INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a relentlessly progressive neurodegenerative disease, which remains incurable due to an incomplete understanding of the underlying molecular pathogenesis. Using the VCP mutant mouse transgenic model of ALS and human post-mortem tissue from sporadic ALS (sALS) patients, we previously showed that spinal motor neurons (MNs) exhibit reduced nuclear to cytoplasmic (N/C) ratios of FUS (Fused in Sarcoma) and SFPQ (Splicing factor Proline and Glutamine rich) proteins. Furthermore, we showed that SFPQ is also mislocalized in SOD1-mutant ALS models, while FUS is not (1,2).

Although these studies revealed novel molecular hallmarks of ALS, they also present important limitations shared by the majority of such traditional neuropathological studies. Firstly, key observations are based on manual
image processing, which is time-consuming (therefore not scalable to large studies) and also introduces potential operator-dependent variability. Secondly, although hundreds of measurements can be extracted from such rich images, previous studies have typically focused on the single measurement deemed to be the most relevant. Furthermore, such analyses are usually based on averaging the signal(s) among all cells originating from identical animals or individuals. However, the degree to which individual MNs follow population-averaged trends in protein mislocalization remains unresolved. Indeed important insights into biological processes such as cellular differentiation and disease progression originate from the characterisation of heterogeneous cell populations coexisting within the same condition (3–8). These studies have been very effective when applied to assays of cultured cells and for cancer subtype classification, however there is not, to our knowledge, an analogous study on tissue sections from the nervous system (9,10). Finally these studies do not provide disease scoring at the cellular or even individual or animal level, which would be advantageous for understanding single cell phenotypic heterogeneity within a disease and, in the longer term, for developing superior patient-specific diagnostic criteria.

Recent developments using deep learning methods have enabled segmentation and object detection from complex imaging data-sets, with exciting prospects within ALS translational research (11,12). Here, by expanding on these methods, we developed a pipeline for image processing of MNs from spinal cord sections, greatly improving efficiency, whilst providing an unbiased approach to data acquisition. We then extracted hundreds of cell measurements and compared different machine learning methods to automatically identify MNs subpopulations from high-content imaging data. Using this approach we reveal that VCP- and SOD1-mutant MNs share phenotypes captured by several related morphological descriptors including Zernike moments, and we validate this finding in human ALS post-mortem tissues. By providing our fluorescence microscopy raw images together with open-source implementations of the methods, we aim to allow others to readily apply these methods in other biological contexts in order to increase the analytical power of tissue section analysis. We propose that such unbiased approaches may substantially deepen our understanding of ALS and other diseases in which we routinely use histopathological analysis.

2 | RESULTS

2.1 | Automated phenotyping of spinal cord MNs from transgenic ALS mouse models

Large variability in phenotype between transgenic ALS mouse models is well described in the literature (13). We previously showed that VCP- and SOD1-mutant ALS mouse models exhibit distinct pathological phenotypes in terms of FUS and/or SFPQ mislocalization (1,2). In these studies, we analysed spinal cord sections from control, SOD1- and VCP-mutant mouse models immuno-labeled for FUS and SFPQ, where nuclear and cytoplasmic compartments were manually identified with DAPI and ChAT (1,2). Using these same images (Table S1), we first aimed to test whether spinal cord MNs from these ALS mouse models exhibit common cellular phenotypes which were not captured by our previous analyses. In order to achieve this, we moved beyond single-protein localisation analysis at the population average level to comprehensive single cell analyses by developing a pipeline for automated image segmentation and morphological profiling combining the open-source Ilastik and CellProfiler softwares (11,14) (Figure 1A and Figure S1A). This allowed for the rapid, automated identification of distinct cellular compartments, and extraction of 750 morphological features for every MN identified (n = 121), thereby greatly improving processing speed and throughput compared to manual processing. Features included fluorescent intensity data and signal distribution (for FUS, SFPQ, ChAT and DAPI), shape and morphometric descriptors (size, shape, perimeter and texture of subcellular structures including the nucleus and cytoplasm).

