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College of Arts & Sciences
Forensic & National Security Sciences Institute

Characterizing stutter in single cells and the impact on multi-cell analysis

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Disclosure

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- The opinions, findings, and conclusions or recommendations expressed in this publication/program/exhibition are those of the author(s) and do not necessarily reflect those of the Department of Justice.

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Overview

- Why are we interested in single cells (sc)?
- Stutter in sc
  - Characterizing sc Stutter
  - Predicting multi-cell stutter

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Questions

- How does stutter manifest when **one diploid copy** is present?
  - Does it **behave differently than bulk** sample amplification?
  - What **level** can we expect and what is the **variance**?
  - How **often** does it occur?
- Are there trends or patterns?
  - Repeat type: **simple** vs **compound** vs **complex**
  - Locus-specific characteristics
- Can we use this information to help bulk sample analysis?
  - At **what DNA quantity** can we expect to see **variability stabilize**

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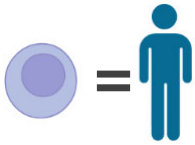
Why Single Cells?



- One cell…one donor → **High resolution**
  - ✓ Assumptions can change (bulk vs. single cell)
- Cell-type known
  - ✓ Profile can be traced back to the specific cell
- No NOC or mixture interpretation
  - ✓ Answers → source (cell type) and sub-source (DNA profile)
- Use to condition mixed profiles

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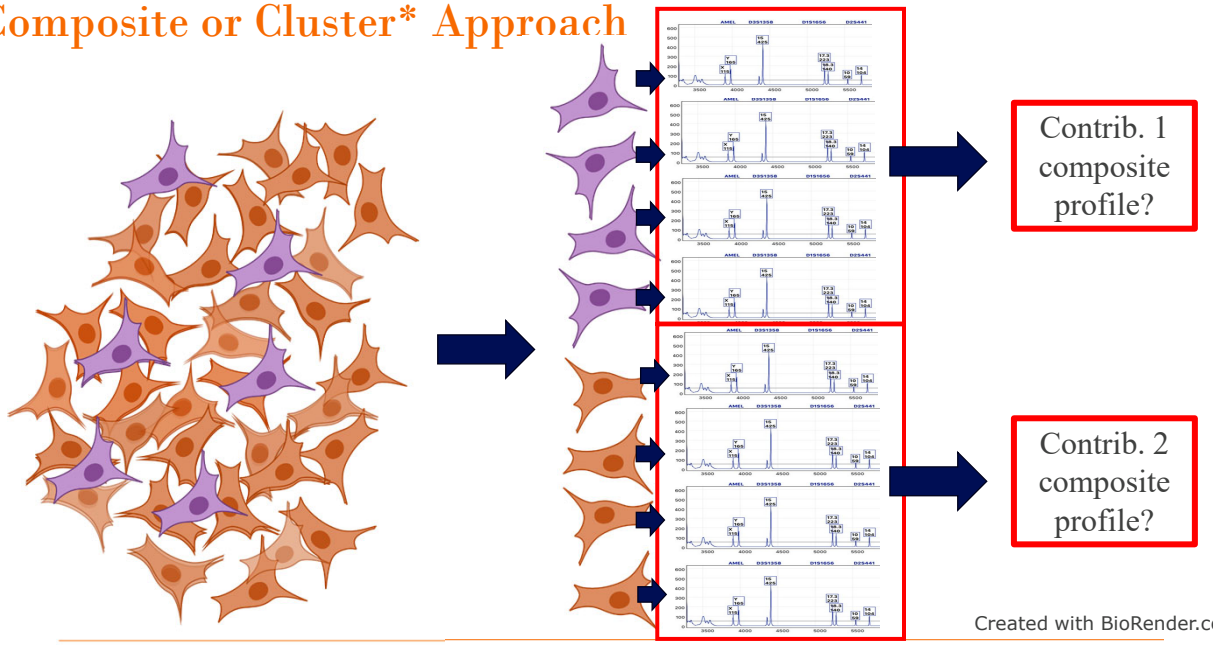
Composite or Cluster* Approach



*Grgicak, C. M., Bhembe, Q., Slooten, K., Sheth, N. C., Duffy, K. R., & Lun, D. S. (2024). Single-cell investigative genetics: Single-cell data produces genotype distributions concentrated at the true genotype across all mixture complexities. Forensic Science International: Genetics, 69(November 2023), 103000. https://doi.org/10.1016/j.fsigen.2023.103000

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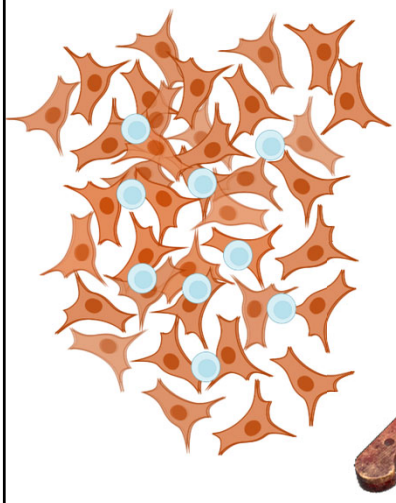
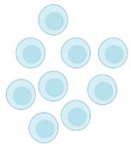
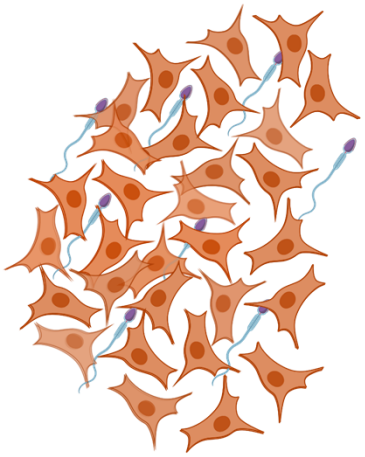
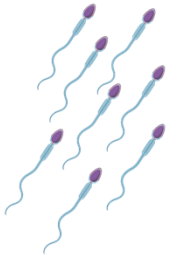
Separating cell types









