AWARD NUMBER: W81XWH-21-1-0094

TITLE: A Scalable Screening Platform to Accelerate Early Drug Discovery for Diverse ALS Subtypes

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CONTRACTING ORGANIZATION: University of California, San Francisco, CA

REPORT DATE: October 2022

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release; Distribution Unlimited

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REPORT DOCUMENTATION PAGE

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1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
October 2022	Annual	30Sep2021-29Sep2022

4. TITLE AND SUBTITLE	
A Scalable Screening Platform to Accelerate Early Drug Discovery for Diverse ALS Subtypes	**5a. CONTRACT NUMBER** W81XWH-21-1-0094
	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	**5d. PROJECT NUMBER**
Steven Altschuler, Ph.D	**5e. TASK NUMBER**
E-Mail:	**5f. WORK UNIT NUMBER**
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) REGENTS OF THE UNIVERSITY OF CALIFORNIA, 1855 FOLSOM ST STE 425 SAN FRANCISCO CA 94143-4249	**8. PERFORMING ORGANIZATION REPORT NUMBER**
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Development Command Fort Detrick, Maryland 21702-5012	**10. SPONSOR/MONITOR'S ACRONYM(S)**
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Development of ALS therapies is challenged by phenotypic and genetic heterogeneity and an incomplete understanding of disease mechanisms. Conducting scalable screens for discovering ALS-relevant druggable targets requires addressing several critical questions. First, which pre-clinical models represent ALS disease states and patient heterogeneity yet allow for tractable, unbiased drug target screens? Mouse or neuronal models may capture hallmarks of ALS pathophysiology but are not tractable for large-scale screens. On the other hand, overexpression or isogenic models in cancer cell lines are highly scalable but have diminished ALS relevance. Second, which patient cell models should be selected for screening? There is no obvious typical "normal" or "ALS" patient cell line. In fact, it is likely that there are multiple ALS subtypes. A method for "intelligently" selecting cell lines for studies and validation is needed. Third, which cell phenotypes will distinguish ALS patient subpopulations? ALS can manifest at the cellular level as aberrant protein expression levels and localization patterns (e.g. TDP-43) as well as in altered organellar structure (e.g. fragmented mitochondria). Identifying ALS subtypes will require profiling dysregulated pathways across many patients. Fourth, how can we identify cellular perturbations that "push" ALS subtypes towards healthy states? Incorporating a broad range of readouts to capture ALS pathophysiology will create high-dimensional datasets. Computational methods are required to identify perturbations that move cells from an ALS to healthy phenotypic regions. None of the challenges raised above can be solved in isolation—they must be addressed simultaneously in an end-to-end screening platform.

15. SUBJECT TERMS

None listed.

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON** USAMRDC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
Unclassified	Unclassified	Unclassified	Unclassified	15	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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1. **INTRODUCTION:**
   How can we accelerate drug discovery for ALS patients? A major challenge in the development of effective therapies is variability from one ALS patient to another, including disease onset, progression and genetics. A key lesson from cancer is that identifying disease subtypes can dramatically accelerate the development and approval of effective therapies. We aim to develop a scalable image-based platform to accelerate early drug target discovery for diverse ALS subtypes. The scalability of the platform, both in speed and cost, will be enabled by the use of: (i) microscopy as our measurement modality, (ii) readily available patient-derived fibroblasts for initial large-scale hypothesis generating screens (subsequent low-throughput validation will occur in neurons), and (iii) machine learning algorithms to assist in the selection of small numbers of cell lines to represent each ALS subtype. We aim to use the platform to identify ALS subtypes and search across the druggable genome for perturbations that push one or more of these disease subtypes towards a phenotypically defined state of health.

2. **KEYWORDS:** Neurodegeneration, ALS, drug discovery, machine learning, systems biology

3. **ACCOMPLISHMENTS:**
   What were the major goals of the project?

Task 1 Develop phenotypic profiling platform that separates ALS fibroblasts from healthy controls (WT). Identify optimal imaging biomarkers and cellular stressors required to separate ALS from healthy control fibroblasts. Develop machine learning algorithms for quantifying imaging-based phenotypes and classifying ALS subtypes vs. healthy controls.

- **Milestone:** development of image-based phenotypic screening platform that distinguishes fALS and healthy patient fibroblasts.
- **Anticipated completion:** July 2022
- **Completed:** July 2022

Task 2 Validate fibroblast phenotypes in paired motor neuron cultures. Establish workflow to generate patient-derived motor neurons (MN). Compare MN and fibroblast phenotypes using phenotypic profiling platform described above.

- **Milestone:** connection of MN and fibroblast ALS phenotypes, validation of early drug discovery platform.
- **Anticipated completion:** October 2022
- **Status:** 10% completed. However, we propose to build upon our recent success with FUS-ALS fibroblast subtype profiling. We would like to continue stress-testing fibroblast models across additional genetic subtypes and markers.

