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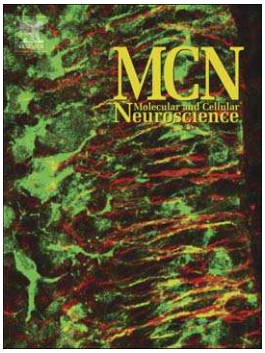

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High Content Analysis in Amyotrophic Lateral Sclerosis

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Abstract

Amyotrophic Lateral Sclerosis (ALS) is a devastating disease characterized by the progressive loss of motor neurons. Neurons, astrocytes, oligodendrocytes and microglial cells all undergo pathological modifications in the onset and progression of ALS. A number of genes involved in the etiopathology of the disease have been identified, but a complete understanding of the molecular mechanisms of ALS has yet to be determined. Currently, people affected by ALS have a life expectancy of only two to five years from diagnosis. The search for a treatment has been slow and mostly unsuccessful, leaving patients in desperate need of better therapies. Until recently, most pre-clinical studies utilized the available ALS animal models. In the past years, the development of new protocols for isolation of patient cells and differentiation into relevant cell types has provided new tools to model ALS, potentially more relevant to the disease itself as they directly come from patients. The use of stem cells is showing promise to facilitate ALS research by expanding our understanding of the disease and help to identify potential new therapeutic targets and therapies to help patients. Advancements in High Content Analysis (HCA) have the power to contribute to move ALS research forward by combining automated image acquisition along with digital image analysis. With modern HCA machines it is possible, in a period of just a few hours, to observe changes in morphology and survival of cells, under the stimulation of hundreds, if not thousands of drugs and compounds. In this article, we will summarize the major molecular and cellular hallmarks of ALS, describe the advancements provided by the *in vitro* models developed in the last few years, and review the studies that have applied HCA to the ALS field to date.

Keywords: Amyotrophic lateral sclerosis, neurodegeneration, high content screening, motor neurons, astrocytes, co-cultures.

Review

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease affecting predominantly motor neurons (MN), with an incidence of 3.9 individuals for every 100,000 people in the U.S. (Center for Disease Control and Prevention - https://wwwn.cdc.gov/als/ALSReportsNew.aspx). Hallmarks of the disease are progressive muscular weakness and dystrophy, caused by loss of upper MNs in the motor cortex and lower MNs in the brainstem and spinal cord (SC). ALS typically progresses rapidly and results in patient death within three to five years after diagnosis; the cause of death for the majority of patients is respiratory failure[1]. Inherited cases of the disease, referred to as familial ALS (fALS), account for 5 to 10% of the patient population; non-inherited cases, as well as those of unknown origin are called sporadic ALS (sALS) and affect the remaining population. Both sALS and fALS have a very similar clinical profile, suggesting common disease mechanisms[2]. Our current understanding of ALS is that the disease is a multifactorial disorder with a cumulative pathology in which more than one cellular mechanism is disrupted, causing a series of detrimental events contributing to MN death. Some of the events characterizing ALS are: the aggregation and misfolding of proteins, high levels of oxidative stress, excitotoxicity, deficient axonal transport, mitochondrial dysfunctions and inflammation[3]. The neurodegeneration occurring in ALS has also been described as a non-cell autonomous occurrence[4], meaning that the MN death is a consequence of a pathological state of other cell types, such as microglia[5,67,8], astrocytes[9–15] and oligodendrocytes[16,17] that either spread their toxicity or simply provide less support compared to a non-pathological condition.

The last 20 years of research have resulted in remarkable advancements in the identification of many genetic factors behind the onset and the progression of the disease. Mutations responsible for the onset of ALS have been identified in various genes; the copper-zinc superoxide dismutase 1 (SOD1) gene was the first to be described in 1995[18], followed by many others including the transactivation response DNA-binding protein 43 (TDP43)[19] and the FUS RNA binding protein[20]. One of the latest to be identified is an expansion in hexanucleotide repeat GGGCC of the chromosome 9 open reading frame 72 (C9ORF72)[21],which account for 50% of familial cases. Other genes have been associated with familial or sporadic ALS such as Optineurin (OPTN)[22], Sequestosome 1 (SQSTM1)[23], Angiogenin (ANG)[24], Survival of motor neuron 1, (SMN1)[25] and NEK1[26], although with a minor epidemiological incidence[27] (for a complete list see https://ghr.nlm.nih.gov/condition/amyotrophic-lateral-sclerosis/show/Related+Gene(s)). These genes code for proteins involved in many unrelated cellular processes confirming the complexity of the disease.

The identification of genetic causes of ALS has allowed the development of *in vivo* and *in vitro* models that recapitulate the biochemical and molecular events occurring in the pathology. More than 20 transgenic mouse models of SOD1 are currently available, expressing either the human wild type (WT) protein or different mutated forms; transgenic mouse strains carrying the human WT or mutated TDP43

and FUS proteins have also been established[28]. More recently, the C9orf72 Bacterial Artificial Chromosome (BAC) transgenic mouse also became available [29–31]. These models have different advantages and reflect the disease in various ways; however, SOD1 mice remain the most used in research. For example, studies conducted on SOD1 mice have revealed that a toxic gain of function rather than a loss in protein function is responsible for the ALS pathophysiology; $SOD1^{-/-}$ mice showed no difference in neurological or morphological levels when compared to littermate controls[32]. Reaume and colleagues have shown that $SOD1^{-/-}$ mice, compared to their littermate controls, show only an increased vulnerability to stress in MNs, while the cell number or functionality was not impaired. Conversely, mice overexpressing mutated forms of the SOD1 protein, such as the $SOD1^{G93A}$ [33], $SOD1^{G37R}$ [34] and the $SOD1^{G85R}$ [35] have shown a selective death of MNs and a progressive fatal paralysis, despite little to no change in SOD1 enzyme activity or, for some mutations, even an increase thereof [36]. Mutant SOD1 protein forms intracellular aggregates, detected in multiple cell types including MNs and astrocytes[37] and cause a series of detrimental cellular events with elevation of reactive oxygen species (ROS) production[38], endoplasmic reticulum (ER) stress[39], inhibition of proteosomal degradation[40], mitochondrial dysfunctions[41] among the most cited. Additionally, studies with ALS animal models also support the hypothesis that non-cell autonomous mechanisms are responsible for MN death in ALS; indeed, when mutated SOD1 was expressed exclusively in motor neurons, animals showed no sign of ALS[42,43] or only mild signs of motor neuropathy[44], suggesting that maybe other type of cells are involved in the pathology. Animal models are a crucial resource in the investigation of disease mechanisms and to test potential treatments; however, their use entails several limitations, as they cannot truly replicate the human disease. In animals, the development of ALS symptoms is dependent on the expression of the transgene as well as the gender and the genetic background of the animal. Transgenic animals are only modeling the disease associated with their specific genome alteration; for example, if we consider the $SOD1^{G93A}$ mouse, which is one of the most frequently used ALS and mutant SOD1 model, it would represent only familial cases of ALS, thus less than 2% of total cases. In addition, some findings could be limited to the population carrying the specific mutation at the glycine 93, which could significantly narrow the patient population benefiting from the treatment. Moreover, animal models typically cannot be used for high throughput studies, which is a great limitation for large-scale therapeutic investigations.