- Replace Diff?
- Low-level male component

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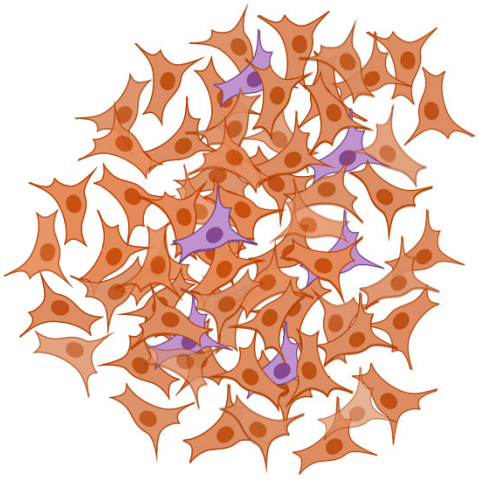
Applications: Random sampling to improve mixture ratio



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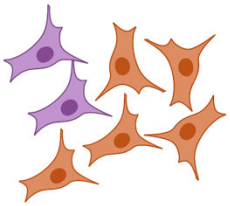

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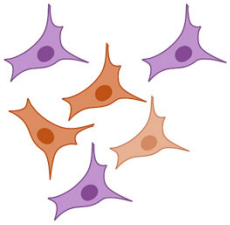

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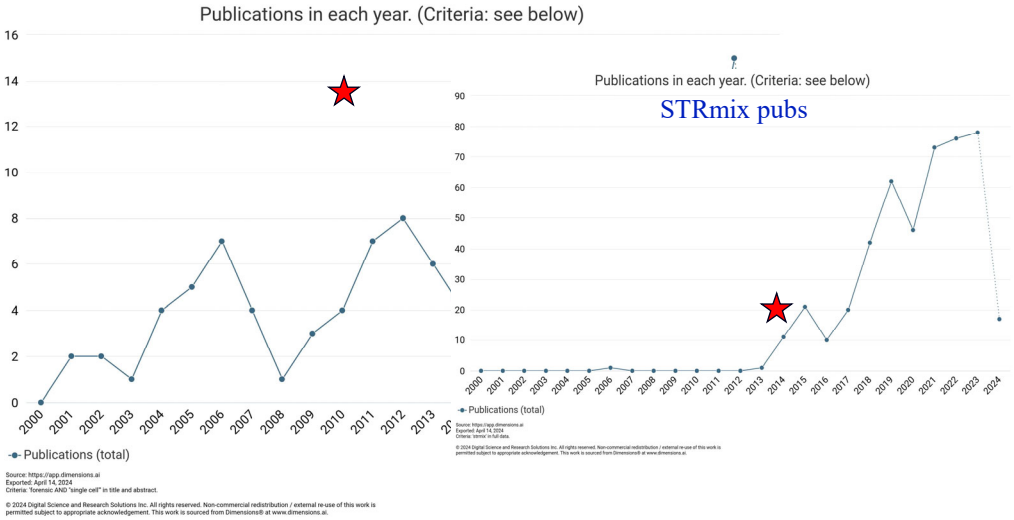
Single Cell Analysis in Forensic Science



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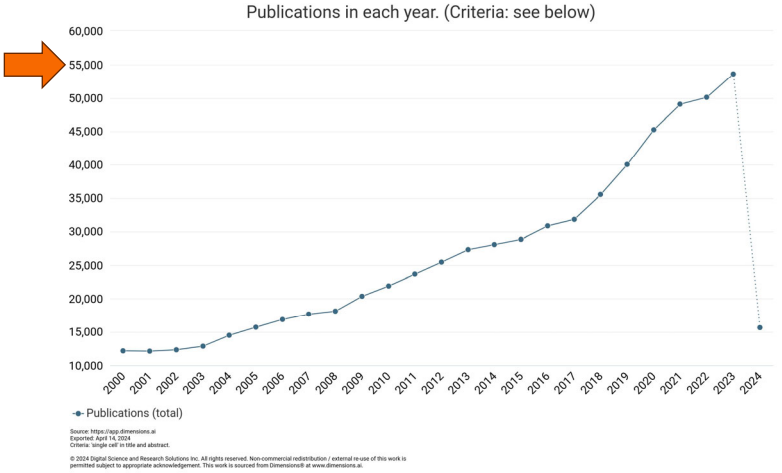
Single Cell Publications – ***Across fields***



…Single cell analysis is a …thing.

Cell Symposia
The conceptual power of single cell biology
August 28–30, 2023 — San Diego, CA, USA

Single-Cell Genomics
Gordon Research Conference

Cold Spring Harbor Laboratory
Single Cell Analysis
June 27 - July 13, 2024

SINGLE CELL GENOMICS 2023
October 9-11, 2023
Engelberg, Switzerland

NIH National Institutes of Health
Office of Strategic Coordination–The Common Fund
Single Cell Analysis Program (SCAP)

