Capturing Ultra-Rare CRISPR-Cas9 Off-Target Editing Events in Single Cells

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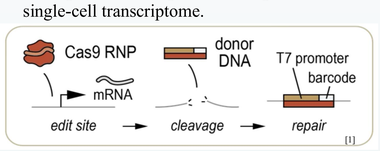
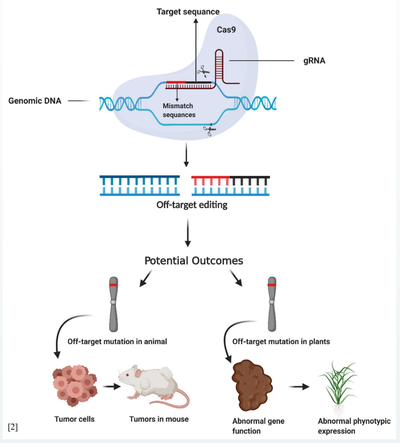
Standard Pipelines Miss Ultra-Rare Edits

- **The CRISPR Off-Target Challenge:** Powerful tool, however, often cuts unintended genomic sites (off-targets).
- **The Technology (Superb-seq):** Inserts barcodes at DNA break sites to simultaneously capture on-target and off-target edits alongside the single-cell transcriptome.



- **The Gap:** Standard analysis filters out edits found in >3 cells, potentially missing rare biological events.
- **Objective:** Develop a pipeline that distinguishes single-cell **off-target edits** from false positives.



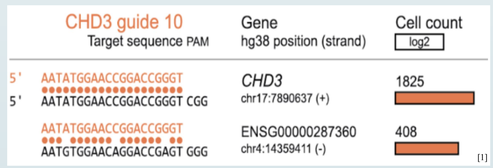
An Adaptive Statistical Pipeline to Filter Noise

Step 1: Candidate Discovery

- **Action:** Relaxed "Sheriff" pipeline filters.
- **Detail:** Retained candidate edit sites appearing in **1 cell** (vs. standard 3) without requiring bidirectional support.
- **Goal:** Capture ultra-rare single-cell editing events.

Step 2: Homology Scoring

- **Action:** Sequence Alignment.
- **Detail:** Scored candidate sites against the intended gRNA using global alignment (Match +1, Mismatch -1, Gap -2).
- **Metric:** Higher scores indicate likely off-target activity rather than sequencing noise.

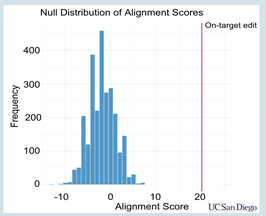


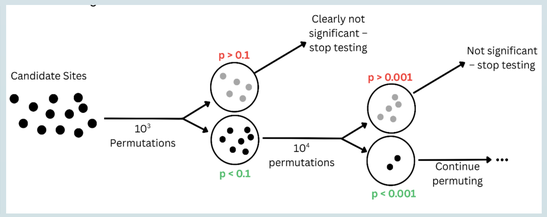
Step 3: Null Model Construction

- **Action:** Scrambled Permutations.
- **Detail:** Generated randomized (scrambled) gRNA sequences and re-scored against the genome.
- **Goal:** Establish a baseline distribution of alignment scores expected by random chance.

Step 4: Adaptive Statistical Testing

- **Action:** Empirical P-Value Calculation.
- **Detail:** Compared observed scores to the null distribution. Used **adaptive permutations** (increasing from 10^2 to 10^6 iterations) to refine p-values for high-significance candidates.