Task 3 Identify clusters/ALS subtypes within familial and sporadic ALS cellular phenotypes. Develop machine learning methods for characterizing subpopulations of ALS patients based on cellular phenotypes. Profile sporadic cell lines and stratify based on ALS subtypes identified above. Characterize phenotypic subtypes and underlying pathway-level defects.

- Milestone: identification and validation of ALS phenotypic subtypes within sALS and fALS.
- **Anticipated completion:** April 2023
- **Status:** Completed July 2022 for FUS-ALS subtype comparison. We would like to extend this over the coming year to other genetic subtypes, pending on Task 2 efforts.

Task 4 siRNA screen of druggable genome in canonical patient fibroblasts. Optimize siRNA dosage and time for gene knockdown. Develop machine learning algorithms for quantifying cellular response to perturbation. Screen druggable genome and identify hits that revert ALS phenotypes to health. Validate hits in MN cultures

- **Milestones:** Identification of gene targets for ALS drug development; publication of 1-2 peer reviewed papers.
- **Anticipated completion:** October 2023
- **Status:** Not yet initiated for siRNA. However, motivated by our recent success evaluating ASO treatment on FUS-ALS fibroblasts, we propose to evaluate additional ALS subtypes in our platform for potential benefit from ASO treatment.

What was accomplished under these goals?

Task 1 Develop phenotypic profiling platform that separates ALS fibroblasts from healthy controls (WT).

We developed a scalable microscopy and machine learning platform to phenotypically subtype readily available, primary patient-derived fibroblasts. Application of our platform identified robust signatures for the genetic subtype FUS-ALS (Kumbier et al.; Fig. 2a-b), allowing cell lines to be scored along a spectrum from FUS-ALS to non-ALS. Our FUS-ALS phenotypic score negatively correlates with age of diagnosis (Kumbier et al.; Fig. 2g) and provides information that is distinct from transcript profiling (Kumbier et al.; Fig. 3a,c). Interestingly, the FUS-ALS phenotypic score can be used to identify sporadic patient fibroblasts that have consistent pathway dysregulation with FUS-ALS. Further, we showcase how the score can be used to evaluate the effects of ASO treatment on patient fibroblasts. Our platform provides an approach to move from genetic to phenotypic subtyping and a first step towards rational selection of patient subpopulations for targeted therapies.

Detailed description of our platform and related results are available in a manuscript posted to bioRxiv: ***https://doi.org/10.1101/2022.09.27.509770***