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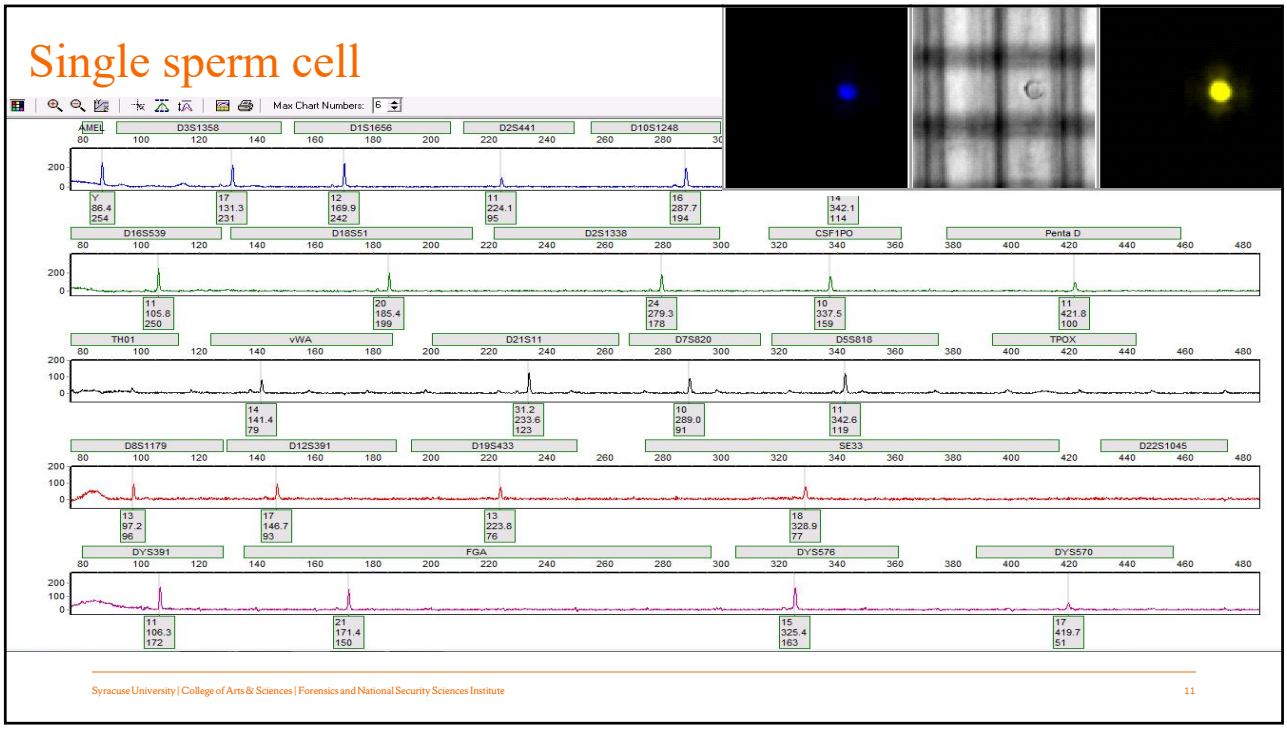
Single sperm cell
Max Chart Numbers: 6
AMEL
D3S1358
D1S1656
D2S441
D10S1248
D16S539
D18S51
D2S1338
CSF1PO
Penta D
TH01
vWA
D21S11
D7S820
D5S818
TPOX
D8S1179
D12S391
D19S433
SE33
D22S1045
DYS391
FGA
DYS576
DYS570
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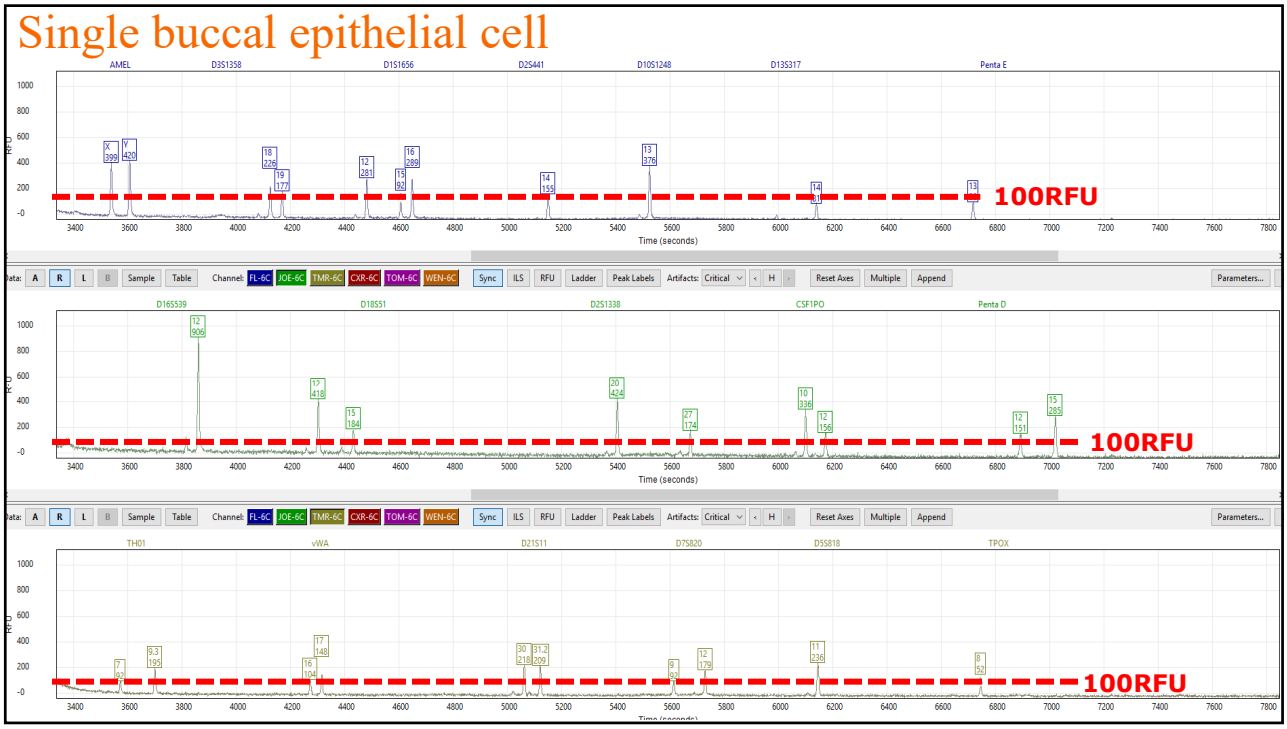
Single buccal epithelial cell
AMEL
D3S1358
D1S1656
D2S441
D10S1248
D13S317
Penta E
100RFU
D16S539
D18S51
D2S1338
CSF1PO
Penta D
100RFU
TH01
vWA
D21S11
D7S820
D5S818
TPOX
100RFU
RFU
Time (seconds)

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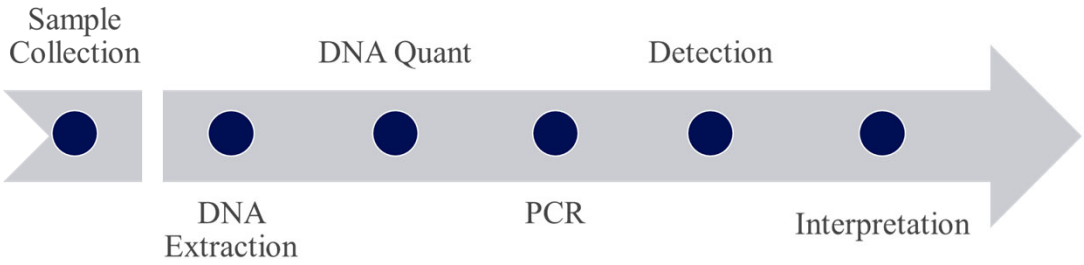
How does it fit into the current process?



