Image-based deep learning reveals the responses of human motor neurons to stress and VCP-related ALS

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Abstract
Aims: Although morphological attributes of cells and their substructures are recognised readouts of physiological or pathophysiological states, these have been relatively understudied in amyotrophic lateral sclerosis (ALS) research.

Methods: In this study, we integrate multichannel fluorescence high-content microscopy data with deep learning imaging methods to reveal—directly from unsegmented images—novel neurite-associated morphological perturbations associated with (ALS-causing) VCP-mutant human motor neurons (MNs).

Results: Surprisingly, we reveal that previously unrecognised disease-relevant information is withheld in broadly used and often considered ‘generic’ biological markers of nuclei (DAPI) and neurons (β III-tubulin). Additionally, we identify changes within the information content of ALS-related RNA binding protein (RBP) immunofluorescence imaging that is captured in VCP-mutant MN cultures. Furthermore, by analysing MN cultures exposed to different extrinsic stressors, we show that heat stress recapitulates key aspects of ALS.

Conclusions: Our study therefore reveals disease-relevant information contained in a range of both generic and more specific fluorescent markers and establishes the use of image-based deep learning methods for rapid, automated and unbiased identification of biological hypotheses.

Keywords
amyotrophic lateral sclerosis, deep learning, human induced pluripotent stem cells, immunofluorescence, motor neurons

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a relentlessly progressive and incurable neurodegenerative disease characterised by the loss of motor neurons (MNs). Key hallmarks of the disease include the mislocalisation and accumulation of ubiquitously expressed RNA binding proteins (RBPs) from the nucleus to the cytoplasm including TAR DNA-binding protein of 43 kDa (TDP-43), Fused in Sarcoma (FUS) and Splicing factor Proline and Glutamine rich (SFPQ) proteins.1–4 What drives pathological mislocalisation and aggregation of RBPs in ALS remains unknown. However, alteration in liquid–liquid phase separation dynamics has been proposed to underlie this process.5–9 RBPs are highly dynamic and have been shown to undergo changes in localisation in response to various stressors.10–16 Notably, mitochondrial dysfunction and oxidative stress are recognised and robust phenotypes in ALS pathogenesis in vitro.17 The role of RBPs in ALS and cellular stress highlights that a diverse and complex interplay exists.

All authors contributed equally to this work.

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Cell shape and morphology are recognised readouts of a cell’s physiological state or phenotype.\(^\text{18}\) We previously reported common morphological descriptors that strongly discriminate sporadic ALS from control post-mortem tissue at single cell resolution,\(^\text{19}\) further indicating that key information related to cellular state might be contained in cell shape in ALS. Dystrophic neurites are a common pathological feature in ALS, and disrupted synaptic integrity has been shown in valosin-containing protein (VCP) mutant human induced pluripotent stem cell (iPSC) cultures of MNs.\(^\text{20}\) Taken together, these studies suggest that the neuronal processes (collectively termed neurites or the ‘neuritome’) may be a good cellular subcompartment to reveal ALS pathomechanisms. However, neurites are challenging to study both in tissue sections (as the arborisation of processes is not captured in tissue sections) and in vitro due to difficulty in accurate segmentation and association of neuronal processes with individual cells. Consequently, neuronal processes remain comparatively understudied in ALS, and it is still unknown how, and to what degree, the neuritome is affected, whether ALS-related stress insults modify this compartment or if cytoplasmic accumulation of RBPs in ALS MNs relates to other aberrant cellular phenotypes such as dystrophic neurites.

We previously generated a high-content imaging dataset of control and ALS-related VCP-mutant iPSC-derived MN cultures co-labelled with a combination of three fluorescent markers, specifically, (i) a nuclear-specific marker (DAPI), (ii) a neuron-specific marker of the neurites (β III-tubulin) and (iii) an antibody against one of five ALS-related RBPs: TDP-43, SPFQ, FUS, heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) or heterogeneous nuclear ribonucleoprotein K (hnRNPK).\(^\text{16}\) In our previous study, we specifically analysed the spatio-temporal responses of the aforementioned ALS-related RBPs to different stressors (oxidative, heat and osmotic). Here, we applied deep learning methods to this rich imaging dataset to test in an automated fashion: (1) Whether aberrant cellular morphological phenotypes, including neuronal processes, associate with ALS; (2) whether these morphological phenotypes correlate to aberrant ALS-related RBP phenotypes; and (3) whether extrinsic stress insults in control MN cultures can recapitulate ALS phenotypic changes. Deep learning models such as convolutional neural networks (CNNs) are now widely used to efficiently perform image classification and image segmentation.\(^\text{21–25}\) Such methods are able to analyse images without prior image segmentation, feature selection or human-directed training and automatically extract features from raw data, removing significant bias from this process. Importantly, CNN-based image classifier performance largely depends on whether sufficient information is contained in the provided set of images. DAPI and β III-tubulin capture complementary and non-overlapping information related to the nuclear shape and neuronal/neurite morphology, respectively. We hypothesised that comparing the performance of different classifiers trained with iterative combinations of fluorescent images can be used to identify which cellular compartment or specific RBP is most affected between any two given culture conditions. Additionally, we hypothesised that similar phenotypes between different MNs culture conditions can be quantified using the trained model predictions. We demonstrate the utility of this approach, which enables the discovery of novel phenotypes in ALS MN cultures and the identification of the relevant extrinsic stress condition that best approximates ALS pathogenesis. The advantage of our method is that it is highly versatile and can quickly guide the scientist towards the most promising hypothesis for further experimental validation. By providing our fluorescence microscopy raw images together with open-source implementations of the methods and trained models, we aim to allow other researchers to readily apply these methods and test additional hypotheses. In summary, we propose the use of deep learning methods to leverage the power of large image databases from ALS-related MN cultures to generate testable biological hypotheses automatically and rapidly, a method that could prove transformational in promoting innovative research directions, diagnostics and therapies.