We next examined whether phenotypic states either associated with ALS-mutant cells or control cells are accompanied by reproducible changes in morphological descriptors. Here we hypothesise that information related to the disease state is self-contained in high-content microscopy data obtained from nuclear and cytoplasmic compartments, thus providing a high-resolution catalogue of specific features of cellular state. Unsupervised hierarchical clustering (Euclidean distance and average clustering) of the 121 MNs using the 750 measured morphological features segregated cells based on the presence or absence of the VCP-mutation rather than segregating SOD1- and VCP-mutant cells together (Figure 1B). This suggests that the ALS phenotype associated with the VCP mutation, hereafter referred to as vcpALS phenotype, dominates a common ALS phenotype shared between SOD1 and VCP mutant MNs, hereafter referred to as comALS phenotype. Hierarchical clustering is, however, often dominated by a single morphological profile and thus can fail to reveal subtle but relevant features in the data (15). In contrast to this, singular value decomposition (SVD) permits deconvolution and ranking of orthogonal morphological profiles (16). Indeed each principal component obtained from the SVD analysis is the weighted sum of all ~800 available microscopy measurements, which capture -in the case of this study- a characteristic cell phenotype. Here we find that the information contained in the data is well distributed among the 121 components derived from SVD analysis (Shannon Entropy = 0.649), and that the
first 31 components capture 90% of the variance in the data (Figure S2). Using linear mixed modelling we find significant association between principal component (PC) 7 (3% of variance) and PC28 (0.4% of variance) with comALS phenotype (SOD1 and VCP mutation), while PC2 (14% of variance) is strictly associated with vcpALS phenotype (Figure 1C). While this analysis confirms the dominance of morphological changes associated with the VCP mutation, as further visualised in the scatter plots (Figure 1D–F), it indicates that SOD1- and VCP-mutant cells together exhibit subtle and consistent morphological characteristics likely to reflect common ALS attributes and that are captured by the aforementioned 800 measurements (Figure 1G,H).

### 2.2 Machine learning methods can identify common phenotypes between SOD1 and VCP mutant ALS mouse models

Having established that high-content MNs profiling data from fluorescence microscopy can capture subtle but consistent phenotypes across VCP and SOD1 genetic
backgrounds, we next aimed to train and compare different machine learning methods to automatically identify MNs exhibiting the *comALS* phenotype in spinal cord sections. Visual inspection of the distribution of the principal components associated with either *comALS* (PC7 and PC28) or *vcpALS* (PC2) phenotype revealed that PC2 and PC28, but not PC7, exhibit bimodal distributions (Figure 2A–C). This observation suggests the presence of at least two distinct subpopulations among the 121 cells. Here we propose that MNs populations can be described in terms of a mixture of two phenotypically distinct subpopulations, namely “healthy” and “sick (diseased)” cells (hereafter referred to as *disMNs*).

Given that healthy cells can exist in spinal cord sections of VCP/SOD1 mutant mice, we selected the probabilistic label-independent Gaussian Mixture Model (GMM) classifier, and compared it with two label-dependent classifiers, namely Logistic Regression (LR) and Multi-Layer Perceptron (MLP). We tested GMM with the following combinations of principal components selected based on their association with either *comALS* or *vcpALS* phenotype: GMM-PC2, GMM-PC2,PC7, GMM-PC7,PC28, and GMM-PC28. Importantly, the a priori distinction between healthy and sick cells is only required for the label-dependent methods (LR and NN) and is based on tissue origin, for example MNs were labeled as sick when they originated from SOD1- or FUS-mutant mice, and healthy otherwise.

When considering accuracy in the ability of the classifiers to discriminate VCP- and SOD1-mutant cells from control cells, LR and MLP performed equally well (AUC$_{LR}$ = 0.96 and AUC$_{MLP}$ = 0.93), while GMM-derived methods resulted in lower scores overall (AUC$_{GMM-PC2}$ = 0.83 > AUC$_{GMM-PC2,PC7}$ = 0.79 > AUC$_{GMM-PC7,PC28}$ = 0.65 > AUC$_{GMM-P7,PC28}$ = 0.63; Figure 2B). AUC (Area Under the Curve) and other related scores are widely used to rank classifiers, however these need to be treated with caution in the current context given the existence of healthy cells in ALS-derived tissue sections (and indeed theoretical disMNs in control mice). We therefore assigned each animal a probability to be sick using the mean probability of their respective cells, as provided by each classifier, and tested whether the VCP and/or SOD1-mutant group exhibited significantly higher probability to be sick compared to the control group.