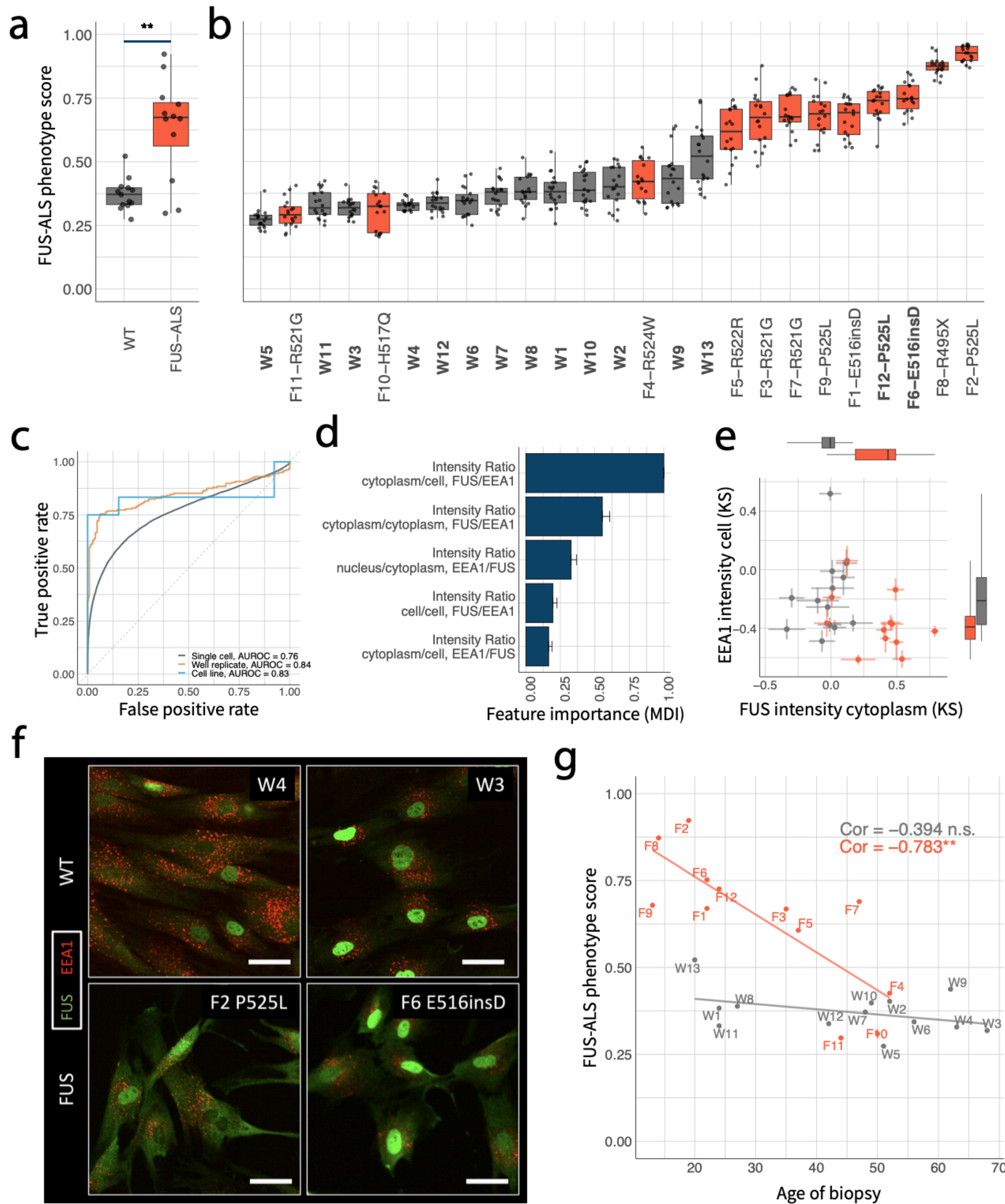


Figure 2: FUS-ALS separation from control WT. a-b, FUS-ALS phenotypic score averaged over cell line (a) or well-level replicates (b). Dots: (a) cell lines or (b) well replicates. p-value: one-sided Wilcoxon rank-sum test; $** < 0.01$. **c,** ROC curve showing single cell, well or cell line classification accuracy based on FUS-ALS phenotypic scores. **d,** Feature importance averaged across all cell line-holdout models and normalized to the top performing feature. MDI: mean decrease in impurity. Error bars: 1 standard deviation across cell line-holdout models. **e,** Two-dimensional projection of FUS-ALS subtype and WT profiles. Dots: averaged Kolmogorov-Smirnov (KS) profiles over well replicates; KS relative to a WT cell line (Methods); lines: 1 standard deviation of KS profiles. **f,** Representative images of cellular phenotypes. Scale-bar: 20um. **g,** Scatterplot of age of biopsy vs. FUS-ALS phenotypic scores. p-value: one-sided t-test, Bonferroni corrected; $** < 0.01$, ns > 0.05. (a-b, e, g) Colors: gray (WT), red (FUS-ALS). (a-b, e) Box plots: median (center line), interquartile range (box) and data range (whiskers).