**RESULTS**

**Repurposing image-based deep learning methods to test biological hypotheses**

We previously studied the spatio-temporal responses of ALS-related RBPs to different stressors in control vs ALS-related VCP-mutant iPSC-derived electrically immature MN cultures using image-based analysis (Figure 1A and Table S1).\(^\text{16}\) These MN cultures have been generated using our previously published protocol for the generation of highly enriched spinal cord MNs\(^\text{20}\) and have been further characterised by the presence of MN-specific markers choline acetyltransferase (ChAT) and SMI-32 (Figure S1A). These cultures were immunolabelled after 1 h of exposure to oxidative stress, heat stress and osmotic stress, along with recovery timepoints from heat stress (2 h) and osmotic stress (1, 2 and 6 h). A combination of three specific markers was used: a nuclear marker (DAPI), a neuronal marker allowing precise identification of neurites (β III-tubulin) and an antibody against one of the following RBPs: TDP-43, SPFQ, FUS, hnRNPA1 or hnRNPK. Using this approach, we generated a large-scale imaging dataset of 156,577 images, which is publicly available in the Image Data Resource (IDR) (Figure 1B–D). In our previous study, we focused on nuclear-to-cytoplasmic ratio measurements of the aforementioned RBPs. Here, we aimed to capitalise on the richness of information contained within this high-dimensional image dataset to
FIGURE 1 Overview of the high-dimensional immunofluorescent image dataset and paradigm to evaluate the relevance of markers in stress and ALS pathogenesis. (A) Experimental design for obtaining immunofluorescence microscopy images of motor neurons (MNs). Control (n = 3 cell lines) and VCP-mutant (n = 4 cell lines) induced pluripotent stem cells (iPSC)-derived MNs in different cellular stress (untreated, osmotic, heat and oxidative) and stress recovery (2 h after heat stress, 1, 2 and 6 h after osmotic stress) conditions were fluorescently labelled with DAPI, βIII-tubulin (BIII) and key ALS-linked RNA-binding proteins (RBPs) and then imaged, resulting in 156,577 images. (B,C) Total number of images in CTRL and ALS cell lines grouped by stress conditions: untreated (UT) in grey, oxidative (OX) in purple, heat (HS, 2-h recovery) in blue and osmotic (OSM, 1-h recovery, 2-h recovery and 6-h recovery) in yellow. (D) Representative images of nuclear marker DAPI, neuronal marker βIII-tubulin and ALS-linked RBPs in iPSC-derived motor neurons. Scale bars = 25 μm. (E) Fifty-two CNN-based classifiers have been trained in this study to discriminate (1) ALS from control MN cultures (isALS test), or untreated MN cultures from MN cultures exposed to (2) oxidative (isOXIDATIVE test), (3) heat (isHEAT test) and (4) osmotic stress (isOSMOTIC test) using 13 combinations of RGB images composed of different channels: Either a single channel was used (DAPI, BIII or RBP), either two channels (DAPI:BIII) or three channels (DAPI:BIII:RBP), and pitch-black images were assigned to the unused channels (Table S2). For each of the four tests (siALS, isOXIDATIVE, isHEAT and isOSMOTIC), the 13 classifiers’ performance as obtained from the area under the receiver operating characteristic curve (AUC) were extracted and compared to uncover the importance of those markers in discriminating two conditions (Table S3). Additionally, 13 model predictions for each of the 4 tests have been extracted for each MN culture (Tables S4–S7).
test whether different MN stressors (including extrinsic stressors and endogenous ALS-causing mutations in the VCP gene) are characterised by detectable phenotypes in cellular compartments and/or RBP fluorescent images. Specifically, we hypothesised that ALS-related phenotypic changes will be recapitulated by one of our aforementioned stress conditions. CNN-based classifiers are powerful deep learning models that can be trained to discriminate images from different conditions by identifying complex relationships between pixels. Here, we trained the following 52 CNNs-based classifiers to recognise cellular phenotypes associated with (i) ALS, (ii) oxidative stress, (iii) heat stress or (iv) osmotic stress using 13 different combinations of immunolabelled images, ranging from DAPI fluorescent images only to the combination of three channels, that is, DAPI, β III and an RBP (Figure 1E and Table S2): 13 classifiers trained to discriminate untreated ALS from untreated control MN cultures (hereafter called ALS classifiers), 13 classifiers trained to discriminate untreated control MN cultures from control MN cultures exposed to oxidative stress (hereafter called Ox classifiers), 13 classifiers trained to discriminate untreated control MN cultures from control MN cultures exposed to heat stress (hereafter called HS classifiers) and 13 classifiers trained to discriminate untreated control MN cultures from control MN cultures exposed to osmotic stress (hereafter called OSM classifiers). The CNN-based classifiers were obtained through transfer learning from MobileNetV2, which has been pre-trained using the ImageNet dataset. The performance of each classifier was evaluated using the total area under the receiver operating characteristic (ROC) curve (AUC). AUC was calculated using 10-fold cross-validation, training on 90% of the dataset, testing on the remaining 10% of the dataset and repeating with 10 different train/test combinations (Table S3). The 52 trained classifiers assigned class (e.g., ALS and stress) probabilities for all the ~10 views from each cell culture (control vs ALS; untreated vs stressed; different timepoints) that were then averaged to obtain a final per culture classification probability (Tables S4–S7 and Figure S1B).

Noting that the information content of images determines the performance of a CNN-based classifier to discriminate between conditions, we harnessed distinct fluorescent markers (DAPI, β III-tubulin and RBPs) to capture different cryptic attributes that reveal cellular state. Against this background, we propose that the performance of the 13 different classifiers trained to identify a specific MN culture condition can reveal the relevant cellular compartment or RBP. During training, a classifier learns to identify a phenotype associated with a specific MN culture condition (ALS vs control; stressed vs untreated). Therefore, we propose that, once trained, this classifier can be used to predict whether similar phenotypes are shared among different conditions. Consequently, we use image-based deep learning methods in two novel ways that are expected to greatly facilitate and accelerate the process of hypothesis testing in biology. In the following sections, we first validate our approach by recapitulating previous findings. We next specifically demonstrate the utility of this approach by testing the following hypotheses: (1) ALS-causing VCP mutations result in previously unrecognised phenotypes contained within the information content of DAPI and/or β III-tubulin fluorescence images alone; (2) addition of ALS-related RBPs immunofluorescence images will improve phenotype detection in an RBP-specific manner; and (3) conventional extrinsic stressors can recapitulate phenotypic aspects of ALS.