Comparison of the classifiers’ ability to discriminate control from ALS-mutant animals using disease probability is expected to better reflect the biological significance of these classifiers. Using this method we showed that only the VCP-mutant group exhibited significantly higher disease probability than the control group when probabilities were derived from either GMM-PC2 or GMM-PC2,PC7 ($P_{GMM-PC2|VCP}$ = 8.6e-03, $P_{GMM-PC2|SOD1}$ = 3.4e-01.

**FIGURE 2** Machine-learning based methods enable automated discrimination of VCP- and SOD1-mutant groups from control groups. (A–C) Density distributions of MNs contributions on PC2 (15% of variance), PC7 (3% of variance) and PC28 (0.4% of variance) respectively. (D) Performance analysis of each classifier in their ability to discriminate ALS-mutant cells from control cells using receiver operating characteristic (ROC) curves and areas under the curves (AUC). (E) The ability of each clustering algorithm to detect SOD-1 or VCP-mutation effect is assessed by comparing the disease probabilities of either the SOD1- or VCP-mutant groups with those of the control group. The disease probability of each animal is obtained by using the mean probabilities of its cells to be sick according to (1) GMM-PC2,PC7, (2) GMM-PC28, (3) Logistic regression or (4) Multilayer perceptron classifiers. Data shown as box plots in which the centre line is the median, limits are the interquartile range and whiskers are the minimum and maximum. Dots are the animal disease profile. p-values obtained from Welch’s t test
GMM-PC2,PC7|VCP = 9.9e-03, and \( P_{GMM-PC2,PC7|SOD1} = 9.6e-02 \); Figure 2E). Furthermore, GMM-PC28 resulted in a significant difference between control and SOD1-mutant groups, and to some extent between VCP-mutant and control groups (\( P_{GMM-PC28|VCP} = 1.1e-02 \), \( P_{GMM-PC28|SOD1} = 1.0e-02 \)). In summary, only GMM-PC28 enabled recovery of comALS effect, however at higher significance for SOD1 group. In contrast, both LR and MLP classifiers resulted in large significant differences between control and ALS mutant groups irrespective of their genetic backgrounds (\( P_{LR|VCP} = 1.4e-04 \), \( P_{MLP|VCP} = 1.2e-04 \), \( P_{LR|SOD1} = 2.4e-03 \) and \( P_{MLP|SOD1} = 3.3e-03 \)). These results suggest that PC2 and PC7 capture a cellular phenotype characteristic of vcpALS. Furthermore, MLP, LR, and to some extent GMM-PC28 are able to capture a unifying phenotype amongst VCP and SOD1 mice, i.e., comALS. In the following sections we focus on the three comALS classifiers, namely LR, MLP, GMM-PC28, and compare these with the vcpALS classifier GMM-PC2,PC7 to study their underlying differences.

### 2.3 LR classifier best captures the broader ALS phenotype at single-cell level

To describe the disease status of an animal or a tissue section, it is important to acknowledge that two cells (or indeed tissues/animals and patients) with similar disease probability can also exhibit different degrees of aberrant phenotype(s). Here we associated each MN with the following two metrics together forming a per-cell ‘disease profile’: (1) the disease probability \( P \) given the observed phenotype as outputted by the classifiers; (2) a ‘severity’ score \( S \) directly derived from \( P \) and expected to reflect on the possibility for a population of cells with identical \( P \) to exhibit different degrees of

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**Figure 3** Comparisons of the different classifiers for predicted percentage of sick cells, disease probability, and disease severity. (A) Comparison of the disease profile similarity among the ALS-mutant cells versus control cells as obtained from GMM-PC2-PC7 (blue rectangle), GMM-PC28 (orange rectangle), LR (green rectangle) or MLP (red rectangle) classifiers using unsupervised hierarchical clustering of the 121 cells. Grey circles = control MNs; magenta circles = SOD1-mutant MNs; green circles = VCP-mutant MNs. (B and C) Heatmap of the animal-level disease probabilities and disease severity scores as predicted by GMM-PC2-PC7 (blue bar), GMM-PC28 (orange bar), LR (green bar) or MLP (red bar) classifiers. The disease probability and disease scores of each animal are obtained by using the mean probabilities to be sick and severity scores respectively of its cells. The different classifiers are hierarchically clustered using the average algorithm on the Euclidean distance between the disease probabilities or disease severity scores across the 10 animals. (D) Bar plots displaying the animal-level percentage of sick cells as predicted by GMM-PC2-PC7, GMM-PC28, LR or MLP classifiers. Grey bars = control mice. Magenta bars = SOD1-mutant mice. Green bars = VCP-mutant mice.
disease phenotypes (see Materials and Methods, and Figure S3).