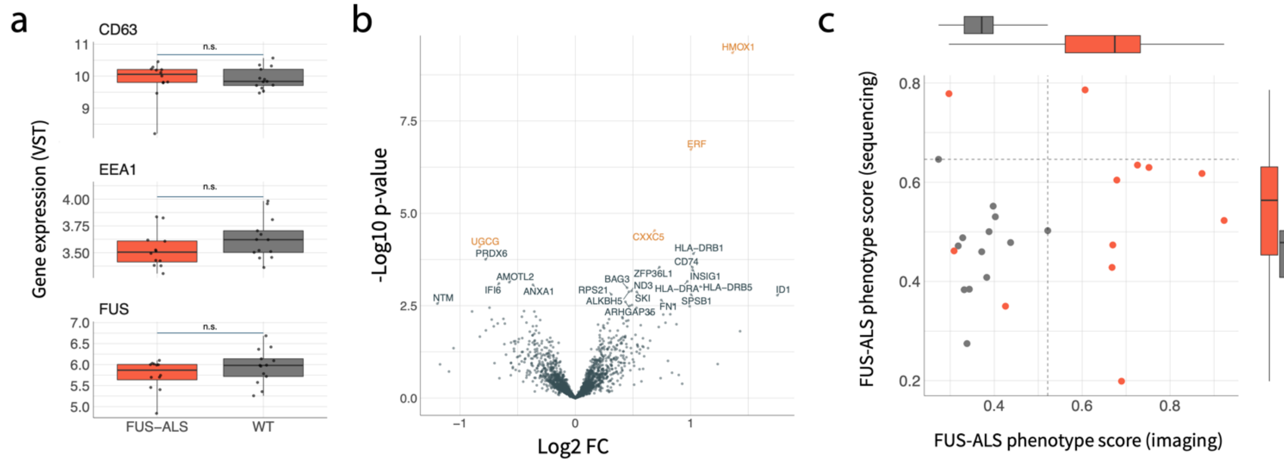


Figure 3: Comparison of phenotypic and transcriptional ALS profiles. a, Gene expression of top biomarkers (variance stabilizing transformation). Dots: individual cell lines. ns: p-value > 0.05, differential expression analysis (Deseq2; Methods). **b,** Volcano plot. X-axis: log2-fold change (FC) FUS-ALS vs WT; Y-axis: -log10 differential expression p-value. Colored genes: corrected BH corrected p-value < 0.05. **c,** Scatterplot of FUS-ALS imaging vs. RNAseq phenotypic scores. Dots: individual cell lines. Dashed lines: maximum scoring WT cell line.

Task 2 Validate fibroblast phenotypes in paired motor neuron cultures.

Departure of lab personnel who were key to the development of motor neuron protocol has been a setback for this aim. As an alternative approach to validating the biological relevance of results in fibroblast models, we have taken advantage of existing collaborations with The New York Genome Center (NYGC) ALS Consortium.

Collaborators at the NYGC have provided us with RNA-seq data on patient-derived spinal cord samples. Over the reporting period, we also generated an RNA-seq dataset on patient-derived fibroblast cell lines used in our phenotypic profiling platform. We conducted an integrated analysis of these data to investigate dysregulation shared between spinal cord and fibroblast samples. Differential expression p-values showed little correlation between spinal cord and fibroblast samples (Kumbier et al.; Fig. E4a-b). However, gene set enrichment analysis highlighted pathway-level dysregulation that was highly consistent across sample type (Kumbier et al.; Fig. 4c).

Moving forward, we propose to build upon our recent success with FUS-ALS fibroblast subtype profiling. We would like to continue stress-testing fibroblast models across additional genetic subtypes and markers.

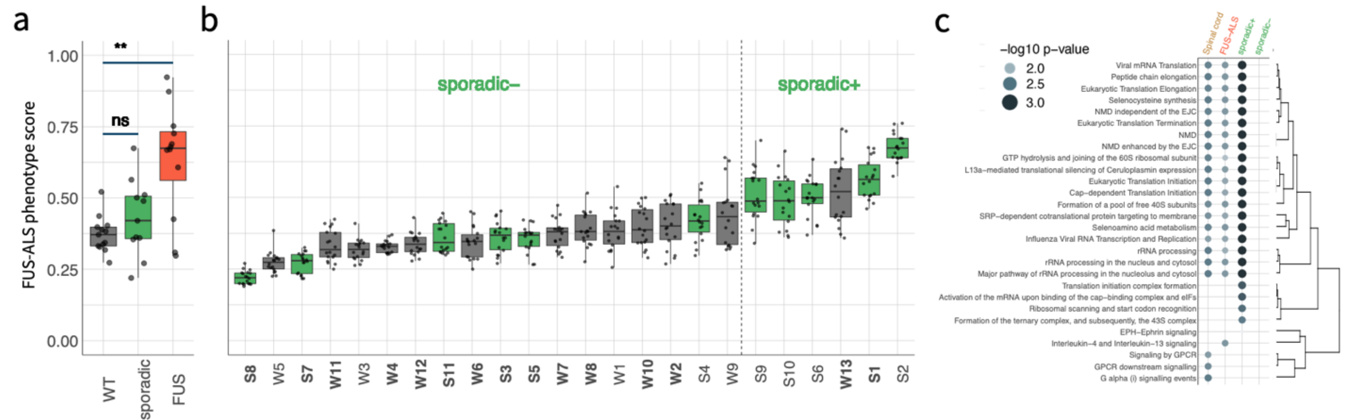


Figure 4: Sporadic ALS separation from control WT. a-b, FUS-ALS phenotypic scores averaged over cell line (a) or well-level replicates (b). Colors: gray (WT), green (sporadic ALS). Dots: (a) cell lines or (b) well replicates. p-value: one-sided Wilcoxon rank-sum test, Bonferroni corrected $** < 0.01$, ns > 0.05. Box plots: median (center line), interquartile range (box) and data range (whiskers). **c,** Pathway (Reactome) enrichment by sample type. NMD: nonsense-mediated decay, EJC: exon junciton complex. Sporadic+: sporadic cell lines with top 5 FUS-ALS phenotype scores. Sporadic-: sporadic cell lines with 6 lowest FUS-ALS phenotype scores. Points size/color: -log10 BH-corrected p-value (gene set enrichment analysis, Methods). Dendrogram: hierarchical clustering using Jaccard distance defined over gene sets associated with each pathway.

Task 3 Identify clusters/ALS subtypes within familial and sporadic ALS cellular phenotypes.