Post-stress recovery of RBP- and neuritome-related phenotypes are closely correlated

Cell shape and morphology are recognised readouts of cell state or phenotype. Here, we first sought to test whether oxidative, heat or osmotic stress are characterised by changes in cell shape. We analysed and compared the performance of CNN-based models trained to discriminate images of untreated control MN cultures from images of stress-treated control MN cultures either using the DAPI staining only (hereafter named stress|DAPI classifier), either the β III-tubulin immunolabelling only (hereafter named stress|BIII classifier) or the combination of two fluorescent markers (hereafter named stress|DAPI:BIII classifier). As shown in Figure 2A, stress|DAPI and stress|BIII classifiers outperform a random classifier (AUC_{DAPI} > 0.5 and AUC_{BIII} > 0.5) across all stress conditions, indicating that DAPI and β III-tubulin fluorescent images capture relevant information related to stressed MNs. This supports the hypothesis that MNs stressed with oxidative, osmotic or heat exhibit previously unrecognised phenotypic changes in both nuclear and neuritome compartments. Furthermore, comparing the performance of these classifiers revealed that the β III-tubulin immunolabelling consistently leads to a significantly higher performance across all three stress conditions, suggesting that the compartment most affected by all three stressors is the neuritome. Although we cannot rule out the possibility that the increase in model performance between stress|DAPI and stress|BIII is due to the larger surface occupied by the neuronal processes compared with the nuclei, the minor increase in model performances across the three stress conditions between stress|BIII and stress|DAPI:BIII supports the hypothesis that DAPI-stained images are not major contributors in these classifiers. The greater performance of osm|DAPI compared with ox|DAPI and heat|DAPI finally suggests larger nuclear-related changes upon osmotic stress compared with the other stress insults.

We previously showed that different stressors affect the localisation of ALS-associated RBPs in control MNs. Thus, we next aimed to test whether our image-based deep learning approach could shed light on the most relevant RBPs to each stress condition in order to replicate these previous findings. Several ALS-causing mutations occur in genes that encode RBPs, including TDP-43, FUS and hnRNPA1, which typically exhibit subcellular mislocalisation of RBPs. Indeed, TDP-43 is mislocalised from the nucleus to the cytoplasm in the vast majority of cases. More recently, we reported widespread SFQ and FUS mislocalisation in various ALS models. Furthermore, additional RBPs have been shown to be mislocalised in models of ALS, including hnRNPK, one of the most abundant hnRNPs. Examining the performance of the stress|RBP models to discriminate untreated MN cultures from those exposed to osmotic, heat or oxidative stress and comparing these with the performance of
stress\_DAPI models revealed significantly higher performances of all five stress\_RBP models compared with stress\_DAPI irrespective of the stress (Figure S2A,B). This result indicates that, although these RBPs mostly localise to the nucleus,\textsuperscript{16} their respective fluorescent images carry information beyond nuclear shape or texture as identified by DAPI. We also find that stress\_TDP43 exhibits the highest AUC across the three stressors. We next compared the performance of the stress\_DAPI:BIII\_RBP models with the stress\_DAPI:BIII models in each stress condition in order to test whether the integration of RBPs fluorescent images together with those of DAPI and β III-tubulin enables the identification of additional stress-related phenotypes. This analysis revealed that TDP-43 significantly increases the ability of the classifier to identify MN cultures under oxidative stress (Figure 2B).

While CNN-based models are not suited to specifically address the subcellular localisation of RBPs, our finding that TDP-43 images, in conjunction with nuclear and neurite fluorescent markers, enable relevant oxidative-stress-related phenotypic information to be captured suggests that TDP-43 exhibits changes in localisation upon oxidative stress. This result is consistent with our prior finding that TDP-43—but not the other four RBPs analysed—exhibits a reduction in nuclear-to-cytoplasmic ratio upon oxidative stress.\textsuperscript{16} We also find that all five stress\_DAPI:BIII\_RBP models perform significantly better than stress\_DAPI:BIII to discriminate untreated from heated MN cultures. Furthermore, we find that the most informative RBPs to heat stress are TDP-43, FUS and hnrRNPK. While in our previous study we detected significant reduction in nuclear-to-cytoplasmic ratio for TDP-43 and FUS upon

**FIGURE 2** Different kinetics of stress recovery related to distinct cellular changes captured by CNN-based classifiers. (A) Boxplots showing the distributions of model performances as evaluated using the AUC for classifiers trained using DAPI, β III-tubulin (BIII) and the combination of DAPI and β III-tubulin markers, respectively, to discriminate untreated from stressed MN cultures. Each classifier was submitted to 10-fold cross-validation in 5 different subsets of the data, resulting in 50 points per classifier. Boxplots display the five-number summary of median, lower and upper quartiles, minimum and maximum values. P-values obtained from a one-sided Mann-Whitney test. (B) Bar graphs representing the increase in performance as obtained from \(-\log_{10}(P\text{-values})\) of one-sided Mann-Whitney test comparing the AUCs from the stress\_DAPI:BIII classifier and the AUCs from individual stress\_DAPI:BIII\_RBP classifiers for oxidative, heat and osmotic stresses. (C) Effect size (mean ± standard errors) of heat\_DAPI-based and heat\_BIII-based classifier predictions of control MN cultures after 1 h of exposure to heat stress and 2 h of recovery from heat stress. Effect size of the treatment at each timepoint is obtained using linear mixed effects analysis accounting for idiosyncratic variations due to cell lines and experiment bias. (D) Effect size (mean ± standard errors) of osm\_DAPI (blue) and osm\_BIII (red) classifier predictions of control MN cultures 1 h after osmotic stress and 1, 2 and 6 h after recovery from osmotic stress. (E) Same as (C) for heat\_TDP43, heat\_FUS and heat\_SFPQ classifier predictions. Solid lines = control MN cultures. Dashed lines = VCP-mutant MN cultures. (F) Same as (D) for osm\_TDP43, osm\_FUS and osm\_SFPQ classifier predictions. Solid lines = control MN cultures. Dashed lines = VCP-mutant MN cultures.
heat stress, we can speculate that the present approach captures more subtle changes beyond the previously studied cellular relocalisation that could explain the detected relevance of hnRNPK to heat stress. Finally, while we previously found that all five RBPs exhibit nuclear-to-cytoplasmic relocalisation upon osmotic stress, here, we find that TDP-43 and SFPQ immunolabelling only contributes to significantly increase the stress]DAPI:BIII performance to identify MN cultures under osmotic stress. It is however important to note that osm]DAPI:BIII exhibits an AUC of ~1.0, implying that a significant improvement is difficult to achieve in this case and that, in the case of osmotic stress, this analysis may underestimate the contribution of the RBP immunolabelling. Altogether, these results indicate that the performance of a classifier is a reliable approach to prioritise which RBPs are most relevant to a specific cell culture condition.