We next performed hierarchical clustering (Euclidean distance and average clustering) of the MNs using the 121 disease profiles to test the ability of each classifier to group cells into disease versus healthy groups. We found that the vcpALS classifier GMM-PC2_PC7 segregated cells in two groups according to the VCP genetic background (Figure 3A). As expected, some MNs derived from VCP-mutant mice cluster with cells derived from either control or SOD1-mutant group; these are potentially healthy cells present in mutant mice reflecting the cellular heterogeneity in tissue. GMM-PC28 classifier segregated MNs in two genetically heterogeneous groups, the smaller group being exclusively composed of cells derived from ALS-mutant mice (4 VCP-mutant cells and 2 SOD1 mutant cells), and therefore called comALS group. LR and MLP similarly clusters cells in three large groups, one group almost exclusively composed of cells derived from control mice (and some SOD1-mutant cells), called the healthy group, one group almost exclusively composed of VCP-mutant cells, called the vcpALS group, and the third group being a mixture of SOD1- and VCP-mutant cells, i.e., the comALS group. This analysis confirms that LR, MLP, and to some extent GMM-PC28, capture the comALS phenotype. This is distinct from the vcpALS classifier GMM-PC2_PC7 that discriminates VCP-mutant cells from the control and SOD1-mutant cells. Notably, using LR we can clearly separate the control group from the vcpALS/comALS groups with the highest degree of confidence (i.e., separation between the groups), whilst MLP groups cells in three clusters, healthy, vcpALS and comALS. This indicates that the MLP classifier first captures vcpALS phenotype whilst LR, and to some extent GMM-PC28, first capture the comALS phenotype.

From the two per-cell disease score metrics, we next derived for each animal (1) the animal probability to be sick, (2) the animal disease severity, and (3) the animal percentage of disMNs, and used them to quantify the similarity between the four classifiers GMM-PC2_PC7, GMM-PC28, LR, and MLP. Using hierarchical clustering based on the per-animal probability to be sick further confirmed the largest differences between the vcpALS classifier GMM-PC2_PC7 and the three comALS classifiers GMM-PC28, LR, MLP are similar (Figure 3B). When the per-animal disease severity was used to compare the classifiers however, MLP was the most divergent classifier, exhibiting large severity for all ALS mutant mice irrespective of their genetic background (Figure 3B). This indicates that although MLP predicts large differences in disease profiles between vcpALS and comALS groups of cells, when looking at the per-animal severity, both VCP- and SOD1-mutant animals exhibit large severity.

Finally we looked at the predicted percentage of disMNs obtained by each classifier and found that the vcpALS classifier GMM-PC2_PC7 predicted disMNs only in VCP-mutant animals (Figure 3C), while the three comALS classifiers (LR, MLP and GMM-PC28) predicted disMNs in ALS-mutant animals (Figure 3A). Notably GMM-PC28 predicted disMNs across all animals although with higher percentages in SOD1-mutant mice and VCP-mutant mice.

In summary, this analysis reveals that MLP, LR and to some extent GMM-PC28 deliver similar results in terms of their ability to discriminate ALS-mutant mice from control animals (similar per-animal scoring metrics). However, for individual cell scoring metrics, LR is the classifier which best captures measurements associated with comALS phenotype rather than dominant vcpALS phenotype, as evidenced by the large component driven by the VCP mutation in MLP classification.