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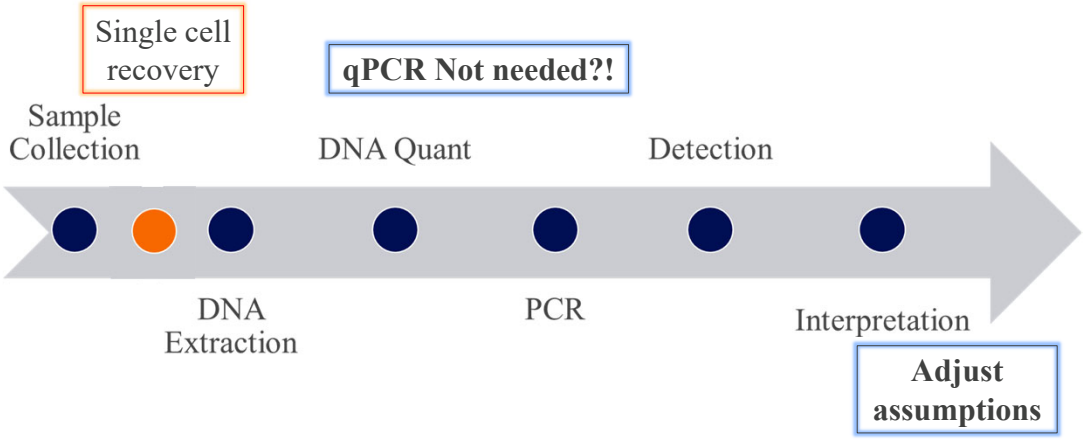
How does it fit into the current process?



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Single Cell Analysis Overview

How to ***interpret*** single cell data

What are the assumptions and how are these different than multi-cell?

- ✓ **Assumption 1**: One cell, one contributor
- ✓ **Assumption 2**: Heterozygote balance → 2 alleles at a locus = homologous pair
- ✓ **Assumption 3**: Stochastic – if a single allele is detected above threshold → assigned to single donor

Vhhp v#hdv|#hqrxjk$ ...

- Allele and locus dropout are expected (the norm)
- How do artifacts manifest in the data?

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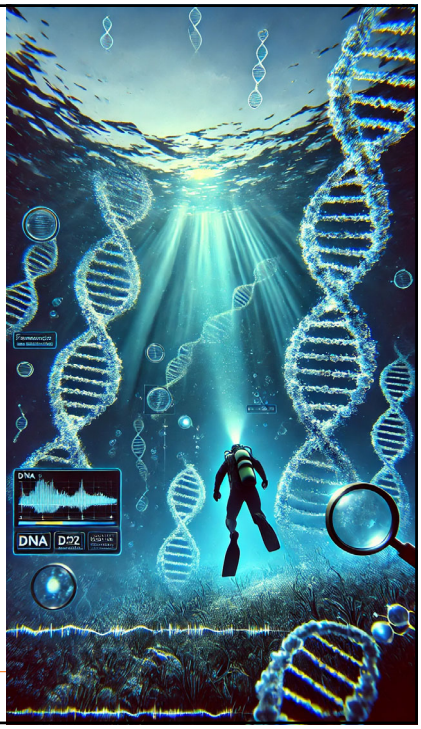
Continue "writing the book on biology…"

- There is still much to understand
- We have observed patterns and created models and to account for uncertainty…
  - How much of the uncertainty is because we don't understand the mechanism fully?
- Deep dive into sc stutter



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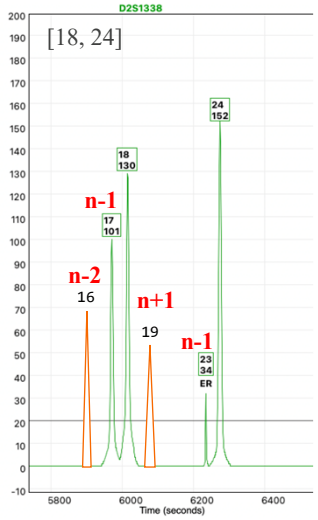
Stutter



- More precise stutter detection → more accurate profile interpretation
- Static, locus-specific, (LUS)-specific models [1-4], *Probabilistic Genotyping* [5]
- Determined for samples with **high levels of DNA** (0.25ng -1.00ng)

overestimating stutter → removal of a true allele

underestimating stutter → misclassification of a stutter product as a true allele [6,7]

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General Methods and Workflow

Targeting and Recovery
(DEPArray™ NxT)

DNA Extraction
(DEPArray™ LysePrep Kit)

Amplification
(PowerPlex® Fusion 6C System)

https://www.promega.com/products/forensic-dna-analysis-ce/str-amplification/powerplex-fusion-6c-system/

Detection
(ABI Genetic Analyzer 3500xL)

https://assets.thermofisher.com/TFS-Assets/LSG/brochures/cms_078314.pdf

Analysis
(Osiris 2.16 AT=20RFU)

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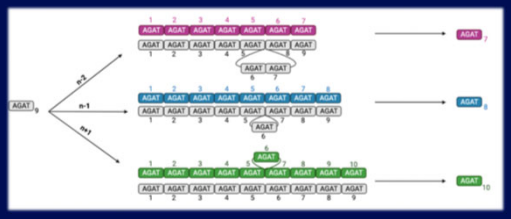
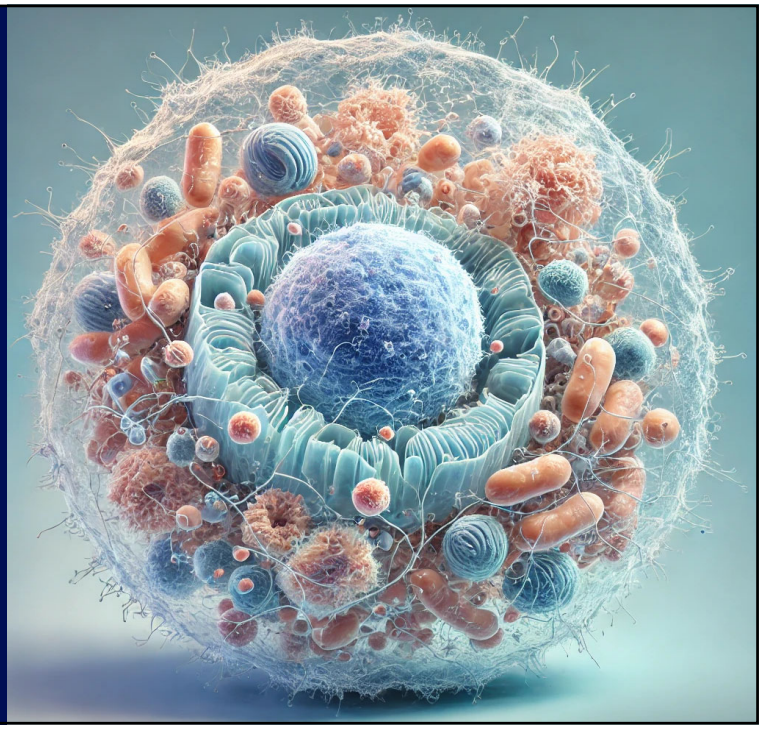
Characterizing Single Cell Stutter