Next, the extent of recovered cellular compartment- and RBP-related phenotypes after heat and osmotic stress were assessed using linear mixed effects analyses of the individual classifier predictions, accounting for idiosyncratic variations due to either individual cell lines or experiments. As shown in Figure 2C, 2 h after recovery from heat stress, the nuclear compartment has fully recovered, as predicted by heat]DAPI, while the neuritome compartment still exhibits some degree of aberrant phenotype, as predicted by heat]BIII. As opposed to heat stress, the nuclear compartment takes longer to recover after osmotic stress compared with the neuritome compartment; however, both compartments exhibit full recovery 6 h after treatment (Figure 2D). Next, looking at the RBP-related phenotypes, we find large heterogeneity in their predicted recovery pattern after both heat and osmotic stresses, with no complete recovery for any of the analysed RBPs 2 h after heat stress (Figure S2C,D) and long-term effects for several RBPs after osmotic stress (Figure S2E,F). In particular, we find that 2 h after heat stress, MN cultures still exhibit high heat]TDP-43 and heat]SFPQ model predictions and lower (albeit still elevated) heat]FUS model prediction (Figure 2E). The results indicate that the TDP-43- and SFPQ-related phenotypes are still present at this stage and that the FUS-related phenotype is only partially resolved, partly reflecting on our previous study, where we did not detect reconstitution of nuclear TDP-43 and FUS to basal levels following 2 h of recovery from heat stress.16 Our previous study also revealed slower nuclear relocalisation dynamics for TDP-43 and FUS after osmotic stress, with FUS exhibiting exceptionally aberrant nuclear-to-cytoplasmic distribution as long as 6 h post-stress.16 Here, we find that TDP-43-related phenotype is fully resolved 2 h after treatment while FUS-related phenotype is not resolved 6 h after treatment (Figure 2F). We also find delayed hnRNPK-related phenotype recovery. Notably, we find that the recovery kinetics for most RBPs after both heat and osmotic stresses correlate over time with the neuritome-related phenotype, suggesting that changes in neuritome relate to change in RBP-related phenotype or vice versa. Finally, and in line with our previous study, we do not find any major difference between control and ALS-related VCP-mutant MNs cultures in their response to stress (Figure S2D,F). While these results at least in part recapitulate our previous findings, thereby confirming the validity of our approach, it is important to note that the trained classifiers do not necessarily capture a phenotype related to the previously studied nuclear-to-cytoplasmic relocalisation and that this may relate to more complex cellular response. Altogether, these results indicate that the performance of a classifier is a reliable approach to prioritise which RBPs are most relevant to a specific cell culture condition and that the CNN-based method can, at least in part, reproduce previous results showing slower TDP-43 and FUS relocalisation dynamics following heat and osmotic stress16 that in some cases these might relate to changes in neuritome.

Heat stress-related changes in the MN neuritome resemble those occurring in ALS

We previously reported common morphological descriptors that strongly discriminate ALS from control tissue at the single cell level,15 indicating that key information related to ALS cellular state might be contained in cellular shape. Having found that our approach is suitable to reproduce prior findings related to stress in MNs, we next sought to test whether ALS-related VCP-mutant MNs are characterised by changes in cellular shape in the nucleus, neuritome or the combination of both. This was achieved by comparing the performances of CNN-based classifiers trained to discriminate images of untreated control MN cultures from images of VCP-mutant MN cultures either using the DAPI staining only (hereafter named ALS]DAPI classifier), either the β III-tubulin immunolabelling only (hereafter named ALS]BIII classifier) or the combination of two markers (hereafter named ALS]DAPI:BIII classifier). As shown in Figure 3A, both ALS]DAPI and ALS]BIII classifiers perform a random classifier (AUC]DAPI > 0.5 and AUC]BIII > 0.5), indicating that, similarly to stress-related conditions, both compartments exhibit phenotypic changes associated with VCP mutation. Further comparing the performances of these classifiers revealed that the inclusion of the β III-tubulin immunolabelling leads to consistently significantly higher performance (AUC]DAPI + BIII = 0.85 > AUC]BIII = 0.83 > AUC]DAPI = 0.65), indicating that the compartment most affected by VCP mutation at this early disease stage is the neuritome. Notably, the consistently high model predictions among the four mutant cell lines according to both classifiers confirm the absence of experimental or cell line bias, as further confirmed by linear mixed model analysis (Figures 3B and S3A). Additionally, the significant correlation between the ALS]DAPI and ALS]BIII model predictions for each MN culture further indicates that the ALS-related changes identified by these classifiers in the nucleus or the neuritome respectively co-occur in the same MNs cultures (Pearson correlation coefficient = 0.67 and P = 2.93e-33; Figure 3C). Notably applying these classifiers to images from TARDBP G298S mutant MNs further validated that the neuritome compartment exhibits phenotypic changes when compared with control MNs (Figure S3B).