Studying molecular mechanisms of the disease as well as testing new drugs or gene-based therapies directly in a wider and human scenario may provide more reliable information on therapeutic approaches that can be more successfully translated to the clinic. For this purpose, researchers have developed methods to isolate cells from postmortem human samples that can be cultured *in vitro* and expanded for a limited time[45]. In the context of ALS, neural progenitor cells (NPCs) from the lumbar spinal cord of ALS post-mortem patients, once differentiated into astrocytes, were shown to dramatically affect MN survival compared to control astrocytes[10,46]. Moreover, reduction of SOD1 levels in astrocytes from sporadic ALS patients that do not carry a mutation in this gene, lead to substantial increase in survival of co-cultured motor neurons. Using a similar in vitro model Re and colleagues, further confirmed that in sporadic cases of ALS, SOD1 toxicity in MNs alone is not responsible for the MN death[46]. A limitation of post-mortem isolated cell based models is that they represent only very late stages of the disease and they are limited in their potential for expansion to large high content analyses

due to limited growth. Additional advancements have come from the advent of reprogramming methods which are based on the de-differentiation of somatic cells, into stem cell-like progenitor cells or induced pluripotent stem cells (iPSCs)[47,48]. Unlike cells obtained from post-mortem samples, fibroblasts can be obtained directly from living patients, and can be used to model the disease at various stages of progression, rather than being limited to the end stage. Once fibroblasts are converted to progenitors or iPSCs, they provide an unlimited source of material thanks to their high proliferative capacity [48]and can be differentiated into many cell types including neuronal cells[48–50]. Several human iPSC lines carrying various ALS mutations are now publicly available and have been successfully differentiated into MNs or glial cells [51–54]. The first iPSC line that had been generated was from an 82 year-old female ALS patient carrying a mutation in the SOD1 gene[51]; derived iPSCs were successfully differentiated to MNs and characterized. Subsequently, a series of other studies derived iPSC lines from both familial and sporadic cases[54–56]of ALS. Although the use of iPSC lines for ALS studies has revealed important hallmarks of the disease, such as the presence of TDP43 aggregates in sALS, there are still many pitfalls in using this system, such as the long time required for the de-differentiation of the fibroblasts into iPSCs and the differentiation of the cells to the desired cell type, the necessity of using more than one clone from the same donor to reproduce the donor's genetic mosaicism[57], and ultimately the loss of the "aging signature" which may be crucial when studying a disease like ALS, for which aging is a major risk factor[58]. Many of these challenges can be overcome using methods of reprogramming patient specific fibroblasts directly to neural progenitor cells (NPCs), which, within 3 weeks allows researcher to obtain a multipotent population of cells that can be expanded as progenitors or differentiated into more specific neural cell types such as neurons (iN), astrocytes (iA) or oligodendrocytes [59–62].The direct reprogramming method, skipping the pluripotent stage, drives fibroblasts to be directly converted into a population of NPCs, likely preserving their aging signature and the donor's mosaicism, thus more closely reflecting the reality of the patient and of the pathology.

Cell type	Reprogramming method	Advantages	Disadvantages
iPSCs	Cocktail of mitogens and morphogens	• Unlimited source of material • Able to differentiate in all cell types • Donor-specific genome identity • Reproducibility within the same clone	• Extensive time for the differentiation • Variability between different clones • Loss of patient mosaicism • Loss of aging signature
iNPCs	Overexpression of cell-specific type transcription factors	• Rapid time for conversion • Donor-specific genome identity • Preservation of the aging signature	• Limited source of material • Differentiation limited to the committed-lineage • Cell variability - experimental variation

Table 1. Advantages and disadvantages of reprogramming methods.

For this matter, iNPCs and iNPCs derived iNs, iAs and oligodendrocytes could be considered as a more valuable system for studying diseases such as ALS; however, compared to iPSCs, iNs and iAs have limited potential for expansion and their mosaicism could lead to experimental variations, which are limiting factors for large-scale screening studies (Table1). Thus, the direct reprogramming method requires further refinements, although it has been successfully used by many laboratories for high-throughput studies and to test compounds in drug screening assays (Fig.2).

Despite the many significant breakthroughs in ALS research in the last few years, Riluzole, approved by the FDA in 1995, is the only pharmacological treatment available to ALS patients. Riluzole is an antagonist of the glutamatergic neurotransmission[63] and reduces glutamate cytotoxicity; however, the exact mechanism of action is still unknown. One possibility is that it may act synergistically on three independent pathways: the inhibition of the glutamic acid release, a non-competitive blockage of the N-methyl-D-aspartate receptor mediated-responses, and a direct action on the voltage-dependent sodium channels[64]. Additionally, some investigations have shown Riluzole also stimulates the release of trophic factors from gliomas, astrocytes, and Schwann cells[65–67]. The efficacy of this treatment was demonstrated in two double-blind, placebo-controlled clinical trials[68,69]. In these studies, Riluzole increased the survival of ALS patients up to three months compared to healthy controls[68–70]. This increase, while precious for the patients and their families, demonstrates a limited effect. Thus, current therapeutic approaches do not represent a real solution, particularly as Riluzole is only effective in slowing disease progression for patients with mild or early symptoms of the disease[71,72]. Patients in advanced stages of the disease do not have suitable treatments aside from symptom management or major medical intervention such as ventilator support[64,73,74]. Although the need is urgent, the search for effective therapies for ALS has been slow and unsuccessful. The heterogeneous and multifactorial nature of ALS poses a serious challenge to understanding the mechanisms underlying the disease pathogenesis.

In the last few years, new approaches showing some efficacy in pre-clinical settings have appeared. In particular, many investigators using different methods proved that the reduction of the human SOD1 protein was beneficial in rescuing the ALS phenotype[75–77], in delaying the onset and/or increasing the life span of animal models of ALS[6,78,79]. Different methods have been applied to achieve direct or indirect reduction of the SOD1 protein through administration of small molecules[80–82] or antibodies[83,84], or the application of RNA technologies, including microRNAs[85,86], RNAi[77,79,87–89] and antisense Oligonucleotides (ASO)[90]. Of great interest is the first human clinical trial conducted by Richard Smith and Tim Miller and his team in collaboration with Ionis Pharmaceuticals, involving the intrathecal delivery of an ASO against SOD1. The compound was reported to be safe and well tolerated by patients[90] and now a new Phase 1/2 study has been initiated to test single and multiple doses in adults affected by familial and sALS (Clinical trial number NCT02623699). A completely different approach to reducing SOD1 protein levels was used in a Phase I pilot study using Pyrimethamine, an FDA approved antimalarial drug; the study is now closed and results have shown a significant reduction of the SOD1 protein in patient leukocytes and CSF[91]. Many other studies are now open, aiming to target different ALS affected pathways such as inflammation (Gylenia trial - NCT01786174 and Rasagiline trial - NCT01786603), cell repairing

mechanisms (Arimoclomol trial - NCT00706147) and mitochondria (Cu(II)ATSM - NCT02870634). The C9orf72 repeat expansion mutation was also targeted by ASOs in MNs differentiated from iPSCs derived from ALS patients; two independent studies have shown the mitigation of the C9orf72-mediated toxicity including a reduction of RNA foci formation, a reversion of the gene expression alteration and a decrease in cell susceptibility to excitotoxicity[92–94]. Recently, Ionis Pharmaceuticals has also announced its partnership with three academic groups to develop antisense therapeutics for C9orf 72 repeat expansion mutations [95].

