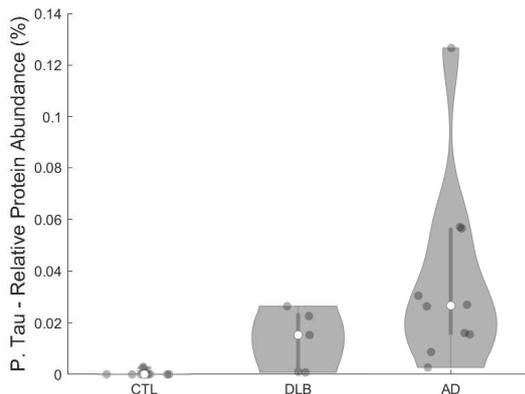


Figure 12: P-tau protein abundance per sample condition stained by AT8.

University of Luxembourg. For this study, cases of neuropathologically confirmed AD and DLB, as well as from age-matched **controls** (CTLs) were selected.

Immunohistochemistry protocol was performed as previously described (Bouvier et al., 2016) (Quesseur et al., 2019). Briefly, sections were immunostained with the primary antibody Mouse anti-4G8 (amyloid-beta) (Biolegend [800712]) or Mouse anti-AT8 (hyperphosphorylated tau) (ThermoFisher [MN1020]). Afterwards, sections were incubated with secondary antibodies Donkey anti-Mouse Alexa Fluor 488 (4G8) or 647 (AT8) (Jackson ImmunoResearch Laboratories, West Grove, PA and Invitrogen, Molecular Probes, Eugene, OR) and mounted. Fluorescent 3D confocal tile scans were captured and stitched on a confocal Zeiss Laser Scanning Microscope710 with a 20x air objective. Tile scans were acquired to cover a larger area of the CA1 subfield of the hippocampus. Identical image acquisition parameters were respectively used for each of the staining on all samples.

4.2 Dataset and Processing

Table 4 reports the number of acquisitions processed in this study grouped by condition.

Table 4: Number of samples per condition and marker.

	Amyloid β	Tau
CTL	11	12
AD	13	12
DLB	4	5

To compute the positive pixel sum coming from the binarized image processed by PRAQA, we used a parameterization of $\theta = 5$ for all images except for three CTL samples with 4G8 staining for which we used $\theta = (8, 8, 10)$ to counteract a higher presence of

noise. Each sample had on average 25 slices separated by $1 \mu\text{m}$ in the third dimension. For some samples, beginning and/or ending slices had to be removed due to tile-related artifacts occurred during the microscope acquisition. For final analysis, the positive pixel sum was normalized to the 3D image size.

4.3 Results

Figures 9 and 10 show a region of interest (ROI) of AT8 and 4G8 stained structures in post-mortem AD brains. The ROI is the CA1 subfield of the hippocampus, a brain region involved in memory consolidation and retrieval and known to be severely affected by AD. AT8 staining reveals intraneuronal fibrillar structures called neurofibrillary tangles (Goedert et al., 1995) and 4G8 staining shows accumulations of the amyloid-beta peptide in intracellular inclusions and extracellular plaques (Hunter and Brayne, 2017). These protein accumulations are considered typical neuropathological hallmarks of Alzheimer’s Disease and are clinically used to assess disease staging. All structural details of both stainings are preserved after PRAQA processing (Figure 9) and the presence of noise is counteracted in the example of the 4G8 stained sample (Figure 10).

The comparison between the original acquisitions and the by PRAQA binarized images demonstrates that PRAQA is capable to segment the qualitatively different protein aggregations successfully as validated by visually inspection of the overlay of the original images and the PRAQA output by a neuropathology expert. For the hyperphosphorylated tau staining, the original image does not exhibit strong background signal and PRAQA resolved also subtle structures (Fig. 9). The original amyloid-beta staining has a stronger background signal and less well-defined structures. In this scenario, PRAQA did not resolve all potential substructures but successfully classified positive pixels that represent the amyloid-beta plaques (Fig. 10).