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Methods

- How does stutter in single cells manifest?
- Focus: n-1, n+1 and n-2
  - Obligate stutter products → only explanation is specific type of stutter
- "Sample size" = 180 cells (41 WBCs, 139 buccal epithelial cells); 6 contributors
  - **29 cycles** n=125; **30 cycles*** n=41

Stutter ratio $S_R = \frac{stutter\ PH}{true\ allele\ PH}$ (eq. 1)

Stutter frequency $S_F = \frac{number\ of\ stutter\ occurences}{number\ of\ true\ alleles}$ (eq. 2)

*D.R.L. Watkins, D. Myers, H.E. Xavier, M.A. Marciano, Revisiting single cell analysis in forensic science, Nature Scientific Reports 2021 11:1 11 (2021) 1–12. https://doi.org/10.1038/s41598-021-86271-6.

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Additional terms

- **St_t – total stutter**
  - all possible stutter events
  - this includes when stutter *does not* occur

Preliminary data

- **St_p** – all **positive stutter** present
  - i.e., all non-zero occurrences of stutter
  - Used to answer question : *When stutter occurs what can be expected*

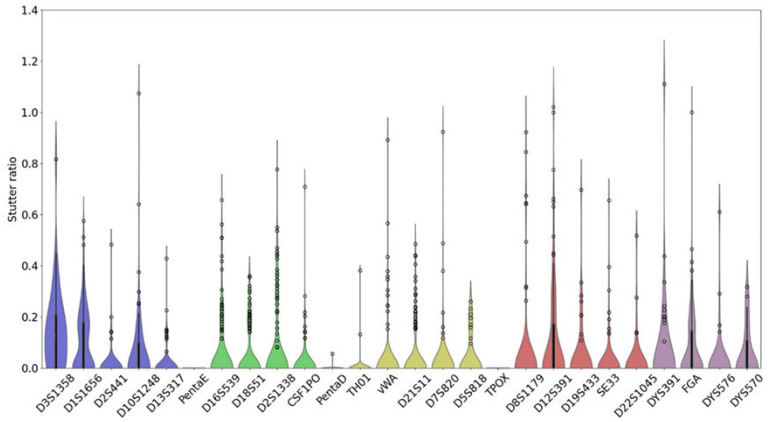
n-1 (29 cycle) stutter

- **St_p (all positive stutter)**
  - *Range*: 5.67% to 56.92 % ± 23.72
  - *Min*: PentaD (n=111) – only one observed stutter peak
  - *Max*: D8S1179
- **St_t (all possible stutter events, whether present or absent)**
  - *Range:* 0 to 14.56 ± 15.90
  - Min: TPOX and PentaE
  - Max: D3S1358
  - When it happens…it is high

Locus	Repeat structure	n	St_t mean stutter (%)	St_p mean stutter (%)
D3S1358	simple	43	14.56 ± 15.90	21.60 ± 14.90
D1S1656	complex	133	10.27 ± 12.64	21.17 ± 9.89
D2S441	simple	68	1.80 ± 6.97	20.38 ± 14.00
D10S1248	simple	105	6.24 ± 14.25	19.71 ± 19.52
D13S317	simple	93	1.92 ± 6.16	16.27 ± 9.61
PentaE	simple	111	0.00	NA
D16S539	simple	131	6.34 ± 13.33	26.84 ± 14.26
D18S51	simple	128	5.08 ± 9.83	22.15 ± 6.38
D2S1338	compound	138	7.30 ± 15.24	32.01 ± 15.08
CSF1PO	simple	103	2.14 ± 8.62	23.26 ± 18.66
PentaD	simple	104	0.05 ± 0.56	5.67
TH01	simple	187	0.27 ± 2.95	25.66 ± 17.71
vWA	compound	145	3.24 ± 11.75	35.41 ± 19.68
D21S11	complex	149	4.60 ± 10.63	25.21 ± 9.94
D7S820	simple	85	2.85 ± 12.30	34.58 ± 28.94
D5S818	simple	41	4.23 ± 8.15	17.97 ± 5.60
TPOX	simple	45	0.00	NA
D8S1179	simple	100	5.17 ± 17.79	56.92 ± 23.72
D12S391	compound	126	10.31 ± 20.44	37.28 ± 22.54
D19S433	simple	47	4.75 ± 12.82	27.90 ± 18.50
SE33	simple	76	2.89 ± 10.08	27.43 ± 17.87
D22S1045	simple	36	2.98 ± 10.02	26.79 ± 17.90
DYS391	simple	47	6.87 ± 18.57	32.27 ± 29.29
FGA	complex	133	7.42 ± 13.55	22.62 ± 14.71
DYS576	simple	38	3.19 ± 11.24	30.28 ± 21.54
DYS570	simple	27	5.75 ± 9.98	19.40 ± 8.19

St_t n-1 (29 cycle)