All the therapeutic strategies reported here are aiming to target single molecules or individual pathways involved in ALS. Similar approaches have been successful in tackling diseases for which we have a strong understanding of their molecular basis[96]. However, as briefly discussed before, the pathogenesis of ALS-genes is still under debate, and many pathways and processes are altered or dysfunctional. The complicated and multi-factorial nature of ALS can definitely affect the success of a single-target based approach and progress towards a therapy with substantive clinical efficacy will most probably call for research strategies that evaluates multi-target approaches[96,97]. The accumulation of protein aggregates, together with excessive cytotoxicity, high level of oxidative stress and inflammation, impaired mitochondria and ER stress[3,98] are all possible targets for designing new therapies. For instance, combining cellular *in vitro* system with automated microscopy may dramatically increase the speed at which we test new drugs, compounds, or other therapeutic approaches influencing MN survival and morphology. The use of HCA technology for disease profiling and treatments screening has the potential to significantly stimulate ALS research and define effective compounds or therapeutic strategies.

HCA in the ALS field: the present

High Content Analysis (HCA) is a relatively new technology that combines the high-throughput acquisition of microscopy images with the automated analysis of those images to generate multiparametric data from different morphological features at the tissue and/or cellular levels[99–101]. Several tools are available to determine the effect of a compound on, for example, cellular survival[102], proliferation [103] or morphology [104–106]. Before automation, these kinds of analyses contained operator based variability, were extremely time-consuming, and dramatically impaired the speed at which a lab could evaluate the efficiency of numerous treatments. Different technological advancements have improved these processes, allowing the increase of the screening power by several orders of magnitude. In the early years after its development, HCA was mostly used by pharmaceutical companies to perform large image-based screenings (we refer to the application of HCA for medium or high-throughput assays as High Content Screening or HCS) for the identification of new drugs and compounds following a phenotypic approach[107,108].

Phenotypic approach to drug discovery using HCA presents various technical advantages over the more frequently used molecular and biochemical assays measuring the activity or expression of a single pre-determined target: the target-based approaches. Extensive reviews of the two approaches, the methodologies associated with them and the differences between them are already available in

literature [100,101,109]. Briefly, utilizing phenotypic assays based on whole cells or tissues it is possible to obtain a large amount of morphological information in one experiment from the quantitative analysis of many features including cell shape, organelle count, compartment size and localization and co-localization of various markers[100,101,110]. The fact that the assays can be non-destructive allows researchers to add spatial or temporal dimensions to the data[100,101]. HCA-based phenotypic screening also comes with various disadvantages, including extensive time and effort required for the development of robust assays[111,112], the cost of the reagents, and the throughput size: while it is possible to screen large numbers of conditions (hundreds to thousands per experiment), these are still yet lower than those allowed by the single read-out biochemical and molecular assays that are predominant in the target-based approaches[100,101,113,114]. Considering the multiparametric nature of HCA, even the management of the massive amount of data generated by this technology can present a concern. Even if data storage issues may be easily addressed by larger databases, the development of correct analytical and statistical methods to obtain the maximum amount of information from these data is still an ongoing process[101,112,113]. This also adds the need for specialized data-analysis expertise to the technical disadvantages.

For these and other reasons, and despite the many advantages of phenotypic screenings using HCA, target-based approaches are still preferred in the drug discovery world. Which of the two approaches is more useful or successful is still a matter of debate[115–117]. Many argue that most common assays used in target-based drug screenings overlook the off-target effects and underestimates the interconnectivity of the cellular mechanisms, thus leading to a high rate of failure in translating to the clinic[110,111,118,119]. First the genome project, and then the spreading of panomics approaches applied to different biological fields demonstrated the complexity of the molecular networks[96,110,119–121]. This is a consideration even more relevant when studying the CNS and its disorders, including ALS. As we discussed before, much is still to be learned about the biochemical and molecular mechanisms of CNS disorders and what is known so far highlights the multi-target nature of these diseases[96,97,122]. HCA-based phenotypic screenings can address these concerns by providing information beyond the function of a single target molecule. The multiple read-outs generated by the cell, tissue or organ-based assays provide a better prediction of the action of the drug or compound on a complex system such as the *in vivo* physiology[96,110,111]. These tests can, therefore, be more relevant to the human pathology and they can more successfully translate to the clinic [96,110,111].

HCA was previously more prevalent in the realm of pharmaceutical industry, and the recent advent of more affordable systems for HCA, has allowed academic labs all around the world to apply multiparametric image analysis to a whole list of diverse fields, from cancer research[124–128], to developmental biology[129,130]. The introduction of HCA to the academic scientific world also opened up new applications beyond screening, headed more towards the idea of profiling[113,131,132] or trying to capture as many properties of a sample as possible. In this way, researchers can approach complex scientific problems from a multi-parametric point of view that embraces and more accurately represents the complexity of biology. In just a few hours, we can now determine the localization of hundreds of different proteins following the exposure of cells to a drug, or changes affecting the morphology of dozens of different cell types after silencing of a gene. In combination with the right *in vitro* models and

gene expression alteration assays, High Content Profiling can be an invaluable tool for functional genomic studies as well as disease phenotyping[112,113,133–135].