The majority of imaging markers (6 / 9 marker sets) evaluated in our platform provided no meaningful separation between ALS (C9orf72, FUS-ALS, TBK1, and sporadic) and healthy controls (Kumbier et al., Fig. 1 b-c). However, three of the nine tested imaging marker combinations separated at least one FUS-ALS cell line from WT (3 FUS lines, 9 WT lines) and two of nine separated at least one C9orf72 cell line from healthy controls. Based on the strength of signal in FUS-ALS, we focused further studies on this subtype. Specifically, we expanded our library of FUS-ALS and WT cell lines and defined a machine learning-based "FUS-ALS phenotype score." Intuitively, this score quantifies where cellular phenotypes fall along a spectrum from "WT-like" to "FUS-like."

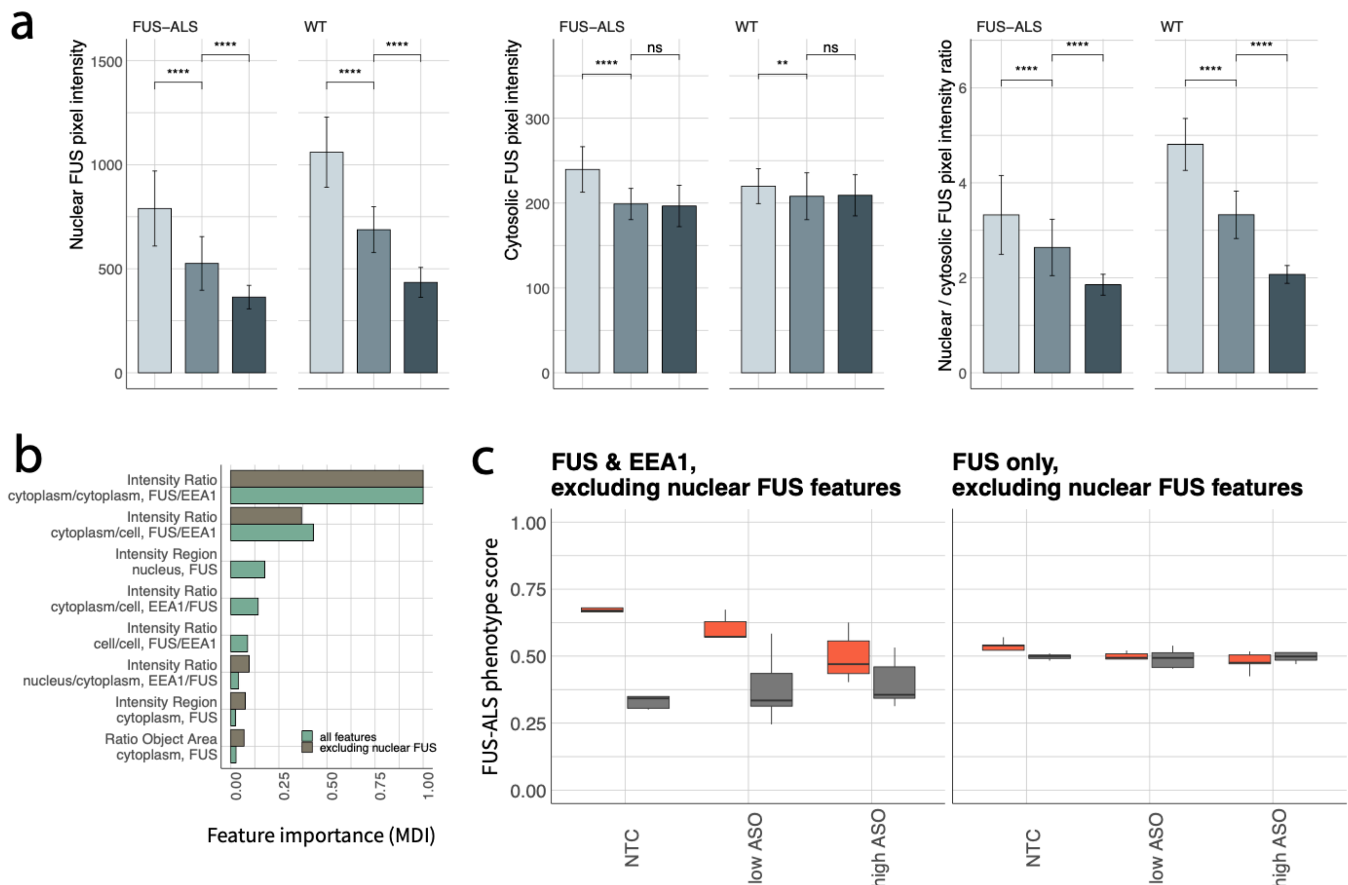
Using our platform, we screened 11 fibroblast cell lines obtained from sporadic ALS patients (along with FUS-ALS and WT cell lines). In addition, we generated RNA-seq profiles for all sporadic, FUS-ALS, and WT cell lines. We evaluated our FUS-ALS phenotype score on the sporadic cell lines. While the sporadic cell lines showed weaker separation from WT than FUS-ALS, five were close to the decision boundary (Kumbier et al.; Fig. 4a-b). We divided sporadic cell lines into high scoring (sporadic+) and low scoring (sporadic-) groups and conducted gene set enrichment analysis for both groups along with FUS-ALS cell lines (Fig. 4bc). Interestingly, sporadic+ cell lines showed consistent pathway enrichment with the FUS-ALS cell lines (Fig. 4c). In contrast, sporadic- showed no pathway enrichment (Fig. 4c). Thus, the FUS-ALS phenotypic score provided a way to identify sporadic subgroups that share pathway dysregulation with FUS-ALS fibroblasts.