### 2.4 Morphometric descriptors are top classifiers for ALS phenotypes

The finding that LR and MLP best capture comALS cell phenotype prompted us to study what combinations of measurements actually carry the relevant information for ALS disease. We therefore looked into which measurements contribute most to the vcpALS versus comALS classifiers. In order to exclude the possibility that divergence in the top contributors between the vcpALS versus comALS classifiers could stem from the different mathematical foundations (GMM, LR and MLP), we trained two additional classifiers (one LR and one MLP hereafter called sLR and sMLP) with a subset of the data composed of control and VCP-mutant cells only, to specifically learn the vcpALS phenotype using these methods (Figure S4A,B). Hierarchical clustering of the classifiers using the predicted disease probability scores across the 121 MNs confirmed the similarity between GMM-PC2_PC7, sLR and sMLP in identifying vcpALS phenotype, compared with MLP, LR and GMM-PC28 in identifying comALS phenotype (Figure S4C). Remarkably, although the comALS or vcpALS classifiers arise from diverse mathematical foundations, the relative contribution of the 750 measurements are very similar among these two groups of classifiers (Figure 4A). This result indicates that the cell measurements which lead to the grouping in comALS or vcpALS groups are robust enough to be independent of the modeling approach.

We next analysed how each of the three categories of measurements, namely area-shape, texture and intensity, contributed to these classifiers using LMM (see Material and Method). We found that area-shape related measurements exhibited higher (although non significant) relative contribution to the comALS classifiers, while texture- and intensity-related measurements contributed more significantly to the vcpALS classifiers (Figure 4B). Extraction of the five top contributors to either LR or
sLR further revealed that the area-shape-related Zernike moments are indeed in the top five contributors of the comALS but not vcpALS classifiers (Figure 4C), as further confirmed in MLP versus sMLP top five contributors (Figure S4D).

We previously showed that MNs from VCP-mutant mice exhibit reduced N/C ratios of FUS and SFPQ proteins and that SFPQ is also mislocalized in SOD1-mutant ALS models, while FUS is not (1,2). Specifically looking at the relative contribution of SFPQ and FUS...
The Zernike moments capture aberrant cell behaviour in human ALS pathological post-mortem tissues. (A) Workflow for generating morphological profiles of MNs in ALS pathological spinal cord sections. Spinal cord sections from healthy donors and sporadic ALS patients were co-stained against ChAT, DAPI, SFPQ or FUS and then imaged (see Table S2 for metadata details). A combination of Ilastik (11) and Cell Profiler (14) enables automatic identification and feature extraction from cytoplasmic and nuclear compartments. (B) Unsupervised hierarchical clustering using 800 scaled measurements of the 66 cells co-immunolabeled with SFPQ or of the 366 cells co-immunolabeled with FUS do not show evidence for sALS phenotype. Grey bars = control MNs; green bars = sALS MNs. Euclidean distance and average clustering method. (C and D) The ability for LR and MLP clustering algorithms to detect the sALS effect in either SFPQ-stained data (C) or FUS-stained data (D) is assessed by comparing the disease probabilities of either the sALS group with those of the control group. The disease probability of each individual is obtained by using the mean probabilities of their cells to be sick according to (1) LR or (2) MLP classifiers. Data shown as box plots in which the centre line is the median, limits are the interquartile range and whiskers are the minimum and maximum. Dots are the individual disease profile. (E) Barplots showing SFPQ (left) and FUS (right) N/C ratios relative contribution to the different classifiers or PCs when applied. (F) Boxplot showing the SFPQ nuclear/cytoplasmic (N/C) ratio per cell from three healthy donors and three sALS patients either grouped according to the patient-based labeling (left) or grouped according to the LR classifiers (right). Grey dots = control MNs; green dots = sALS-derived MNs. p-value obtained using Welch’s t test. (G) Boxplot showing the FUS nuclear/cytoplasmic (N/C) ratio per cell from eight healthy donors and ten sALS patients either grouped according to the patient-based labeling (left) or grouped according to the LR classifiers (right). Grey dots = control MNs; green dots = sALS-derived MNs. p-value obtained using Welch’s t test. (H and I) Boxplots showing the distributions of the relative contributions of area-shape (light grey), texture (grey) and intensity (dark grey) related measurements in LR and MLP classifiers either using SFPQ data (H) or FUS data (I). Z-scores obtained by a permutation test to assess the significance of the measurement category to each classifier are shown above each boxplot. Data shown as box plots in which the centre line is the median, limits are the interquartile range and whiskers are the minimum and maximum. (J) Barplots showing the relative contribution of the top five measurements in LR in either SFPQ or FUS data. Zernike moments either in the nucleus or the whole MNs contribute largely to both LR classifiers. Bars are color-coded according to the ranking in contribution for a given classifier, from dark blue to white for high to low ranking.
N/C ratios' to the classifiers showed a stronger impact on the vcpALS classifiers compared to the comALS classifiers (Figure 4D and Figure S4E,F). This corroborates our previous findings that showed increased protein mislocalization in VCP-mutant compared to SOD1-mutant ALS mouse models (1,2). Visual inspection of randomly chosen tissue sections from example animals further confirmed that aberrant SFPQ protein mislocalization in VCP mutant MNs can easily be captured by eye and that SOD1- and VCP- mutant cells share features which are more challenging to pick up by inspection, hence reinforcing the importance of machine learning methods which can identify subtle changes from high-content data (Figure 4E).