We next aimed to understand what information is used by these ALS classifiers to discriminate images from control and VCP-mutant MN cultures. Integrated gradient (IG) is one popular approach for CNN model interpretation enabling the visualisation of the relevant
Looking at the IGs of randomly selected images with high ALS\textsubscript{DAPI} model predictions showed relevant pixels mostly overlap with the outline of the nuclei, with some contribution from pixels located inside the nuclei (Figure 3D). This indicates that the ALS-related phenotype identified by the ALS\textsubscript{DAPI} classifier primarily relates to nuclear
shape (including the size) rather than to other DAPI-related measurements such as texture or intensity. Next, looking at the IGs of randomly selected images with high ALS|DAPI:BIII model predictions showed relevant pixels primarily located at the edges of the neurites, indicating that relevant information mostly arises from the outline of the neurites rather than from the texture or the intensity of the β III-tubulin immunolabelling (Figure 3E). Altogether, these results indicate the network of neurites carries most ALS-related phenotype information.

Mitochondrial and oxidative stress are recognised and robust phenotypes in ALS development in vitro, and thus, in vitro models of cellular stress are important tools to investigate ALS. However, it remains unknown which type of cellular stress is most physiologically relevant to study ALS pathogenesis. Thus, we next sought to test whether heat, osmotic or oxidative extrinsic stress insults induce similar nuclear and/or neuritome-related phenotypic changes in control MNs cultures as those captured by ALS|DAPI and ALS|BIII classifiers. First, looking at the scatter plot of these two model predictions for individual MN cultures colour-coded according to the stress conditions showed that heated MN cultures consistently score high according to both classifiers forming a coherent cluster, as opposed to oxidative and osmotic conditions (Figure 3F). We next quantified the extent of ALS-related nuclear vs neuritome phenotypes induced by each individual treatment (oxidative, heat and osmotic) by analysing the ALS|DAPI or ALS|BIII model predictions of individual stressed MN cultures using linear mixed modelling (see Section 4). This analysis revealed that heat stress, and to some extent osmotic stress, induces a minor however non-significant ALS-related nuclear phenotypic change in control MN cultures, as indicated by the increase in ALS|DAPI model predictions for these two MNs cultures (Figures 3G and S3C). This is in contrast with the neuritome compartment which ALS|BIII model predicts significant phenotypic changes in heated MNs cultures only (Figures 3H and S3D). Altogether, these results indicate that the neuritome is the compartment most affected by ALS-related VCP mutation and that heat stress induces similar neurite-associated changes in control MN cultures.

**FUS immunolabelling best captures ALS-related phenotypes that are recapitulated by heat stress**

Two thirds of RBPs are expressed in a cell type-specific and temporally regulated manner. While previous studies showed RBP-specific mislocalisation in various ALS models, it remains unknown which RBP is most predictive of ALS at a particular disease stage. Thus, we next sought to test whether different ALS-related RBPs capture distinct ALS-related information in VCP-mutant MN cultures by analysing the model performances of ALS classifiers trained with images immunolabelled with antibodies against TDP-43, FUS, SFPQ, hnRNPK or hnRNPA1 only (hereafter named ALS|RBPs classifiers). Because all five aforementioned RBPs exhibit predominant nuclear localisation, we first tested whether ALS|RBPs classifiers exhibit significant improvement compared with the ALS|DAPI classifiers. This analysis showed that all five ALS|RBPs classifiers outperform ALS|DAPI classifier (AUC_{ALS|RBPs} > AUC_{ALS|DAPI}), ruling out the possibility that the phenotypic changes captured by these classifiers simply overlap with those identified by the ALS|DAPI classifier and indicating that they identify ALS-related phenotypes beyond changes in the nuclear shape (Figure 4A). Comparing their individual performances further revealed large differences in the individual RBP-based classifiers’ ability to discriminate ALS from control MN cultures, with ALS|TDP-43 exhibiting the best performance and ALS|SFPQ the least (AUC_{ALS|SFPQ} = 0.7 < AUC_{ALS|hnRNPK} = 0.73 < AUC_{ALS|FUS} = 0.79 < AUC_{ALS|hnRNPA1} = 0.85 < AUC_{ALS|TDP-43} = 0.9). Examining the IGs for randomly selected images with high ALS|RBPs model predictions indicated that the relevant pixels in all five ALS|RBPs classifiers are excluded from the nuclear areas as opposed to the most relevant pixels of the ALS|DAPI classifier that are most commonly localised at the inner nuclear membrane or inside the nucleus (Figure 4B). This demonstrates that the better the performance of the classifier, the less relevant the intranuclear pixels. For example, relevant pixels in the ALS|TDP-43 classifier are fully excluded from the nuclear area. Altogether, these results suggest that the different performance of ALS|RBPs classifiers in identifying ALS MNs cultures result from distinct RBPs localisation rather than nuclear shape. We previously showed that considering DAPI and β III-tubulin together significantly increases the performance of both ALS|DAPI and ALS|BIII classifiers. We next analysed how the performance of the ALS classifier would change by adding a third channel composed of RBPs immunolabelling and compared the performances of the ALS|DAPI|BIII classifier with the five ALS|DAPI|BIII|RBPs classifiers. This analysis indicates that all five RBPs significantly increase the ability of the trained CNN to discriminate VCP mutant from control MN cultures; however, while hnRNPK leads to the most modest improvement, TDP-43 and FUS immunolabelling lead to the highest increases in classification performance (Figure 4A). These results support the hypothesis that TDP-43 and FUS exhibit changes in localisation that are detected by these classifiers. Indeed, our prior studies have shown that in the same experimental model used here and at the same development stage, TDP-43 and FUS mislocalisation already occur, thus, we can expect similar RBP mislocalisation. These results further suggest that all five RBPs exhibit mislocalisation at different degrees, which previous studies could not demonstrate possibly due to lower sensitivity.