Neuroscience has long relied on advanced imaging techniques. Naturally, HCA rapidly found its place in this field and was successfully used for applications such as basic cell counting in studies of neurogenesis[136] or progenitor proliferation[137], or in more complex morphological analyses to understand axon growth[138–141], dendrite extension[142] and synaptogenesis[143]. It's not the intention of this article to review the possible applications of HCA, many of which have already been reviewed by other authors in this journal issue. Instead, we will focus our attention on the most relevant studies that have applied HCA in ALS research, reviewing how they used this technology and how they helped to advance the ALS field. The first report of a direct application of automated microscopy to the field of ALS came in 2004 from Corcoran and colleagues[144]. In the study the authors designed a screening assay using COS1 cells (immortalized monkey kidney cells) mutated by adenovirus infection to overexpress wild type as well as mutant forms of the human SOD1 protein fused with a Green Fluorescent Protein (GFP). Upon inhibition of the proteasome machinery, COS1 cells showed the appearance of SOD1 containing aggresomes when overexpressing the G85R mutant form of the protein. The assays consisted of identifying compounds that reduced the number of cells containing GFP-positive aggresomes in COS-1 cells overexpressing G85R-SOD1-GFP. The screening library consisted of 20,000 small molecules of various origins (NINDS, NIC and Chembridge collections). The authors focused their attention on two particular chemicals that showed some significant activity in their assay: an HDAC inhibitor named Scriptaid and a Flavin analog of unknown cellular target. While these two compounds seem to have limited or no effect on the expression and distribution of the wild-type protein, they do reduce the aggregation of the mutant protein without affecting its expression level. This first study presents several caveats which include the cellular model used. The expression of the human protein is driven by a strong CMV promoter and followed by inhibition of protein degradation, which suggests the amount of misfolded cellular protein might be higher compared to the human pathological conditions leading to different kinds of pathogenic processes[145]. COS1 cells are not neuronal and not of human origin, however they are derived from monkeys thus complicating predictions of off-target effects of the compounds. Furthermore, translation to clinical therapies can fail when the background species does not match that of the target protein. Moreover, the study focused on one unique target pathway (mutant SOD1 aggregation). As previously noted, the multifactorial nature of ALS suggests that targeting one unique pathway might not change the clinical outcome. These considerations raise questions on the relevance of the model. The information that the assay did not function with other ALS-associated mutant forms of the proteins strengthens these concerns. Finally, while the study took advantage of automated microscopy for image acquisition, the analysis was still performed manually with the images "scored by eye for enhancement or absence of aggresomes". Operator-based analyses introduce the possibility of errors or inconsistencies during the data analysis step. These caveats aside, this paper has the undeniable merit of demonstrating to the field the possibilities of HCA technology reporting such a large amount of conditions screened in a single study. It also brought to the attention of the ALS community HDAC inhibitors as modulators of aggresomes formation, which led to more studies trying to translate their application to clinic[146].

Years later, Benmohamed and colleagues published one of the earliest reports on the use of fully-automated HCA in an ALS model[147]. In this study, researchers used a cellular model very similar to the one previously reported[144] but with a few modifications. A rat neuronal cell lines (PC12 cells) engineered to conditionally express different pathogenic mutant forms of the SOD1 protein was fused with a yellow-fluorescent proteins (YFP) to screen a large library of more than 50,000 compounds. Even in this model the overexpression of mutant SOD1, followed after one week by inhibition of the proteasome, induced the appearance of protein aggregates and cell death. The group designed a well-thought screening composed of different assays based on two main output readings: cell viability for their primary screening, and YFP-G93A-SOD1-containing aggresome count as a second filtering step. 50,000 compounds were individually administered to the cells in culture one day before proteasome inhibition. Sixty-eight compounds passed the various steps of the initial screenings for their ability to restore cell viability in culture for more than 60% of the DMSO treated control. Most of these compounds proved active as well in the second screening, by reducing or blocking protein aggregation. The authors used a chemical analysis approach to group the active compounds into cohorts of similar scaffold structures that resulted in 3 main groups selected for further work: the Arylsufanyl pyrazolones (APY), the Cyclohexane-1,3-dione series and the Pyrimidine 2,4,6-trione series. This study showed the implementation of a very robust screening system. Each compound underwent several steps of screening, singly and in duplicate, with a six point dose-curve first and a twelve point dose-curve after. For the first time, the authors presented the ALS field with the correct statistical methods for quantifying their screening robustness, by calculating Z-factors and Z'-factors, as representation of the size of the effect induced by a treatment[148,149], for each assay used in this study. The automation of the screening process (performed with a POLARstar – BMG Labtech and an Arrayscan – Cellomics system) as well as the analysis allowed researchers, not only to count the number of cells with aggregates but also to measure the number of aggregates/cells over hundreds of thousands of images. Despite the strength of the screening design and results, the model used by the author is similar to the one reported by Corcoran and therefore raises similar concerns about its relevance. Indeed the cells used in this study are closer to a neuronal phenotype, but the species (rat) is even more genetically distant from the human than the monkey. Inhibition of the proteasome is still required to induce a strong pathological phenotype and the assay seems to be effective only with one of the pathological mutations tested (the G93A). The study is again focused on only one of the many cellular processes affected by the pathology, at least in their secondary screening, which is protein aggregation. The authors themselves acknowledge the possibility that some of these compounds work through an off target or secondary target effect, in light of the differences in active concentrations between the survival and aggregation assays. In that case the non-human nature of the PC12 cells would become even more concerning, and the study less suitable for clinical translation. These inconsistencies suggest that these chemicals should be tested in more relevant *in vitro* models before being taken any further to the clinic.

The Brown lab at University of Massachusetts used the same cell line, PC12, to develop assays for compound screening in an ALS setting[150]. In one assay, cells were stably transfected with a vector expressing GFP under the control of the human SOD1 promoter. In the other assay, they were transfected with a vector expressing a fusion protein of a mutant form of SOD1 and GFP. In two separate studies,[82,150] these assays were used in combination with a HCA approach to screen various libraries for

more than 100,000 compounds for their ability to: 1) reduce SOD1 promoter activity or 2) increase the degradation of the protein, following the assumption that reducing the cellular amount of SOD1 would ameliorate the ALS phenotype. Interestingly, despite the large number of chemicals screened the studies reported very few positive hits. In one screening, most of the drugs that were able to reduce the promoter activity also reduced cellular viability while no drug resulted in specific reduction of SOD1 protein[150]. In the second study only 3 compounds showed significant effect in reducing the promoter without affecting cell survival[82]. Compound number 7687685 was the only one able to reduce the amount of SOD1 mRNA and protein also when tested in a human cell line (HeLa cells). However, when G93A-SOD1 mice were injected with a large dose of the compound only a very small decrease in SOD1 protein was observed in spinal cord extracts. Larger doses proved toxic to the animals, discouraging any further investigation into the clinical application.

Boyd *et al.* further extended the search by screening a library of 75,000 compounds, this time with PC12 cells overexpressing the wild-type form of the TDP-43 protein fused with GFP[151]. In their assay, formation of the ALS-associated TDP-43-containing aggregates was stimulated by the induction of oxidative stress through cellular exposure to Sodium Arsenite. The screening, performed with an INCell Analyzer 1000 instrument (GE Healthcare), was aimed at identifying compounds that could reduce the number of GFP positive aggregates. After several rounds of screening, with different thresholds and number of dose-points, only a small number (16) of positive hits were identified. One of them, LDN-0130436, was chosen for further validation by western blot analysis of HeLa cells protein extract. All of the 16 hit compounds were then tested *in vivo* in a C. Elegans model of ALS, overexpressing the A315T mutant form of TDP-43. One of the hits, LDN-0130436, demonstrated the ability to protect cells from TDP-43 mediated toxicity and to improve movements.