2.5 | Morphometric descriptors also capture phenotypes in human tissue sections from sporadic ALS cases

We next sought to validate our findings in human post-mortem tissue (PMT) from ALS patients. We previously analysed spinal cord sections from healthy and sporadic ALS (sALS) PMTs, immuno-labeled for either FUS or SFPQ, where nuclear and cytoplasmic compartments were manually identified with DAPI and ChAT (1,2). Using these same images (Tables S2 and S3) and a combination of Ilastic and CellProfiler (see Materials and Methods and Figure S1B), we automatically identified 432 MNs (66 stained for SFPQ and 366 stained for FUS) from which we extracted 850 morphological features (Figure 5A). Unsupervised hierarchical clustering (euclidean distance and average clustering) using the morphological profiles across either the 66 MNs stained for SFPQ or the 366 MNs stained for FUS did not lead to clear segregation of the cells based on disease status of the human samples (Figure 5B), suggesting that ALS phenotype is more subtle in human PMT compared to ALS mouse models. Nevertheless, SVD analysis of either the SFPQ data or FUS data, both rich-content data as shown by the high Shannon Entropies (Figure S5A), confirmed the presence of disease-related phenotype with the association of three principal components in either SFPQ or FUS data with sALS (Figure S5B).

We next trained a combination of GMM with sALS related components, as well as LR and MLP for sALS automated identification. While the different methods showed very similar accuracy for the SFPQ data (Figure S5C), LR and MLP led to the highest accuracy for FUS data (Figure S5D). This would suggest that the sALS phenotype is supported by weighted linear combinations of all measurements in FUS data and that a restricted list of morphological profiles captured by two principal components is sufficient to capture cellular characteristics associated with sALS in SFPQ data. Further comparison of the abilities of each classifier to discriminate control from sALS groups using the per-individual probability to be sick confirmed our prior finding in mice that LR and MLP are the most efficient classifiers to capture sALS phenotype in high-content imaging data (Figure 5C,D and Figure S5E,F). We next looked at the relative contribution of either SFPQ or FUS N/C ratio in these classifiers. We found that SFPQ localisation has comparatively more weight than FUS in discriminating sALS from control cells in LR and ML (Figure 5E). This result is in line with our previous findings showing that, while both SFPQ and FUS proteins exhibit significant mislocalization in ALS, the extent of mislocalization is larger for SFPQ than for FUS (1,2).

We next sought to assess the effect of MNs re-labelling on the significance of SFPQ and FUS protein mislocalization. We therefore performed Welch’s t-test comparing the protein N/C ratios between two groups, either using the patient-based labels or the healthy versus disMN labeling derived from the classifiers. Remarkably LR-based re-labeling decreased p-values in both data-sets, confirming the increase in signal-to-noise ratios upon re-classification of the cells (Figure 5F,G).

Using a permutation test, we next tested the relationship between the relative contribution of each category of measurements (area shape, texture and intensity) in the classifiers that best discriminate sALS from control cells, namely LR and MLP. We found that area-shape related measurements significantly contribute to these models in both SFPQ and FUS data (Figure 5H,I). Further looking at the five top relative contributors in LR in either data-sets revealed that area-shape-related Zernike moments are again among the top five measurements that contribute to the LR classifier in both SFPQ and FUS data (Figure 5J), as well as in MLP classifiers (Figure S5G). Cumulatively, these findings suggest that common cellular disease phenotypes occur in human and mouse ALS models and are captured by the Zernike moments.