We next sought to test whether the ALS-related changes identified by the individual ALS|RBPs models are recapitulated in any of the extrinsic stressor cultures conditions. We first considered the effect size, as obtained by linear fixed effect analysis (Section 4), of each extrinsic stressor culture condition on the predictions of the seven ALS classifiers (ALS|DAPI, ALS|BIII, ALS|hnRNPK, ALS|SFPQ, ALS|FUS, ALS|hnRNPA1 and ALS|TDP-43) trained using a single channel. This revealed that oxidative and osmotic stress recapitulate one or two ALS-related phenotypes out of the seven captured by the ALS classifiers. However, heat stress induced more than a 10% increase in ALS prediction across five out of seven classifiers (Figure 4C). ALS|TDP-43 classifier, which performs best in ALS MN classification,
indeed the unique model that leads to significant, however modest, model prediction across the three stress conditions (Figures 4D and S4B,C). Additionally, we find that heat stress is the unique condition that leads to similarly high disease model prediction in control MN cultures and in untreated VCP-mutant MN cultures, given the ALS/JFUS and ALS/JhnRNPK classifiers (Figures 4E and S4B,C). Hierarchical clustering of the untreated ALS MN cultures together with the three stress conditions according to the effect size of each classifier (Euclidean distance and Ward clustering) eventually confirmed that heat stress induces overall the most similar cellular changes to ALS (Figure 4F). Altogether, these results confirm that MNs exposed to heat stress most closely resemble ALS cells with respect to phenotypes captured by the majority of ALS classifiers. Additionally, it shows that while TDP-43 is the RBP that carries the strongest information related to ALS, it is the FUS immunolabelling that captures most similar phenotypes between heat stress and ALS MNs cultures.

DISCUSSION

In this study, we combine multichannel fluorescence high-content microscopy data with deep learning imaging methods to unveil—directly from unsegmented images—novel neurite-associated morphological perturbations. This approach can be used to leverage existing high-content imaging datasets to gain new phenotypic insight into the original biological questions asked, as established by this study. We uncovered a surprising degree of previously unrecognised disease-
relevant information in broadly used and often considered ‘generic’
biological markers of nuclei (DAPI) and neurons (β III-tubulin) in the
context of our human stem cell model of VCP-related ALS. Addition-
ally, we reveal changes associated with ALS-related RBP immunofluo-
rescence imaging that can be captured in VCP-mutant MN cultures. We
were also able to systematically examine whether heat, oxidative
or osmotic stress induce similar modifications that could therefore
reinforce their utility in modelling aspects of MN dysfunction in ALS.
Our study establishes the use of CNN-based methods for rapid, auto-
mated and unbiased testing of biological hypotheses.

CNN-based methods are now widely used for image classification
and segmentation and have been successfully applied to medical
imaging data for disease detection and prediction.36–38 Here, CNN-
based image classifiers have been trained to identify stress- and ALS-
related phenotypic changes in unsegmented images of multiple MN
cultures. We further showed that the performance of such classifiers
is a reliable approach to prioritise which RBPs are most relevant to a
specific cell culture condition, although refined analysis will be
required to interpret their precise relevance to the underlying dis-
ease/stress process. CNN-based classifiers face challenges with inter-
pretability and are not suited to specifically address the subcellular
localisation of RBPs as previously described with conventional
methods based on image segmentation.16,35 Nevertheless, we showed
that the phenotypes identified in the DAPI or β III-tubulin fluorescent
images are indeed contained in the outlines of the nuclei and in the
edges of the neurites, respectively. Furthermore, we could demon-
strate that training a classifier with fluorescent images of a given RBP,
in conjunction with nuclear and neurite fluorescent markers, enables
the recapture of previously found phenotypes related to RBP cellular
localisation. Specifically, we could reproduce previous results showing
TDP-43 and FUS mislocalisation in ALS iPSC-derived MNs.3,20,35
Additionally, our study suggests that SFPQ, hnRNPA1 and hnRNPK
also exhibit mislocalisation however at different degrees. Notably,
hnRNPA1 is a component of RNA transport granules in neurons,39
and we can speculate that the extent of cytoplasmic relocalisation for
this primarily nuclear RBP 16 may be too subtle to be captured by
analysing its nuclear-to-cytoplasmic ratio.

DAPI and β III-tubulin are often considered ‘generic’ biological
markers, and the usage of their fluorescent images are most often
intended for nuclear or neuronal segmentation. Our study uncovers
previously unrecognised disease-relevant information that is con-
tained within DAPI and β III-tubulin fluorescent images. Given that
DAPI and β III-tubulin are broadly used markers, and given that our
method can be easily implemented in other biological conditions,
future studies might use DAPI and β III-tubulin fluorescent images from
different biological systems and experimental paradigms to reveal innovative research directions. For example, this approach
could be useful in interrogating the presence and onset of aberrant
cellular morphologies in time course experiments and across neurolog-
ical disorders.

Morphological attributes of cells and their substructures are
recognised readouts of physiological or pathophysiological states18; however, these have been relatively understudied in ALS research.

Here, we demonstrate that the neuritome compartment exhibits
aberrant phenotypes in ALS pathogenesis, as evidenced by the high
efficiency of deep learning classifier to identify ALS MN cultures
uniquely based on β III-tubulin fluorescent images. We also show
modest (albeit significant) perturbations in the nuclear compartment
given the predictive value of the DAPI fluorescent images in identify-
ing ALS MN cultures. While it remains unclear whether these are
strictly pathogenic events, the similar phenotypes detected in the
neuritome of MN cultures exposed to heat stress suggest that these
events relate to a form of MN stress. Through a thorough comparison of
heat, oxidative and osmotic stress-induced changes in both cellular
shape and ALS-related RBP immunolabelling, we further demonstrate
that neuritome-associated perturbations were also detected in control
MNs cultured in three different stress conditions. These findings sup-
port the notion that the neuronal processes exhibit large perturba-
tions across various stress conditions and argue for increased focus
on this cellular subcompartment in future research, such as testing
these methods on ALS human pathological tissue sections. Another
striking finding is the correlation between recovery kinetics of the
neuritome compartment after osmotic and heat stresses, and those of
several RBP-related phenotypes. Assuming that the RBP-related phe-
tonotypes captured by the CNN-based classifier relate to an RBP
change in cellular localisation, this result suggests that previously
observed stress-induced RBPs mislocalisations are coupled to global
changes in the neuritome.16 Indeed, neurite degeneration has been
shown to occur upon oxidative stress through the cytoplasmic
sequestration of two proteins (PRMT1 and Nd1-L) in in vitro models
of FUS mutant-related ALS.40 Furthermore, TDP-43 mislocalisation
and aggregation has also been demonstrated in dystrophic
neurites,4,33 while we recently reported an increase in wild-type FUS
within neuronal processes in VCP-mutant MNs.35 Finally, a regulatory
role for FUS has also been shown in synaptic formation and
function,40–43 and aberrant FUS activity in the axonal compartment
has been evidenced in a FUS mutant ALS mouse model.64 Altogether,
these studies support the hypothesis of an association between RBP
mislocalisation and aberrant neuronal processes in ALS. The finding
that several RBP-related phenotypes present similar recovery patterns
as the neuritome further suggests that additional ALS-related RBPs
might exhibit similar aberrant neurite localisation in ALS. Future work
will directly address the nature of these perturbations using classic
approaches that necessitate nuclear and neurite segmentation and
the acquisition of hundreds of measurements from each cellular
compartment.