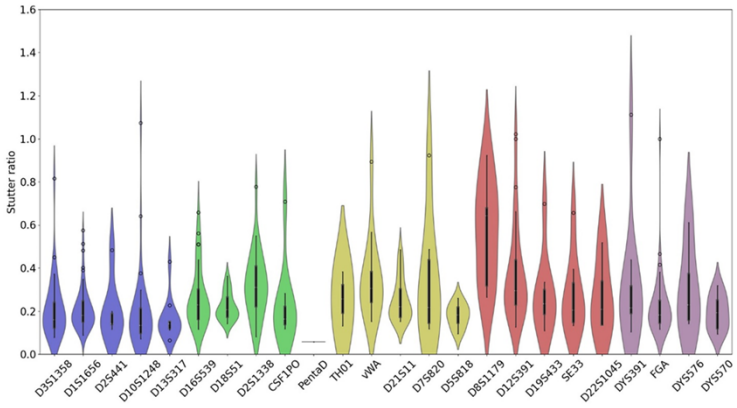
- Generally, as locus size increases stutter decreases



Stt violin plots, 29 cycles, n-1 stutter. Distribution of n-1 stutter ratios of all possible occurrences of stutter across loci (by channel) when amplified using 29 cycles.

St_p n-1 (29 cycle)

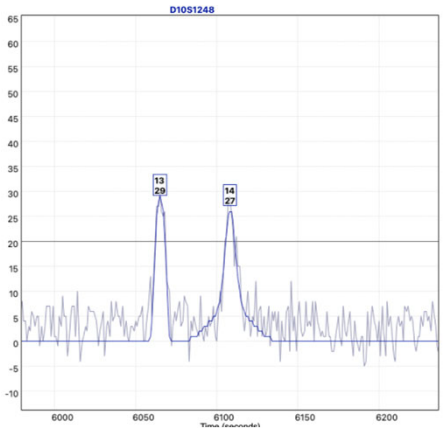
- Generally, as locus size increased stutter decreases
- TH01, D8, D22
  - High stutter when it occurs…more frequent



Stp violin plots, 29 cycles, n-1 stutter. Distribution of n-1 stutter ratios of all positive occurrences of stutter across loci (by channel) when amplified using 29 cycles.

Stutter Ratio ≥….. n-1 (29 cycle)

- 2439 instances of possible stutter
  - 13% (> S_R= 15%)
  - 6.2% (> S_R= 25%)
  - 1.4% (> S_R= 50%)
- Equal to or greater than the stutter causing peak?
  - 0.2% (5/2439)
    - D10S1248 locus S_R= 107%,
    - FGA S_R=100%,
    - DYS391 S_R= 111%,
    - two observations at D12S391 S_R= 100% and S_R= 102%



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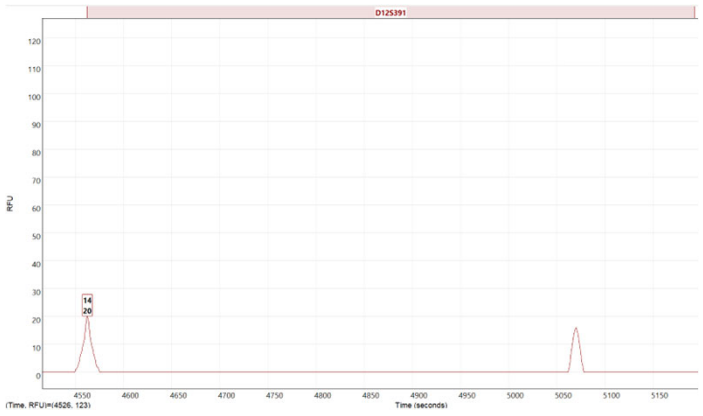
Where'd the true allele go?

- N-1 (29 cycles)
- D12S391
- expected genotype
  - [15,20]
  - 14 peak is the observed stutter at this locus



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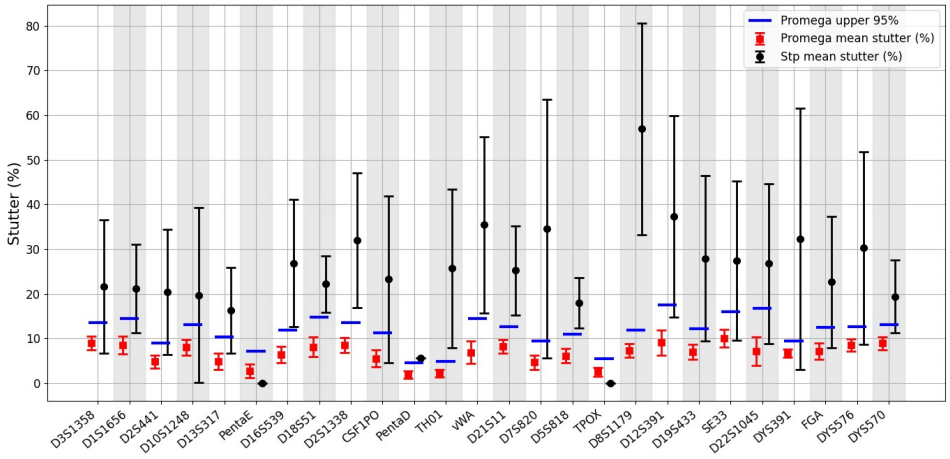
Promega PPF6c stutter vs. sc Stp
n-1 (29 cycle)

- Promega data based on 0.5-1ng samples [9]
- Sc stutter higher
- 95% upper limit (Promega stutter threshold) NOT appropriate for sc samples



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n+1, n-2 (29 cycles)

- n+1
  - St_p range 14.02% to 81.48%
  - 14/26 loci had no n+1 stutter observed
  - not observed in both PentaE or PentaD (n=111 and n=104, respectively)
  - one instance of a stutter peak of 172% at TH01 was observed
- n-2
  - St_p range →8.64% to 86.67%
  - 10/26 loci had no n-2 stutter observed
  - PentaE and PentaD did have 1 occurrence each of n-2 stutter
  - no instances of a stutter peak over 100% observed