3 | DISCUSSION

3.1 | Novel pipeline for rapid processing and automated analysis of pathological sections

Our previous studies and those of others have relied on manual segmentation of pathological sections followed by analysis of single measurements. Here we developed a pipeline that couples fluorescence microscopy with highly quantifiable, automated and reproducible morphological profiling of MNs to allow fast and unbiased recovery of single-cell morphology profiles. By applying SVD analysis to the hundreds of cellular measurements derived from this platform we uncovered independent complex cellular phenotypes that associate with ALS MN populations. By showing that ALS phenotypes naturally emerge from such
molecular mechanisms that include aberrant protein
regression (21,22).
Most studies related to ALS development report on
adaptation this method to analyse ALS astrocytes which
have been shown play a central role in the disease pro-
gression and MNs automated classification. Importantly given
the mapping of an image onto a set of complex orthogo-
nal Zernike polynomials (30) and have been proposed
to provide a complete basis function representation of
cell shape that preserves all the information about the
shape of the cell (31). These quantitative single cell shape
descriptors have been shown to be reliable indicators of
various cancers (28,32,33). Our study suggests that area-
shape measurements including Zernike moments may
provide a means to read out the phenotypic state of ALS
cells which cannot be easily captured by visual micro-
scopic examination, hence the advantage of combining
high-content microscopy data with machine learning
methods to identify ALS sick cells. An important future
avenue of this work will investigate the relationship be-
tween these area-shape measurements and intracellular
molecular phenotypes.

3.3 | Future perspectives

Our work provides a framework for future studies with
larger sample sizes, greater numbers of patients, and
higher numbers of markers to rigorously test questions
about the nature of heterogeneity between (i) cells within
a tissue and (ii) patients. We envision that the ability to
identify and interpret information contained in patterns
of cellular heterogeneity in pathological sections will
provide insights into physiology and disease that may
be missed by traditional population-averaged or small-
cell number studies. In particular, there are two specific
areas where we envisage this method to be particularly
informative for ALS, as well for other disorders. The
first one is the study of the disease spatio-temporal evo-
lation of the disease at the single cell level. In addition
to progression in its severity, ALS also spreads spatially
across the rostro-caudal axis (34). At present we do not
have good data on what is the progression of changes
that occur at the cell level, which could inform on the
molecular pathways involved in spreading across ana-
tomical regions. To address this we will be able to use
the pipeline we developed here for a future study of serial
sections along the spinal cord of individual animals to
investigate spatio-temporal progression at different dis-
ease stages, as well as comparing the same anatomical
regions of the spinal cord across the disease spectrum.

3.2 | Morphometric descriptors capture common cellular phenotypes across human and mouse ALS models

Most studies related to ALS development report on
molecular mechanisms that include aberrant protein
homeostasis (ER stress and autophagy) and/or changes
in RNA processing (23–26). Although cell morphol-
ogy is intimately related to the physiological state of
cells and intracellular mechanisms (27–29), there is not,
to our knowledge, a comprehensive description of cell
morphological changes in ALS. Here we show that area-
shape related measurements, such as Zernike moments,
are key discriminators of aberrant disease phenotypes
across human and mouse ALS models. We also show
that FUS and SFPQ N/C ratios have varying but lower
weights in ALS MNs classifiers compared to the cellular
characteristics described above. Zernike moments are
remarkably the re-classification of MNs into healthy and
sick cells using these label-independent classifiers, as op-
opposed to patient-dependent labeling, led to an increase in
signal-to-noise ratio for specific markers as well as an in-
crease in significance in protein mislocalization. We also
propose a combination of two single-cell disease scoring
metrics derived from these classifiers that will be instru-
mental for future investigations of disease development
using histopathological tissue sections.