Taken together, these results highlight how our platform provides an approach to move from genetic to phenotypic subtyping. Phenotypic subtypes enable the grouping of patients with distinct genetic backgrounds, and most importantly sporadic ALS, into subpopulations that share pathway-level dysregulation.

Task 4 siRNA screen of druggable genome in canonical patient fibroblasts.
Over the reporting period, we used our phenotypic profiling platform screen a library of small molecules and quantify their effects on FUS-ALS cell lines. While several compounds "reverted" the FUS-ALS phenotype to WT, it was not clear whether this reversion represented disease-relevant modulation or simply reflected known targets of the compound.

Excitingly, we had an opportunity to make a slight course correction and test an antisense oligonucleotide (ASO) therapy developed for FUS-ALS. We selected the five youngest FUS-ALS patient cell lines, as they showed the greatest separation in our platform, and age matched them to the five youngest WT cell lines. Cell lines were electroporated with both a low (1uM) and a high (10uM) dose of ASO. Cells recovered for 48h before being stained and imaged with the optimal imaging marker set. Analysis of these treatments using our platform revealed that the overall lowering of FUS levels through the ASO led to a more WT-like cytoplasmic phenotype in mutant cells (as measured by cytosolic EEA1 and FUS intensities; Kumbier et al.; Fig. 5c-d). Interestingly, the separation and reversion obtained from using both EEA1 and cytosolic FUS features was considerably more pronounced than using cytosolic FUS features alone (Kumbier et al.; Fig. 5c).

Motivated by our recent success evaluating ASO treatment on FUS-ALS fibroblasts, we propose to evaluate additional ALS subtypes in our platform for potential benefit from ASO treatment.