Several lines of evidence support the hypothesis that cellular
stress is one central mechanism by which MN death occurs in ALS
and in vitro models of cellular stress are therefore important tools to
investigate ALS disease.17 It remains however unknown which type of
extrinsic cellular stress most closely approximates ALS pathogenesis,
and relatively little is known about the effect of thermal stimulation,
hyperosmolarity or arsenite-induced oxidative stress on the neuritome
compartment. Here, we find that iPSC-derived MNs exposed to heat
stress, as opposed to hyperosmolarity or arsenite-induced oxidative
stress, closely recapitulate the phenotypes of ALS MN cultures
captured by several classifiers. This result suggests that heat stress more closely approximates ALS pathogenesis compared with osmotic and oxidative stressors. This is in line with previous non-mammalian studies that have defined heat stress as being relevant to the study of neurodegeneration. Heat stress-induced stress granules sequester more misfolded proteins, are less dynamic and have increased protein poly-ubiquitination compared with arsenite-related stress granules.43–48 The lack of similarity between oxidative stress and ALS here may be attributed to the use of arsenite, which although widely used as an inducer of oxidative stress, may not truly approximate physiological oxidative stress that has been reported in ALS. As the pathogenic cascades underlying ALS are multifactorial and not fully determined, heat stress could indeed be a useful cellular model to study disease mechanisms. Our study demonstrates the ability of heat stress to induce subtle ALS-related cellular changes associated within the neuritome compartment and within the FUS immunofluorescent images. In particular, our study demonstrates the ability of heat stress to induce ALS-related cellular changes associated within the neuritome compartment and within the FUS fluorescent images. Interestingly, we previously found that heat stress alone caused cell death in an iPSC-derived model of MNs.16 Furthermore, heat stress,49,50 ageing and neurodegeneration51–53 all associate with intron retention.2,4,54,55 Future research will investigate the molecular mechanisms by which elevated temperatures lead to similar responses as those observed in patient-specific ALS MN cultures.

MATERIALS AND METHODS

Compliance with ethical standards

Informed consent was obtained from all patients and control controls in this study. Experimental protocols were all carried out according to approved regulations and guidelines by UCLH’s National Hospital for Neurology and Neurosurgery and UCL’s Institute of Neurology joint research ethics committee (09/0272). Cell culture, stress treatments, immunohistochemistry and image acquisition were performed as in Harley and Patani.16 Indeed, these data are utilised in the current manuscript and no additional experiment was required.

High-content imaging dataset

The imaging dataset used in this study consists of fluorescence microscopy images of iPSC-derived MNs as previously reported.16 The neurons either came from control cell lines or cell lines with the ALS-related VCP mutation and underwent experimentation after 6 days of terminal differentiation. Details of iPSC lines are provided in Table S1. To induce stress, the cultures were subject to 1 h of oxidative stress, 1 h of osmotic stress and 1 h of heat stress. To examine recovery, the cultures were subject to 1 h of stress and then returned to untreated conditions for 2 h following heat stress and 1, 2 and 6 h following osmotic stress. Following stress treatments or recovery, cultures were fixed and then immunostained with a combination of three markers, specifically a nuclear-specific marker (DAPI), a neuron-specific marker allowing to outline the neurites (β III-tubulin) and an antibody against TDP-43, SPFQ, FUS, hnRNPA1 or hnRNPK. The dataset is divided in different experiments (repeats done different days), each with several 96-well plates. Each well corresponds to one cell line, one stress condition and one combination of fluorescent markers. Each well has several non-overlapping fields of view (ranging from 10 to 12), and each field of view has several planes or z-stacks (ranging from 3 to 5) with 1-μm steps, generating a large-scale imaging dataset of 156,577 images (Figure 1A,B). The dataset is publicly available in IDR and can be found under study idr0112 and using the direct link https://idr.openmicroscopy.org/webclient/?show=screen-3001.

Image pre-processing

All images went through pre-processing steps described in Figure S1. Raw images are 16-bit images. Sixteen-bit raw z-stack images (1080 × 1080 pixels) from the same field of view were first merged using Maximum Intensity Projection (MIP), where the pixel with maximum intensity across all z-stacks is selected at each location in the image. Following conversion of MIP images to 8-bit images, channels were merged together to form an RGB image. We created 13 types of RGB images, either composed of one, two or three channels, to train image classifiers with 13 different combinations of immunostained images (Figure 1E). For images with three channels, DAPI was assigned to blue channel, β III-tubulin to the red channel and the RBP to green channel. For images with one or two channels, pitch-black images were assigned to the remaining channels so that the image would still be considered RGB. Images were then enhanced using Python Image Library Pillow ImageOps auto contrast function, to normalise image contrast. This function calculates a histogram of the input image, removes 0.1% of the lightest and darkest pixels from the histogram and remaps the image so that the darkest pixel becomes black (0) and the lightest becomes white (255). In the fourth step, the enhanced images were divided into 16 smaller images of size 270 × 270 pixels, which allowed better resolution and more images. Structures at this scale proved to be more distinguishable with IGS and yielded similar results than with whole images. This division also made sense for the fifth step, which consisted in resizing images to 224 × 224 pixels. Finally, images were normalised using mean intensity = [0.485, 0.456, 0.406] and std = [0.229, 0.224, 0.225] across the images from the ImageNet dataset. The last two steps were added in order to fulfil the requirements when using pre-trained models, which expect input images to be normalised in the same way as the dataset on which they were trained.