The numbers of compounds and genes screened in these early reports of HCA applied to the ALS field perfectly represent the incredible potential held by the technology. These studies pioneered the development of methods and procedures for ALS-associated screenings; brought attention to possible confounding factors, and suggested new therapeutic targets.

However, all of these studies suffer from the lack of better and more relevant models of the disease when they were performed. The assays were based on cell lines of non-human origin, with a forced overexpression of an exogenous mutant protein. They focused on only one pathological form of only one ALS-associated gene, with little regard to the complex multi-factorial nature of the pathology. These studies followed a target-based approach to the problem, overlooking the multi-parametric possibilities offered by HCA. All of these inconsistencies could be the reason some of these screenings were not able to identify any significant hits[150]. Or, more importantly, the reason that, in other studies the hit compounds fail to successfully translate to an *in vivo* model[82]. It would still be interesting to repeat some of these screenings with more recently developed cellular models, or testing some of the hit compounds.

The studies reviewed so far all used HCA as a way to screen libraries of compounds. We discussed before the possibility of using this technology to profile pathological features. One such study was reported by the Tibbet lab that using an HCA based approach to test the influence of more than 18,000

RNAis on the localization of TDP-43 and FUS protein in HeLa cells[152]. Protein visualization was obtained by immunostaining, thus eliminating the requirement for exogenous expression of a tagged protein. The screening was performed on a BD Pathway Bioimaging System (BD Biosciences), and measured the ratio between the cytoplasmic and the nuclear fluorescent signal. Using this approach, they were able to identify more than sixty genes that, when inhibited, increased the amount of cytoplasmic TDP-43 signal over the nuclear signal and are therefore assumed to promote the cytoplasmic translocation of the ALS-associated protein. As previously discussed, accumulation of cytoplasmic TDP-43 aggregates is cytotoxic and one of the hallmarks of ALS[153]. The study then focused on the specific role of one of these genes: ITPR1. Inhibition of this gene also rescued ALS-like phenotypes in a Drosophila model of TDP-43-dependent ALS. In this work, Kim *et al.* used a more relevant human cell line to identify factors that can influence one known pathological mechanism. Even so the use of transformed cell lines represents a caveat in data interpretation as all the possible biological processes linked to the immortalization of the cells most likely affect the gene expression profiles[154].

In 2007, the afore-mentioned protocols to derive iPS cells directly from patient fibroblasts and subsequently differentiate them into MNs were developed[48,51,54]. Egawa *et al.* were the first to use an HCA system (IN Cell 6000, GE Healthcare) to analyze phenotypical features of MNs derived from fibroblasts of ALS patients. In particular they focused on cells from three individuals carrying mutations in the TDP-43 gene[155]. In this study, aggregate formation, neurite length and TDP-43 co-localization with other proteins were assessed, and these clearly demonstrated that this model recapitulates several hallmarks of the disease. They observed that when oxidative stress was induced in the cells by exposure to Sodium Arsenite, there was an increased amount of insoluble TDP-43 that affected survival more in the ALS-derived MNs than those derived from healthy controls. The researchers used this observation to establish an assay in order to perform a small drug screen. They tested four drugs known to target RNA processing, a process in which TDP-43 is strongly involved. When MNs carrying TDP-43 mutations were treated with one of the 4 drugs, Anacardic Acid, their survival rate was restored to the level of healthy MNs. This study was the first one to apply automated microscopy and image analysis to cellular and molecular phenotypes of ALS in patient-derived MNs. Without a doubt, this work was very important in the process of establishing the best possible assays for HCA-based analysis in ALS, demonstrating that this system can successfully quantify pathology-associated differences in a relevant cellular model. Of note is the focus on extrapolation of data from various parameters like cell survival, axon length and aggregate number, rather than a unique molecular target. Indeed, the throughput of their drug screening assay is very low, but the authors' priority was set on determining the relevance of the model.

In their work Egawa and colleagues studied MNs derived from patients affected by fALS, and in particular those carrying mutations in a very specific gene. Applying similar reprogramming protocols, the Javaherian group extended observations to MNs derived from fibroblasts of three sALS patients[56]. These cells present *de novo* TDP-43 pathological hallmarks very similar to those observed in fALS-derived MNs as well as in postmortem fALS tissue. Cells from one of these three lines were used to develop an HCA assay that would identify reduction in the number of TDP-43-containing aggregates visualized by immunohistochemistry. They then tested in this assay an FDA approved library of more than 1,700 compounds and identified several that were able to reduce the number of TDP-43

aggregates in MN nuclei. This work helped in strengthening the position of the fibroblasts conversion protocol as a good model for *in vivo* studies of ALS and therefore for high throughput phenotypic screenings. However, of the initial eight familial and sixteen sporadic fibroblasts lines converted, only three sALS showed the TDP-43 aggregation. The authors advance many hypotheses for this discrepancy, both technical and biological for why this discrepancy happened, most of them focusing on the undeniable heterogeneity of the clinical symptoms of ALS and the varying forms of TDP-43 associated pathology. Indeed, this is an issue that still affects our ability to identify the best *in vitro* model.

Heterogeneity is an issue not only at the molecular level but also at the cellular level. Despite the complexity of the Nervous System, all of these studies have focused on one unique cell type: MNs. As mentioned before, other cell types such as microglia, astrocytes and oligodendrocytes have been shown to be major contributors to MN death in ALS. Partial excision of mutant SOD1 from cells of the myeloid lineage, including microglia, greatly extended survival of SOD1 mutant mice by slowing disease progression[6]; similarly, using the same mouse model in a different study, the complete replacement of cells of the myeloid lineage significantly slowed ALS progression[156]. Dysfunctional oligodendrocytes have been described in ALS patients as well as in mutant SOD1 mice, and interestingly, in ALS mice a loss of oligodendrocytes has been reported to occur even before disease onset[16]. One of the mechanisms best described in familial and sporadic ALS cases is the alteration of the astrocyte glutamate transporter activity which lead to a decrease in the glutamate clearance from the intersynaptic space, glutamate excitotoxicity and neuronal death[3]. ALS patients and ALS animal models have a reduced activity of the Glutamate transporter (GLT-1), and the upregulation of the GLT1 transporter has been shown to extend the survival of ALS mice[157]. Our laboratory has also played a significant part in investigating the non-cell autonomous contribution to MN death in ALS with the help of HCA technology. The assay we developed is based on an *in vitro* co-culture system of healthy MNs, differentiated from embryonic stem cells isolated from transgenic mice expressing GFP under the control of the MN specific Hb9 promoter, and patient derived astrocytes. In the first study we published in 2011 we derived astrocytes from postmortem spinal cord NPCs of one fALS as well as seven sALS patients[10]. We then tested survival of GFP positive MNs in co-culture with ALS or healthy control derived astrocytes. We demonstrated that healthy murine MNs show a reduced survival rate when co-cultured with human astrocytes derived from post-mortem spinal cords of sALS or SOD1-fALS patients[10]. This work, as mentioned before, helped to establish the role of astrocytes in MN loss during ALS, for the first time extending these observations to a human setting. In contrast to the previous studies on converted MNs, we applied our analysis to both familial and sporadic forms of the disease. However, even if having screened a larger number of patient lines than previous studies, we still had not started exploring the potential of an automated imaging system. Moreover, our methodology raised the question of whether the isolated NPCs might be affected by stress conditions induced in a postmortem setting. Indeed, at this stage in life, these cells have already been through years of oxidative insults[158].