As a demonstration how PRAQA can be used for disease classification, Figures 11 and 12 show the relative protein abundance in dependence on the sample condition. The analysis of amyloid-beta peptide abundance within the CA1 hippocampal subfield shows in agreement with literature an enrichment overall of amyloid-beta level in AD samples (Marti Colom-Cadena, 2013). Interestingly, a lower and residual amyloid-beta presence is also revealed in age-matched controls as well as in samples from patients with DLB. The relative abundance is here comparable to AD samples with low Amyloid burden (Marti Colom-Cadena, 2013). This finding may indicate a general age-dependent increase of amyloid-

beta aggregation even in cognitively healthy controls which is also coherent with previous findings (Rodríguez, 2012).

The comparison of hyperphosphorylated tau aggregation among samples exhibits an even stronger disease dependence (Fig. 12). While CTL samples were consistently negative for tau aggregation, AD samples exhibited a very strong enrichment to up of 0.13%. Strikingly, some DLB samples also showed considerable amounts of phospho-tau proteins compared to the age-matched control samples with levels similar to AD condition (Marti Colom-Cadena, 2013).

Overall, this first application of PRAQA to human brain samples demonstrated that the targeted approach allows for quantification of protein aggregation within heterogeneous tissue. Furthermore, PRAQA analysis of amyloid-beta and hyperphosphorylated tau distribution in CA1 subregions of AD and DLB cases confirms that the two conditions share common mechanisms of protein aggregation and may represent a more continuous spectrum of cellular pathologies than typically anticipated.

5 CONCLUSIONS

Protein function is essential for cellular and tissue homeostasis and impairments are linked to diverse diseases. Here, we introduced PRAQA as an efficient tool to quantify relative protein abundance and localization in complex tissues from 3D fluorescent images. For this purpose, PRAQA specifically targets luminescence variability caused by optical constraints along the z dimension and by inter-sample variability by applying a Median Absolute Deviation approach for each 2D (x - y) plane of the image (Section 2).

Extensive benchmarking of PRAQA against alternative algorithms for synthetic scenarios has demonstrated that the targeted approach allows for accurate, robust and efficient quantification of relative protein abundance in cases where noise and intensity changes play an important role in the measurement (Section 3). In particular, we have shown that PRAQA performs well when dealing with images compromised by intensity changes (either intra-acquisition or inter-acquisition) and is also resilient up to a considerable amount of noise. The key advantages of this algorithm are its simple parameterization (1-parameter), computational efficiency and direct applicability that does not require any preprocessing. These features prevent typical biases of alternative approaches based on versatile parameters which may compromise measurements and thus the results.

However, in cases of extreme noise, dedicated multi-parametric pipelines might be used to deal with these peculiar situations but care has to be taken for sample comparison.

Finally, we have used PRAQA for a real application in neurodegeneration and quantified protein aggregation in human brain samples (Section 4). In this application, we quantified amyloid-beta and hyperphosphorylated tau protein aggregation in immunostained brain samples of AD, DLB and control subjects where PRAQA was able to segment reliably the qualitatively different structures. The first comparison of protein aggregation between the disease conditions substantiates the essential role of amyloid-beta plaques in AD but also indicates some shared properties of protein aggregation in neurodegenerative diseases.

Overall, the targeted approach of PRAQA represents an efficient and easy-to-use tool to investigate spatially resolved protein abundance in health and disease conditions.

5.1 Future Work

We plan to extend the algorithm by an additional module to estimate an optimal range for the threshold parameter θ . This automation may be achieved by statistically inferring when false positive pixels start to appear in a *salt and pepper* manner throughout the acquisition by progressively lowering θ . Furthermore, we will test PRAQA with other staining markers leading to different structures and potentially adapted SEs for ad-hoc situations (e.g. filaments or branches) also in the context of the neurodegenerative samples. In this respect, PRAQA will be also a valuable extension to combine morphological characterizations (Salamanca et al., 2019) with protein abundances for investigations of multicellular mechanisms in neurodegeneration.

CONFLICT OF INTEREST

The authors declare no competing financial interests.

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