Thus our study represents a significant advance in
ALS histopathology as it presents an integrated pipeline
for automatic identification and classification of MNs in
pathological sections from raw image processing to the
figures presented here. To facilitate the ready applica-
tion and future development of methods for automatic
identifications and scoring of ALS cells, we provide our
complete raw image data sets, as well as open-source im-
plementations of the various methods including the open-
source workflow to carry out the segmentation, profiling
and MNs automated classification. Importantly given
reasonable tissue and immunolabeling quality, and a
sufficient number of cells, our approach is theoretically
applicable to any type of cell irrespective of their size
or other characteristics. Given increasing recognition
of non-cell autonomous effects in ALS, we are currently
adapting this method to analyse ALS astrocytes which
have been shown play a central role in the disease pro-
gression (21,22).
This will offer a unique and crucial dataset on ALS progression. Another area where this method might be used, is in associating molecular phenotypes with cellular phenotypes at the single cell level. Specifically, by being able to reliably and automatically discriminate between cells that present a disease phenotype and cells that are still healthy (even within the same patient) we will be able to effectively create a framework for “digital molecular pathology” for ALS. Utilisation of these automated image analysis techniques can inform on which cells are (i) most likely to be affected at any given time in individual patients, and therefore, (ii) help to determine both the precise molecular pathomechanisms and potentially therapeutically treatable targets. Finally adapting this method to other relevant cell types may shed light on previously unrecognized glial phenotypes in ALS pathology.

ACKNOWLEDGMENTS
This work was supported by the Idiap Research Institute and by the Francis Crick Institute which receives its core funding from Cancer Research UK (FC010110), the UK Medical Research Council (FC010110), and the Wellcome Trust (FC010110). R.P. holds an MRC Senior Clinical Fellowship [MR/S006591/1]. A.S acknowledges the support of the Wellcome Trust [213949/Z/18/Z].

AUTHOR CONTRIBUTIONS

DATA AVAILABILITY STATEMENT
We provide raw images and complete source code (which is not a software but rather a compilation of R and python) to readily reproduce figures, tables, and other results that involve computation in order to facilitate the development and evaluation of additional profiling methods. We also provide the measurements of each of the ~600 cells whose origins are annotated. The raw images, metadata and single-cell measurements provided as comma-delimited files have been deposited Zenodo under the accession number 3985099, together with the image processing pipelines. The scripts for automated detection of MNS subpopulation can be freely accessed on Github in the following repository: https://github.com/RLuisier/ALSdisMN.

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 REFERENCES


Linear mixed effects analysis of the relationship between the type of classifiers (comALS versus vcpALS) and the relative contribution of the measurement categories to account for idiosyncratic variation due to classifiers. Data shown as box plots in which the centre line is the median, limits are the interquartile range and whiskers are the minimum and maximum. Green = vcpALS classifiers; grey = comALS classifiers

**Figure S5** (A) Fraction of explained variance captured by the first 24 and 40 principal components that captures 90% of the signal in SFPQ and FUS data respectively. (B) Barplots showing the association between principal components and ALS in SFPQ (left) and FUS (right) data. Linear mixed effects analysis of the relationship between ALS phenotype and each of the 24 and 40 first principal components to account for idiosyncratic variation due to individuals shows significant association of PC1, PC4 and PC7 and ALS in SFPQ data, and association between PC4, PC15 and PC17 with ALS in FUS data. (C, D) Performance analysis of each classifier in SFPQ data (C) or FUS data (D) in their ability to discriminate sALS MNs from healthy MNs using receiver operating characteristic (ROC) curves and area under the curves (AUC). (E, F) The ability for each clustering algorithm to detect sALS effect is assessed by comparing the disease probabilities of sALS group with those of the control group. The disease probability of each individual is obtained by using the mean probabilities of its cells to be sick according to individual classifiers in SFPQ data (B) or FUS data (F). Data shown as box plots in which the centre line is the median, limits are the interquartile range and whiskers are the minimum and maximum. Dots are the individual disease profile, *p*-values obtained from Welch’s t test. (G) Barplots showing the relative contribution of the top five measurements in MLP in either SFPQ or FUS post-mortem tissue data. Zernike moments either in the nucleus or the whole MNs contribute largely to both MLP classifiers. Bars are color-coded according to the ranking in contribution for the given classifier, from dark blue to white for high to low ranking.

**TABLE S1** List of images used for FUS and SFPQ cellular localisation in (1,2); mouse data

**TABLE S2** List of images used for FUS and SFPQ cellular localisation in (1,2); human data

**TABLE S3** Description of the donors from which PMTs were obtained

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**How to cite this article:** Hagemann C, Tyzack GE, Taha DM, et al. Automated and unbiased discrimination of ALS from control tissue at single cell resolution. *Brain Pathology*. 2021;00:e12937. [https://doi.org/10.1111/bpa.12937](https://doi.org/10.1111/bpa.12937)