Following our investigation, Serio and colleagues used a similar HCA approach to test if astrocytes converted from fibroblast derived iPS from a single TDP-43 fALS patient showed a similar reduction in survival of co-cultured MNs[12]. In their assay, MNs were also derived from iPS cells either of healthy individuals or ALS patients, and then transfected with Hb9-GFP expressing vector for fluorescent

visualization. Since they could not observe a similar impact on healthy MN survival when cultured with mutant astrocytes, they suggested the possibility of a difference in astrocytic involvement in the pathology depending on the form of ALS. The same heterogeneity issue was hypothesized to be behind inconsistencies in the work of Burkhardt et al[56]. Thus, while human studies are indeed more relevant to the disease, they also present many challenges, predominantly due to the variability between individuals linked to different genetic backgrounds, as well as diverse patient histories and environmental factors. For these reasons, it is important to confirm findings in large cohorts of patients.

It is also important to notice that the work published by Serio and colleagues used astrocytes derived from fibroblasts and not from postmortem NPCs with all the pros and cons discussed in the previous paragraph. As reported before, in 2014, we published a study in which we used a new protocol for the direct conversion of human fibroblasts collected from living patients into iNPCs and then into astrocytes, thus skipping the iPS stage[11]. Using this novel reprogramming method we generated astrocytes from different backgrounds: one SOD1 fALS, three C9orf72 ALS and three sALS. We then used the same assay design previously described[10], coupled with an INCell Analyzer 6000 HCA system (GE Healthcare) to test Mouse Hb9-GFP positive MNs survival when in co-culture with ALS or healthy astrocytes. Interestingly the presence in culture of the disease-derived astrocytes significantly impacted MN survival and caused up to 80% of MN death. Indeed, the introduction of the HCA system in our lab, with the automation of the process of image analysis, provided the technological means to extend our findings to large cohort of patients in a reasonable time-frame. The larger screening possibilities helped us to solidify our findings and find commonalities between the different pathological forms of ALS. There is still much to understand about the phenotypic features of neuronal cells derived from progenitors through conversion protocols, but the remarkable amount of work accomplished by multiple groups in just a few years would have been far more time-consuming and less feasible with traditional methods of imaging and analysis.

While only a handful of studies have used HCA technology for screening or profiling in the ALS field, they have already pointed to few novel compounds that may modulate disease progression and in more recent years, with the advent of more relevant cellular models, provided new insights into the etiology of the disease. These latest findings raise hope in the community and demonstrate the growing importance of HCA in this field. It is interesting to notice that earlier studies focused on the technology and the development of statistically robust high-throughput screening: large portion of the research reports were spent describing the screening methods and design and its results with large compound libraries[82,147,150,151]. As a screening readout, these works mostly utilized, the drug effect on one specific target, leaving aside the possible application of HCA for phenotypic multi-parametric studies. This was probably due to the lack of better cellular models of the disease. Indeed in the last few years all of the studies that used HCA in an ALS setting focused mostly on the validation and profiling of the newest human-derived cellular models[10–12,54,155]. In these articles, the part dedicated to the HCA application was marginal and its application limited to a small portion of the study, a possible indicator of the natural integration of this technology in basic biology and the more common availability of HCA systems in academic settings. While a full consensus on the phenotypic aspects of human stem cell-derived cellular models for ALS has yet to be achieved, these models undoubtedly represent a step forward from the

immortalized cell lines. Even so, no large compound or drug screenings has been reported that took advantage of these models, from the hundreds of thousands of compounds screened in the early days, to the relatively small screenings performed more recently.

HCA in the ALS field: the future.

Despite the limited use of HCA in the study of ALS to date, it has already proven its potential to accelerate the search for a treatment; to be sure, there are many other unexplored possibilities to exploit high throughput image analysis to the benefit of research. In the last part of this review, we will advance some suggestions for how HCA can contribute to our progressive understanding of ALS.

In the course of our investigations, we as well as others, have shown that patient-derived cell based assays are a robust method to mimic disease mechanisms *in vitro* and HCA has allowed the identification of subtle changes in cell morphology in a multiplicity of these samples. This is crucial, particularly with regard to neurodegeneration, where morphological changes often precede neuronal death. For example we were also able to observe a significant reduction in the number and complexity of MN neurites prior to cell death[11]. Another relevant morphological change in ALS is the appearance of cellular inclusions. Both sporadic and familial cases of ALS present accumulation of SOD1 and/or of TDP43[159] proteins; it is yet to be determined whether they are a source of toxicity for the cells or a defense mechanism adopted to segregate toxic material. HCA protocols for quantifying cellular aggregates have already been successfully validated for Huntington's[160] and Alzheimer's[161,162] disease. As demonstrated by some of the studies reviewed in this article, HCA systems can perform automated, unbiased quantification of aggregates in ALS settings at low throughput. With such large screening potential, though, these protocols could be applied to hundreds of patient-derived samples in only a few hours. Moreover, the association of HCA with immunofluorescence techniques allows the detection and quantification of increase or decrease of markers of the pathological state, such as ER stress, oxidative stress, mitochondria dysfunctions, misfolded proteins, and cellular apoptosis. By screening a large number of patient samples, we can evaluate similarities and differences in the cellular features of sporadic and familial ALS cases, or those associated with specific gene mutations, thus providing more efficient biomarkers of the pathology. Grouping patients by biologically relevant pathological hallmarks even has the potential to refine the approach taken by clinical trials. Currently, all ALS patients are treated as one homogenous population, without regard to the possibility that individuals from different disease subpopulations might respond to treatments in different ways. An efficient stratification based on data collected from hundreds of patients could dramatically improve the success and the rate of new therapies tested.