Data augmentation

In order to improve accuracy and reduce overfitting, we performed five augmentations on each image of the training set as follows and
as previously described\textsuperscript{57}, (1) 90° rotation, (2) one horizontal mirror, (3) one vertical mirror, (4) 90° rotation of horizontal mirror and (5) 90° rotation of vertical mirror. This results in a sixfold increase of the number of available images for training (five rotations + original).

**CNN-based image classifiers training**

We trained 52 CNN-based classifiers to discriminate (1) ALS from control MNs cultures (isALS test), untreated MNs cultures from MNs cultures exposed to (2) oxidative (isOXIDATIVE test), (3) heat (isHEAT test) and (4) osmotic stress (isOSMOTIC test) using 13 combinations of RGB images composed of different channels: Either a single channel was used (DAPI, BIII or RBP), either two channels (DAPI:BIII) or three channels (DAPI:BIII:RBP), pitch-black images being assigned to the unused channels (Table S2). Instead of training a full new neural network, we performed transfer learning that can be used to address the relatively small number of available images by introducing information from another domain.\textsuperscript{58} For training, images were fed into torchvision MobileNetV2 model, which has been pre-trained on ImageNet.\textsuperscript{59,60} MobileNetV2 is a CNN based on a streamlined architecture that uses depth-wise separable convolutions to build lightweight deep neural networks and that is effective for fine-grained image classification. MobileNetV2 is a lightweight neural network of 3.5 million parameters, as opposed to the widely used ResNet that contained 11.7 million parameters, making it suitable for fine-tuning with limited number of images.\textsuperscript{61} All layers of the pre-trained CNN classifier were fine-tuned on our dataset, allowing the training of a highly accurate model with a relatively small training dataset.\textsuperscript{62} The last layer was modified so that it turned the features into predictions for two classes instead of the thousand classes from ImageNet. Training was performed by stochastic gradient descent with learning rate 0.001, batch size 32, using the cross entropy loss function. The training was stopped after 10 epochs. A 10-fold cross-validation scheme has been used to evaluate the accuracy of the classification predictions generated by the trained classifiers: The images were shuffled randomly and divided into 10 stratified folds, preserving the percentage of samples for each class; each fold was used once as a test dataset, while the remaining folds were used for the training dataset. ROC curves were generated to evaluate the model’s ability to distinguish two cell culture conditions. ROC curves plot the true positive rate (sensitivity) vs the false positive rate (1 – specificity). The area under the ROC curve was used as the performance measure or classification accuracy. The classification accuracy over all folds is reported in Table S3. We evaluated the 52 trained models on all MN cultures when the right combination of markers were available (typically FUS, DAPI and BIII are available for some MNs cultures while SFPQ, DAPI and BIII are available for others). The probabilities to belong to one of the four tested conditions (iALS, isOXIDATIVE, isOSMOTIC and isHEAT) outputted by the classifiers are then aggregated by computing the average probability over all of the 16 cropped images originating from a single image, thereby obtaining a single probability per original-sized images. A single probability per MN culture is reported for each of the four tests by averaging the signal over all images (typically seven per MN culture) as reported in Tables S4–S7.

**Model explainability and IG**

The IG is a widely used interpretability algorithm that allows to identify what pixels of an image have the strongest effect on the model’s predicted class probabilities and therefore allowing to visualise which parts of the image are important for classification,\textsuperscript{31} by computing the gradient of the model’s prediction output to its input features. We used the Captum Insights method\textsuperscript{63} to obtain the IG for randomly selected images associated with high classifier prediction scores.

**Model prediction data analysis**

We used R and lme4\textsuperscript{64} to perform linear mixed effects analysis of the relationship between individual model predictions and either stress MNs culture condition (at the corresponding time after treatment) or VCP-mutant MNs cultures, accounting for idiosyncratic variation due to either cell line or experiments. As fixed effects, we either entered the stress condition or the VCP-mutation variable into the model. As random effects, we had intercepts for either cell lines (CTRL1, CTRL2, CTRL3, MUT1, MUT2, MUT3 and MUT4) and experiments. P-values were obtained by likelihood ratio tests of the full model with the effect in question against the model without the effect in question, that is, comparing a full linear model fitting the classifier predictions using both the fixed effect (the stress or disease) and the random effects (cell line and experiments), with a reduced linear model, which only considers the random effects.

**Data and software availability**

We provide raw images, complete source code and trained models to readily reproduce figures, tables and other results that involve computational methods. The image data that support the findings of this study will be uploaded to the IDR.\textsuperscript{65,66} As this requires some more time given the size of the data (1 TB), we have uploaded a subset of the data on Zenodo under the accession number 4664177. The 52 trained deep learning models have been uploaded on Zenodo under the accession number 4664252. The code for obtaining the models as well as the Jupyter notebooks are available at https://github.com/idiap/als-classification.

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CONFLICT OF INTEREST
The authors declare no competing interests.

ETHICS STATEMENT
Experimental protocols were all carried out according to approved regulations and guidelines by UCLH’s National Hospital for Neurology and Neurosurgery and UCL’s Institute of Neurology joint research ethics committee (09/0272).

AUTHOR CONTRIBUTIONS
Conceptualisation: R.L., R.P.; formal analysis: C.V., R.L.; investigation: C.V., J.H.; writing—original draft: R.L., R.P.; writing—review and editing: R.L., R.P., C.V., J.H.; resources: R.P.; visualisation: C.V., J.H.; funding acquisition: R.L., R.P.; supervision: R.L., R.P.

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DATA AVAILABILITY STATEMENT
The data that support the findings of this study are openly available in IDR at https://idr.openmicroscopy.org/webclient/?show=screen-3001, reference number idr0112.

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SUPPORTING INFORMATION
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