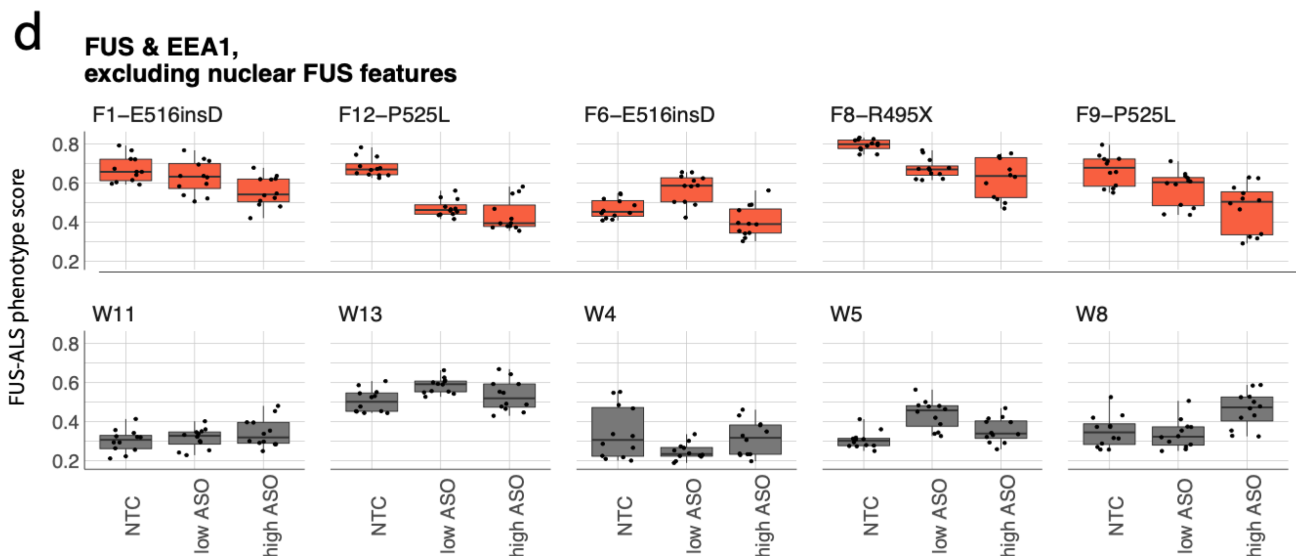


Figure 5: ASO treatment modulates FUS-ALS vs. WT phenotypic separation. a, Effect on average FUS intensity in different cellular compartments due to ASO FUS-knockdown. Bars: average intensity over group; whiskers: standard deviation across well replicates. p-value: one-sided Wilcoxon rank-sum test; ns > 0.05, $** < 0.01$, $**** < 0.0001$. Shading: light grey (no-targeting control), medium gray (low ASO treatment, 1um), dark gray (high ASO treatment, 10um). **b,** Feature importance normalized to the top performing feature. Color: green, models trained using all features; brown, models trained with nuclear FUS features excluded. MDI: mean decrease in impurity. **c,** FUS-ALS phenotypic scores averaged within cell lines and treatments. Models trained on EP cells treated with H2O only. Left/right: models trained with/without nuclear features of FUS. **d,** FUS-ALS phenotypic scores averaged within well replicates (dots) by treatment using models without nuclear FUS features. (c,d) Colors: gray (WT), red (FUS-ALS). (c,d) Box plots: median (center line), interquartile range (box) and data range (whiskers).

What opportunities for training and professional development has the project provided?
The project has allowed for training of a new graduate student, an postdoctoral fellow, and two technicians. Please see section 7.

How were the results disseminated to communities of interest?

- Dr. Kumbier met bi-weekly with members of the New York Genome Center ALS Consortium to discuss results from this work and related areas.
- Over the course of the reporting period, the team met multiple times with the Project ALS therapeutics core at Columbia University to discuss results from this work.
- At the end of the reporting period, the team posted a draft of our manuscript detailing results from this work to bioRxiv in the hopes that this work will be useful to the ALS community. We will submit this work for publication in a journal in the coming weeks.

What do you plan to do during the next reporting period to accomplish the goals?

1. Explore the generalization of our platform to additional ALS subtypes.
   a. Our initial screens provided moderate evidence for separation of C9orf72 cell lines. We plan to further evaluate this evidence across larger libraries of C9orf72 cell lines using the optimal imaging marker sets identified from studies of FUS-ALS, which revealed separation among a subset of C9orf72 cell lines. We will

investigate whether this separation generalizes to new C9orf72 cell lines, and further, whether younger onset C9orf72 patients display the strongest disease phenotypes.
    b. Using RNA-sequencing data from healthy control, FUS-ALS, and sporadic cell lines to guide the selection of improved imaging markers that generalize to new ALS genetic subtypes.

2. Conduct ASO perturbations to ALS subtypes to assess cellular response
    a. For sporadic ALS, we will test FUS ASO on the sporadic subset that exhibits FUS-like phenotypes and look for signs of "reversion" to health
    b. As additional subtypes become separable, we will evaluate the effects of appropriately selected ASO treatment.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Our profiling platform provides an approach to move from genetic to phenotypic subtyping in ALS using primary patient-derived fibroblast cell lines. Phenotypic "ALS subtype scores" quantify a disease spectrum by measuring a distance of each cell line to WT. Our work shows how the FUS-ALS phenotype score can be applied to reveal meaningful information about the ALS patient cell lines:

1. FUS-ALS phenotypic scores negatively correlated with age of FUS-ALS diagnosis: earlier onset patients tended to fall further from WT. This finding suggests that scores may serve as a proxy for quantifying disease severity and may useful for identifying patients that would respond best to therapies.
2. FUS-ALS phenotypic scores suggests a quantitative approach to stratify sporadic ALS patient cell lines. Sporadic ALS patients make up ~90% of reported cases. Most of these patients have no known genetic cause and exhibit clinical heterogeneity (e.g., age/location of onset and disease progression) that is difficult to study. Our Scores groups the sporadic cell lines into subsets that are phenotypically similar or dissimilar with FUS-ALS. Interestingly, the phenotypically similar group shares pathway-level dysregulation with FUS-ALS cell lines as well as spinal cord tissue samples. Such stratification provides a first step towards the development of therapies targeted towards specific ALS subtypes.
3. Observing how cellular perturbations affect the FUS-ALS phenotypic scores can be used to evaluate therapeutic candidates and study disease mechanisms. Fascinatingly, different modeling strategies based on the EEA1 and FUS imaging markers revealed that ASO knockdown both "pushed" WT cells towards FUS-ALS and "reverted" FUS-ALS towards WT. The push from WT to FUS-ALS depended on reduced nuclear FUS levels, consistent with mislocalization of mutant FUS to the cytosol due to a loss of nuclear localization signal. In contrast, the reversion of FUS-ALS towards WT depended on reduced cytosolic FUS levels, consistent with observed patient cases with higher cytosolic levels. Decreased nuclear and increased cytosolic FUS levels in FUS-ALS have led to hypotheses of loss- and/or toxic gainof-function (respectively) for FUS.