On the topic of identifying efficient therapeutic treatments, HCA technology was originally designed to accelerate drug discovery and is therefore, with the urgency of finding an effective therapy for ALS, an ideal platform to make pre-clinical studies faster. As previously discussed, large-scale screening of compounds with potential benefits for ALS is already a reality, as are more relevant models to the human disease such as the cells directly derived from patient fibroblasts. With the increasing consensus

of opinion on the non-cell autonomous nature of the disease, drug screens should take advantage of more complex models. As an example, the co-culture system developed in our lab provides a good opportunity to test compounds on more than one cell type at the time. Yet, as discussed before, no large compound screening since the appearance of the patient-derived cellular models has been reported on these newly developed assays. It is important to consider that working high-throughput with patient-derived cells is much less convenient and more complicated than using cell lines, due to the fact that the rate of duplication of the cells is much lower compared to immortalized cell lines[163]. Extra efforts in miniaturization of the assays can help in making these models appealing for HCS applications. To give an example of how we can increase the screening power of patient-derived cellular models, we proceeded to optimize the format of our co-culture assay in order to allow more compounds to be tested at the same time. In previous studies, we generated data by co-culturing iAstrocytes and Hb9-GFP+ MNs in 96-well dishes; we have now determined the optimal conditions to replicate results in 384-well dishes. We followed previously described methods[11] to obtain homogenous base layers of iAstrocytes, derived from either fibroblasts of healthy individuals or patients affected by fALS or sALS. On top of these, we then cultured MNs and monitored survival, average number of branch points, and the average neurite area after 4 days in culture. Differences between co-cultures in the presence of non-ALS iAstrocytes and ALS iAstrocytes were found in all analyzed features (Fig. 2A). The results are strongly consistent with observations found in the 96-well plates (Fig. 2A), demonstrating that this assay can successfully be scaled up.

After confirming the results from our assay in a larger culture setting, we treated co-cultures of sALS-iAs and MNs with Riluzole (see Methods section). Riluzole treatment increases MN survival (Fig. 2B) in the presence of ALS-iAs when compared to dymethil sulfoxide (DMSO) control treatment. We used these data to calculate the robust Z-factor, a statistical measure of the size of the effect induced by a treatment. The Z-score measure is considered the best available parameter to evaluate the quality of an assay and to assess its suitability for HCS applications[148]. The “robust Z-factor” is a variation of the Z-factor that is less sensitive to outliers[149]. We used results from Riluzole treatment as positive control and DMSO treatment as negative control to calculate a robust Z-factor of 0.69, which qualifies our assay as “very good” as defined by Birmingham and colleagues[149]. These data confirm the robustness and reliability of our model and present it as a possible available tool for large drug screens in the ALS field. They also demonstrate how new cellular models and *in vitro* assays can be increasingly miniaturized to make them more convenient for large screenings.

Published studies have already explored large numbers of compounds; however, with the innovations newly provided by HCA and the new available cellular models, we should consider ALS drug testing to be merely at its beginning. With the drastic reduction in time needed to collect large amounts of data, the ability to screen libraries of containing thousands of compounds with limited personnel requirements and the ever-decreasing costs of the technology, we believe HCA will be a key player in the future of ALS research, as well as many other neurodegenerative diseases. The joint efforts of academia and industry can help to provide new therapeutic approaches so desperately needed by the ALS patients.

Materials and methods

Co-culture Assays

Human tissue collection, fibroblast isolation and conversion were performed as described in Meyer et al.[11] Briefly, biopsy skin samples were enzymatically dissociated with 0.05% Trypsin/EDTA (Invitrogen) to obtain fibroblasts. To convert fibroblasts into iNPCs, a mixture consisting of the retroviral vectors Oct3/4, Sox2, Klf4, and c-Myc was applied to the cell culture with a multiplicity of infection of 10 for each viral vector. The cells were incubated overnight in a final volume of 700-µL medium/viral vector. To differentiate them into astrocytes, the iNPCs were seeded at low density in a 10-cm dish. The day after seeding, the medium was switched from iNPC medium (DMEM/F12, 1% N2, 1% B27, 20 ng/mL FGF2, 20 ng/mL EGF, and heparin, 5 µg/mL; Sigma-Aldrich) to iAstrocyte medium consisting of DMEM plus 10% FBS and 0.3% N2. Astrocytes were allowed to mature for at least 7 days. For the co-culture assay 96-well plates (Corning) and 384-well plates (BD Falcon) were coated with human fibronectin (5 µg/mL; Millipore) as previously described (Meyer et al.). 10,000 iAstrocytes per well were used in the 96-well plates; in the 384-well plates, 2,500 iAstrocytes per well were used. Hb9-GFP+ MNs were differentiated from mESC as previously described[11]. The day after iAstrocyte plating, GFP positive FACS sorted (on a BD FACSVantage/DiVa sorter) MNs were added in culture; 10,000 per well in the 96-well plates and 800 per well in the 384-well plates. Co-culture medium consisted of DMEM/F12, 5% horse serum, 2% N2, 2% B27 plus GDNF (Invitrogen; 10 ng/mL), BDNF (Invitrogen; 10 ng/ mL), and CNTF (Invitrogen; 10 ng/mL.

Riluzole Assay

Riluzole (Sigma) was dissolved in 100% DMSO (Sigma) at a concentration of 10 mM. It was added to the plate at a final concentration of 10 µM 2 hours after iAstrocyte plating. For this experiments iAs deriving from a fibroblasts from a patient affected by sALS were used. Riluzole was administered 2 hours after MN plating, and then again 2 days later.

High Content Analysis

Plate scanning and analysis were performed daily with an IN CELL 6000 confocal plate reader (General Electric). Image analysis was performed with the IN CELL Developer and Analyzer software, which identified cell bodies and neurites based on GFP fluorescent signal. Data visualization and statistical analysis were performed with GraphPad Prism and R.

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Figure legend:

Figure 1. A schematic representation of the co-culture assay developed in our laboratory. iAstrocytes are differentiated starting from iNPCs deriving from fibroblasts of healthy individuals or ALS patients. Hb9-GFP positive stem cells are differentiated into MNs, sorted and added to the iAstrocytes in culture.

Figure 2. A) Differences in survival and morphological changes between MN cultured with healthy or ALS iAs are consistent between 96 and 384-well plates, demonstrating the robustness of the assay. n=3 in each condition. Error bars represent SEM. B) Images captured by the HCA system reveal positive effect on MN survival in culture with ALS affected astrocytes exerted by Riluzole administration (Scale bar = 50 µm). C) The graph reports quantification of normalized MN survival at 4 days *in vitro* in the presence of sALS affected astrocytes after DMSO (negative control) or Riluzole (positive control) administration. n=5 for both conditions. Data were used to calculate a Robust Z-factor (0.679) qualifying our assay as a good assay for different therapeutic screening.