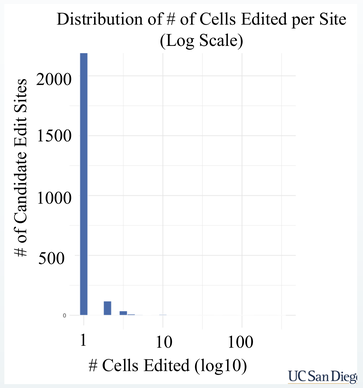


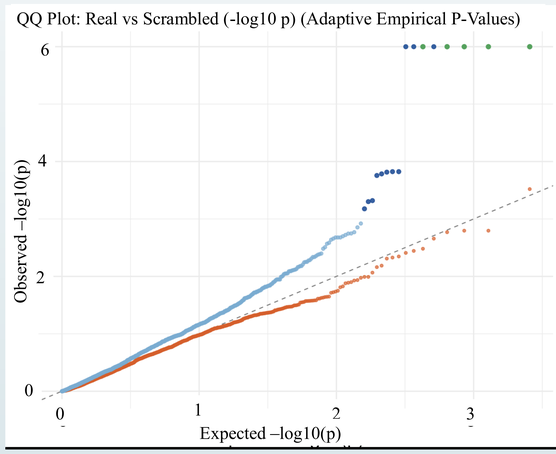


Identification of 11 Novel Off-Targets on Chr 1

Sheriff Setting	# Edits Detected
Strict (default)	6
Relaxed (mine)	2562

Figure 1: Relaxed filtering reveals a hidden population of single-cell edits. Standard pipelines detect minimal off-target activity. Relaxing filters reveals thousands of candidate sites (x-axis) occurring in only 1–2 cells (y-axis), necessitating a sensitive statistical approach to distinguish signal from noise.





Significant Sites	Count
Confirmed (prior work)	5
New (this study)	11

Figure 2: Identification of 11 Novel Off-Targets. The QQ plot compares observed alignment scores against a scrambled null distribution. Points marked in dark blue represent significant off-target events ($p < 0.001$). We identified 11 new ultra-rare sites (blue) missed by standard analysis.

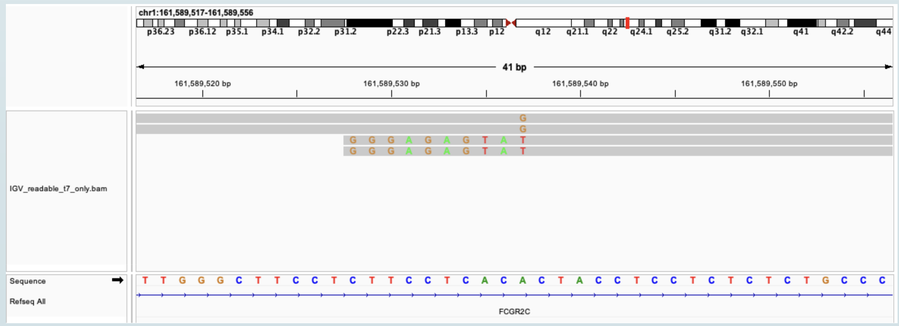


Figure 3: Validation of a Single-Cell Off-Target Edit. A confirmed edit in the FCGR2C gene. This event was present in just 1 cell but excluded by standard pipelines due falling under the 3-cell minimum filter. Our pipeline successfully recovered it with high statistical confidence.

Deep Safety Profiling at Single-Cell Resolution

- **Discovery of Novel Off-Targets:** We identified **11 new ultra-rare off-target sites** on Chromosome 1 that were missed by the standard Sheriff pipeline.
- **Validation of Single-Cell Events:** We manually validated a specific off-target edit in the *FCGR2C* gene. This event occurred in only **one cell** and lacked bidirectional sequencing support, proving that biologically real edits exist below standard detection thresholds.
- **Methodological Success:** The **adaptive permutation strategy** successfully distinguished true biological signal from sequencing noise without the computational cost of running millions of permutations for every candidate site.

Significance & Impact

- **Therapeutic Safety:** Standard pipelines filter out single-cell events to reduce noise. However, in clinical contexts like gene therapy or stem cell engineering, a **single off-target mutation** in a pro-oncogene could theoretically lead to clonal expansion and malignancy.
- **Deep Safety Profiling:** This pipeline establishes a new framework for "deep safety profiling" of gene editing tools, demonstrating that it is possible to monitor genotoxicity at the single-cell resolution.

Future Directions

- **Genome-Wide Expansion:** The current analysis was restricted to Chromosome 1 (approx. 8% of the genome). We plan to scale the adaptive permutation pipeline to the **full human genome**, where we expect to uncover hundreds of additional rare off-target sites.
- **Functional Consequences:** Since Superb-seq simultaneously captures the transcriptome, our next step is to link these rare off-target edits to **differential gene expression** in the affected single cells to assess phenotypic impact.

Acknowledgements

- **Mentors:** Special thanks to **Brad Balderson** for guidance on the Sheriff pipeline and troubleshooting, and **Graham McVicker** for project direction and support.
- **Lab Support:** The entire McVicker Lab for helpful discussions and feedback.
- **Funding:** This work was supported by the **UC LEADS** and **STARS** programs.

References

[1] Lorenzini MH, Balderson B, Sajeev K, Ho AJ, McVicker G. Joint single-cell profiling of Cas9 edits and transcriptomes reveals widespread off-target events and effects on gene expression. bioRxiv [Preprint]. 2025 Aug 28:2025.02.07.636966. doi: 10.1101/2025.02.07.636966. PMID: 40909645; PMCID: PMC12407703.

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