What was the impact on other disciplines?

Our work may inspire the larger ND community to build similar platforms the enable profiling of easily obtainable patient samples and stratification of sporadic disease.

What was the impact on technology transfer?
Nothing to report

What was the impact on society beyond science and technology?
Nothing to report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change
Personnel changes in our lab slowed progress on the development of MN protocols. Our success with fibroblasts mitigates this deficit.

Actual or anticipated problems or delays and actions or plans to resolve them
Nothing to report

Changes that had a significant impact on expenditures
Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects
Nothing to report

Significant changes in use or care of vertebrate animals
Nothing to report

Significant changes in use of biohazards and/or select agents
Nothing to report

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

Kumbier K, Roth M, Li Z, Lazzari-Dean J, Waters C, Huang P, Korobeynikov V, Phatnani H, Schneider N, Jacobson M, Wu L. *A scalable screening platform for phenotypic subtyping of ALS patient-derived fibroblasts*. bioRxiv. 2022. Available from: https://doi.org/10.1101/2022.09.27.509770

Acknowledgement of federal support: yes

Books or other non-periodical, one-time publications.
Nothing to report

Other publications, conference papers and presentations.
Nothing to report

- **Website(s) or other Internet site(s)**

Our work is posted on the bioRxiv and linked to on our public lab website:
https://www.biorxiv.org/content/10.1101/2022.09.27.509770
https://www.altschulerwulab.org/publications

- **Technologies or techniques**

The research activities have produced a scalable microscopy and machine learning platform to phenotypically subtype readily available, primary patient-derived fibroblasts. The platform includes both a robust high-content microscopy screening protocol and machine learning pipeline to iteratively search for separation across a broad spectrum of ALS patient subtypes and library of imaging markers

- **Inventions, patent applications, and/or licenses**

Nothing to report

- **Other Products**

  - High-content microscopy imaging database for healthy control and ALS patient-derived fibroblast cell lines. We are currently exploring locations to host this database.
  - Supervised learning models for imaging-based classification of ALS vs. healthy control patient-derived fibroblast cell lines.
  - Optimal imaging marker sets for classification of FUS-ALS vs. healthy control patient-derived fibroblast cell lines.
  - RNA-sequencing and whole genome sequencing data for healthy control and ALS patient-derived fibroblast cell lines. These data will be posted upon publication of our manuscript.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	*Steven Altschuler*
Project Role:	*Principal Investigator*

Researcher Identifier: *saltsc*
Nearest person months worked: *0.6*
Contribution to Project: *Dr. Altschuler supervised the project, providing guidance on overall direction, experimental design, and analysis.*

Name: *Lani Wu*
Project Role: *Principal Investigator*
Researcher Identifier: *0000-0002-0052-7537*
Nearest person months worked: *0.223*
Contribution to Project: *Dr. Wu supervised the project, providing guidance on overall direction, experimental design, and analysis.*

Name: *Karl Kumbier*
Project Role: *Postdoctoral researcher*
Researcher Identifier: *0000-0001-6521-1173*
Nearest person months worked: *3.5*
Contribution to Project: *Dr. Kumbier provided analytical input to experimental Design, developed computational analysis methods, and Analyzed imaging and sequencing.*

Name: *Maike Roth*
Project Role: *Lab technician*
Researcher Identifier: *0000-0003-1003-7002*
Nearest person months worked: *3.5*
Contribution to Project: *Ms. Roth provided input into experimental design and performed work in the areas of culturing cell lines, microscopy screens, and sample prep for sequencing*

Name: *Zizheng Li*
Project Role: *Graduate student researcher*
Researcher Identifier: *0000-0002-6953-7733*
Nearest person months worked: *4*
Contribution to Project: *Mr. Li provided input into and conducted antisense oligonucleotide experiments.*
Funding Support: *UCSF's Graduate Division funding.*

Name: *Ping Huang*
Project Role: *Lab technician*
Researcher Identifier: *0000-0002-7345-2927*
Nearest person months worked: *10*
Contribution to Project: *Ms. Huang helped Maike Roth with culturing and screens of patient derived cell lines and microscopy-based screens.*

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Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report

What other organizations were involved as partners?

Organization Name: *Project ALS*
Location of Organization: *New York*
Partner's contribution to the project: *Financial support, collaboration*

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *Nothing to report*

QUAD CHARTS: *Nothing to report*

9. APPENDICES:

See attached pdf.