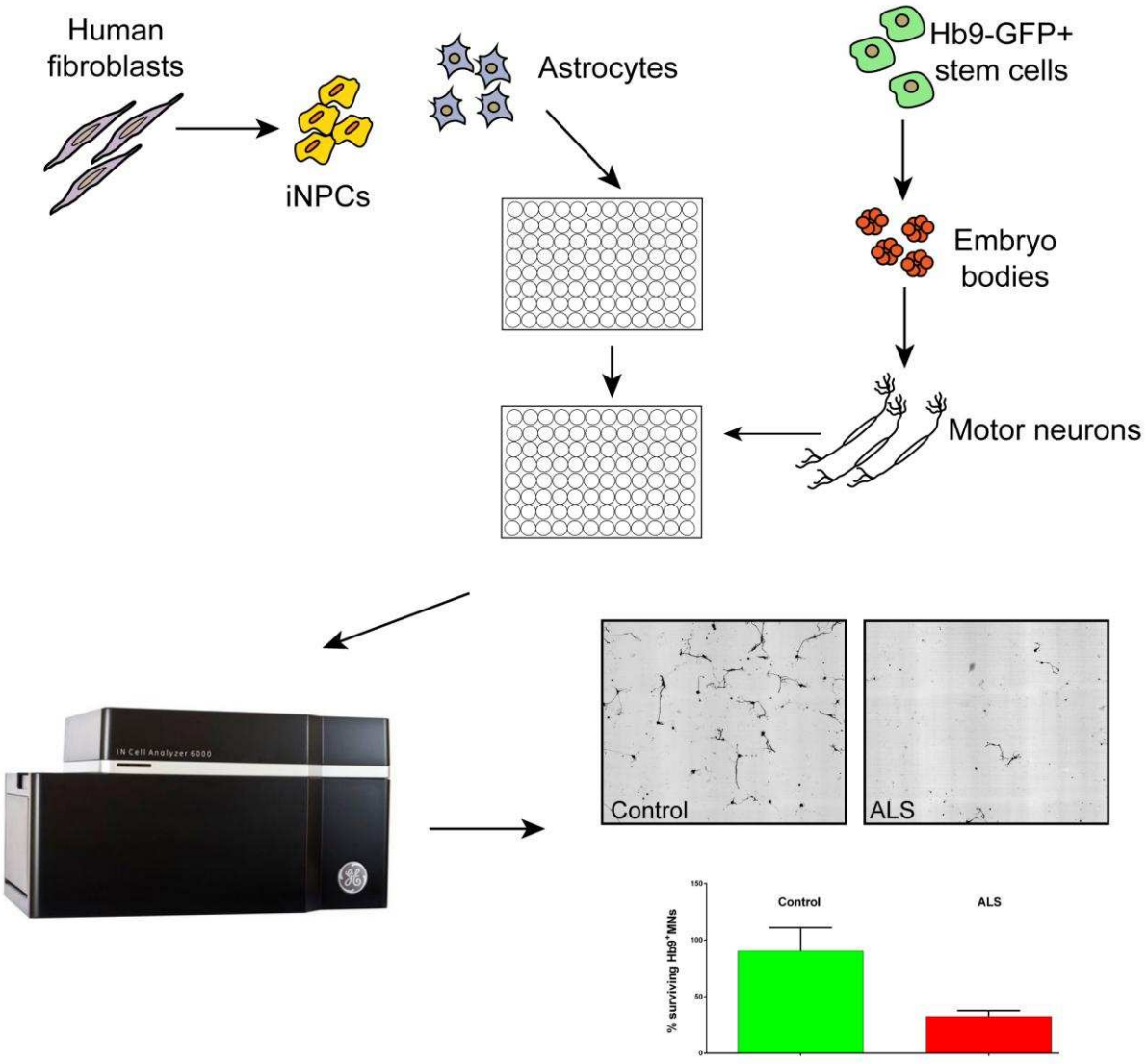

Fig. 1

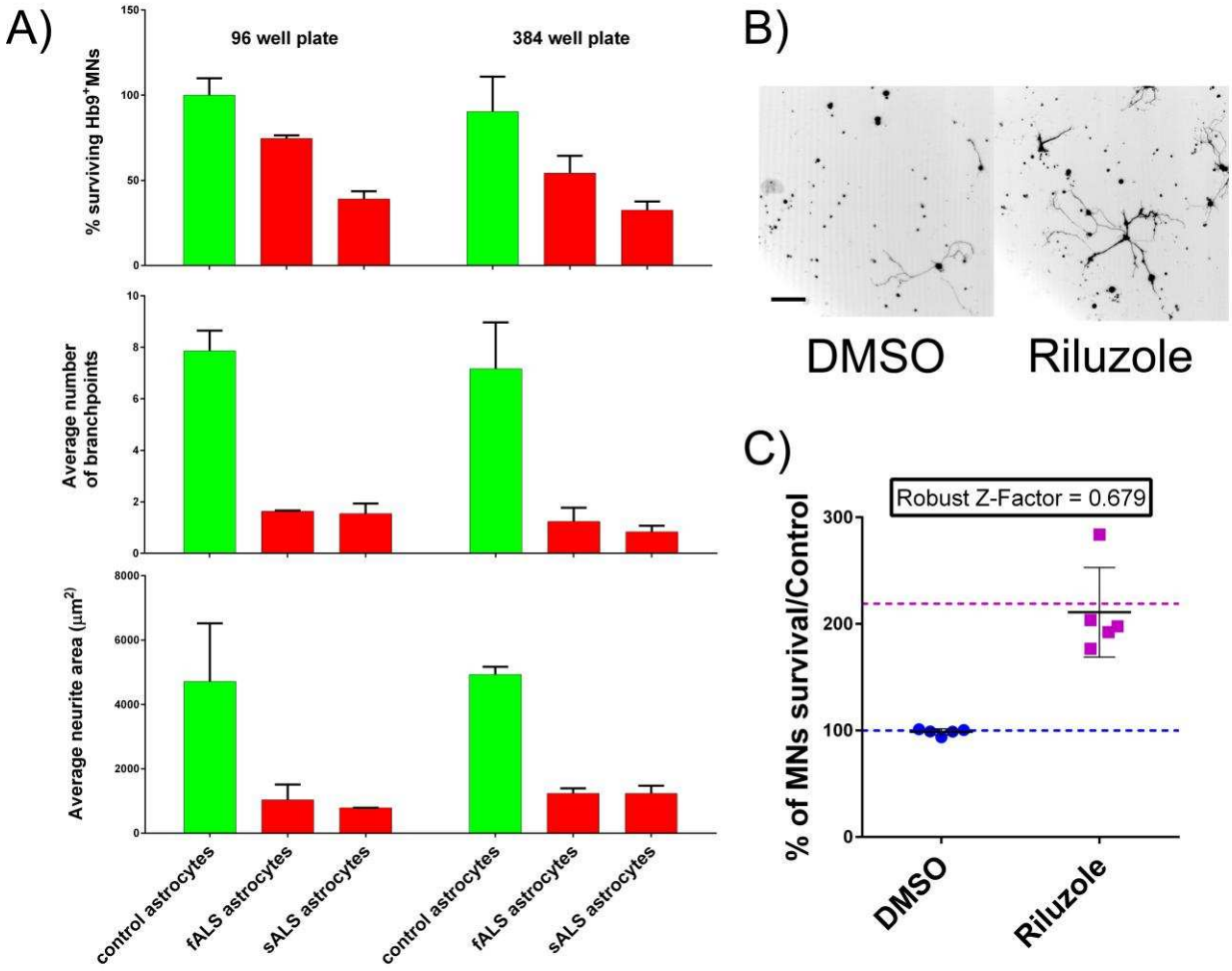

Fig. 2