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30 cycles

- n-1 at 30 cycles,
  - St_p decreased →29 cycles 26.20% to 19.16%, at 30 cycles
  - PentaD and PentaE did exhibit stutter, but stutter was present at the lowest percent and frequency compared to all other loci.
  - No stutter was observed at 30 cycles at D13S317.
  - A one-way ANOVA (α=0.05) - evaluate the locus-specific stutter at 29 and 30 cycles
    - indicate that **cycle dependent variations** in stutter percentages are largely not statistically significant (18/26 loci),
    - Significant differences were observed in 8 loci: D1S1656 PentaE, D16S539, D18S51, TH01, vWA, D21S11, TPOX and FGA
- n-2 and n+1
  - 29 and 30 cycles were similar (no ANOVA)
  - St_p
    - n-2 stutter rate across all loci: 29 cycle: 36.15% 30 cycles: 13.72%.
    - n+1: 29 cycle: 29.29% and 30 cycles 14.38%

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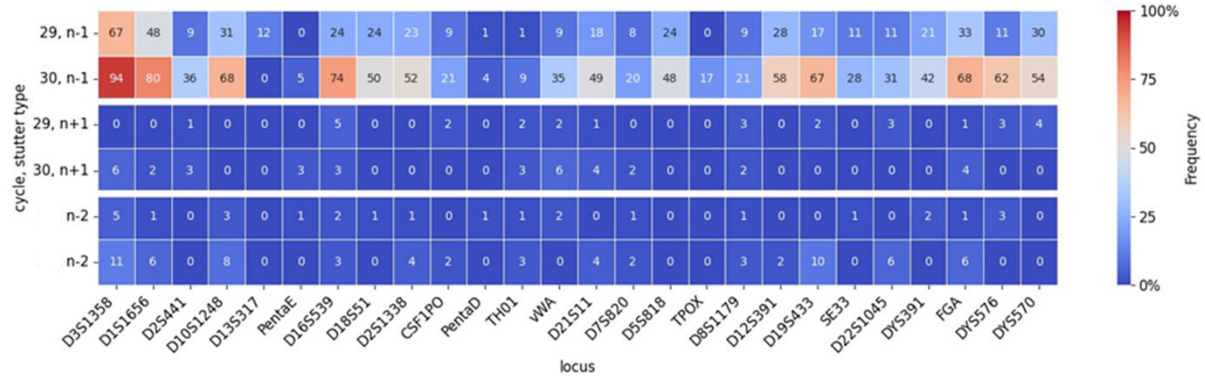
Frequency of stutter (sc) – 29 and 30 cycle

n-1	• Average Δ →23.6 ± 14.8% • Range – 29 cycles→ 0% to 67% – 30 cycle→0% to 94% • >50% : D1, DY576, D16 • D13 →decreases 12%
n+1	• Average Δ→ 0.35 ± 2.33% • Range – 29 cycles→ 0% to 5% – 30 cycle→0% to 6% • Max increase - D3 (6%)
n-2	• Average Δ→ 1.65 ± 3.09% • Range – 29 cycles→0-5% – 30 cycles→2-11% • Max increase - D19 (10%)



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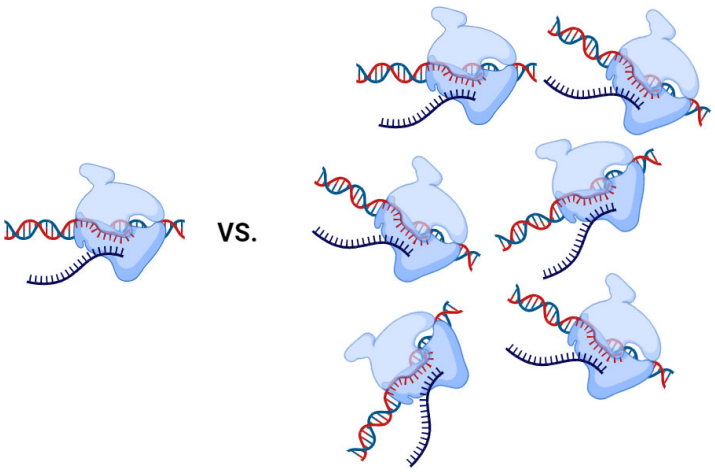
Single cell stutter to inform multi-cell analyses



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Methods – resampling (1 of 2)

- Question: At what point does variance normalize, stutter peak height become more predictable, and large "swings" in stutter are not observed?
- Resampling - Overall, repeat type and individual
  - **1 thru 10, 15, 20 and 50 cells (29 cycle only) - 5000x**
  - Estimating the mean stutter and variance for cell counts ranging from
  - ANOVA ($\alpha = 0.05$) and the post-hoc Tukey's: evaluate the statistical sig. between each simulated cell grouping
- Based upon the following assumption
  - a single DNA template strand - grow exponentially by a power of two during the PCR process –
    - i.e., the total number of resulting strands for *x* number of cycles for a single template strand will be equal to 2^x and the total number of DNA strands in *n* number of cells for those x number of cycles

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Methods – resampling (2 of 2)

- Given a set of parent/stutter peak pairs from single cell stutter data, P = $\{p_1, p_2, \ldots, p_m\}$ and S = $\{s_1, s_2, \ldots, s_m\}$, where p is a single parent peak height RFU value, s is a corresponding stutter peak height value and m is the number of parent/stutter peak pairs in the experimental data set
- Resampling can be performed from this initial set through randomly selecting parent/stutter peak pairs (k), where k ranges from 1 to N, to simulate N new "single cell" samples . This process can be repeated c times (where c represents the number of cells being simulated) and the resulting peak heights summed to estimate parent and corresponding stutter peak heights for c starting cells for the k^{th} sample in N simulated amplifications:

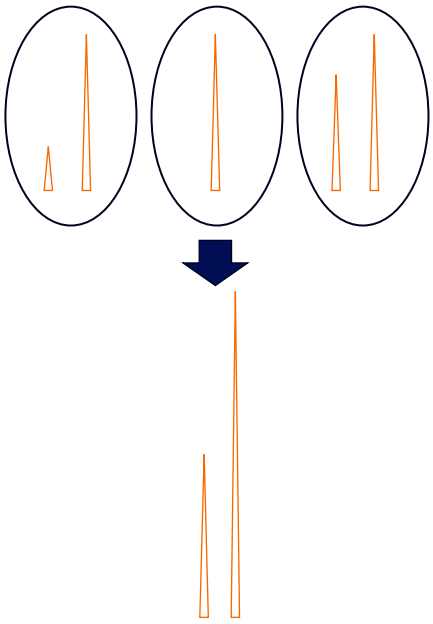
$$PH_p = \sum_{j=1}^{c} p_{kj} \qquad \text{(eq. 4)}$$

$$PH_s = \sum_{j=1}^{c} s_{kj} \qquad \text{(eq. 5)}$$

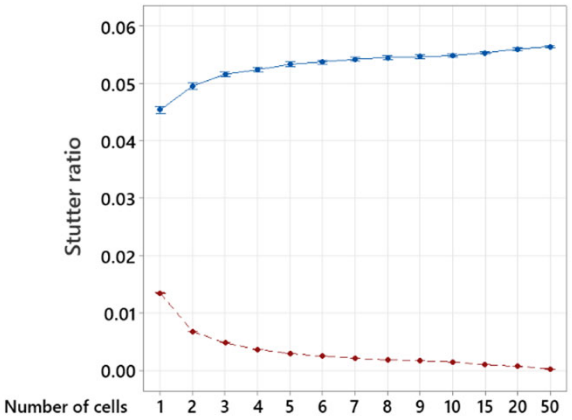
- where PH_p and PH_s are the estimated parent and stutter peak heights. These peak heights can then be used to estimate stutter ratios for an amplification of c number of cells.

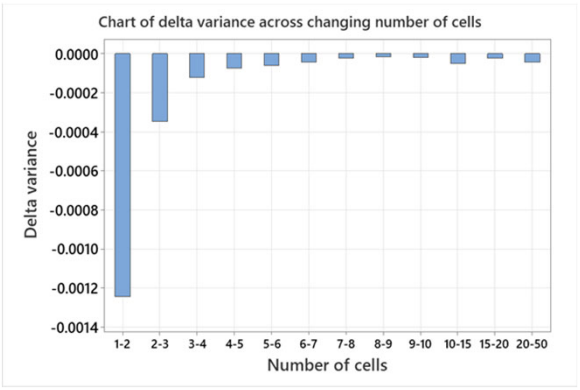
Let's simplify…

- Sc data → generated many stutter and parent peak combination
- **Randomly** selected these pairs depending on # of cells
  - To generate 3 cell samples → select 3 stutter/parent pairs and add them
- "Ideal" PCR model



n-1 – locus wide





- Most significant 1→2
- *At ~7* ***cells*** *variance appears to normalize*

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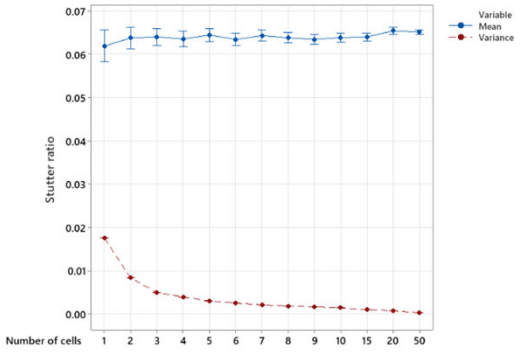
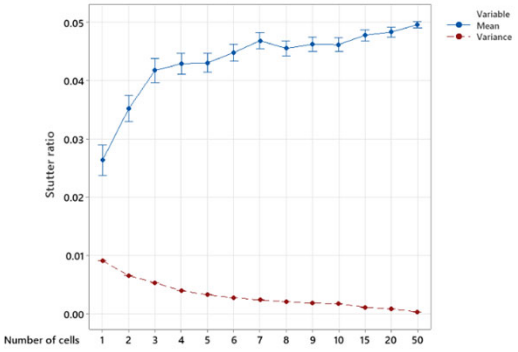
But not all loci are created equally

D16S539 Interval Plot of Stutter, Variance



$[GATA]_n$

SE33 Interval Plot of Stutter, Variance



$[AAAG]_n$

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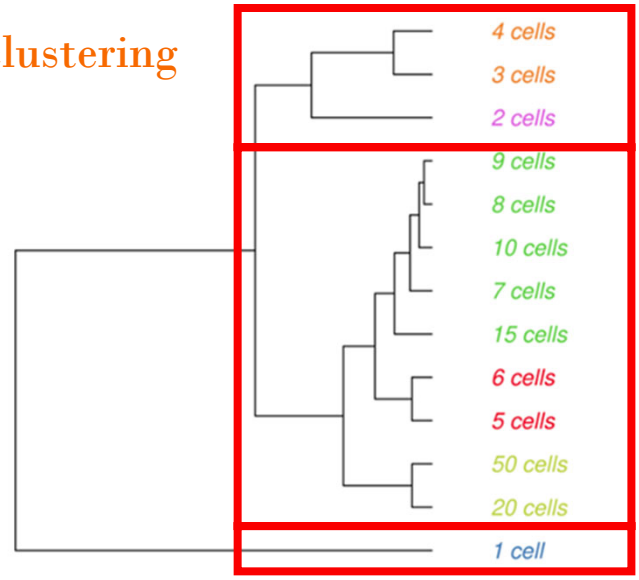
n-1 (29 cycle): Hierarchical clustering

- Locus-wide
- Summary of ANOVA and Tukey's test (α=0.05)
- 3 primary groupings
- ***Stabilization of stutter → 5-7 cells***
  - ***–33 – 46pg (0.033ng – 0.046ng)***



N-1 Hierarchical clustering dendrogram of cell numbers across all loci combined

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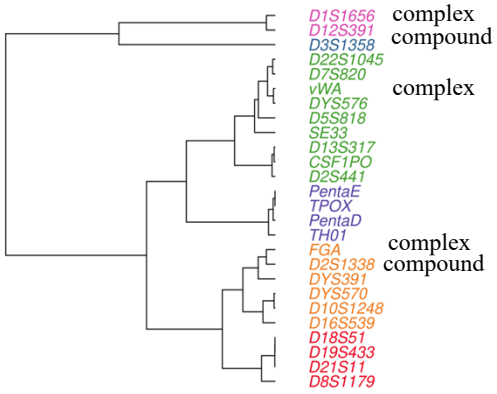
n-1 (29 cycle) hierarchical clustering of loci at 1 cell



- ANOVA and Tukey's test were performed on 1 cell simulated data to show any potential clusters or similarities across loci.
- 4 primary groups
  - –On going work to characterize the relationship

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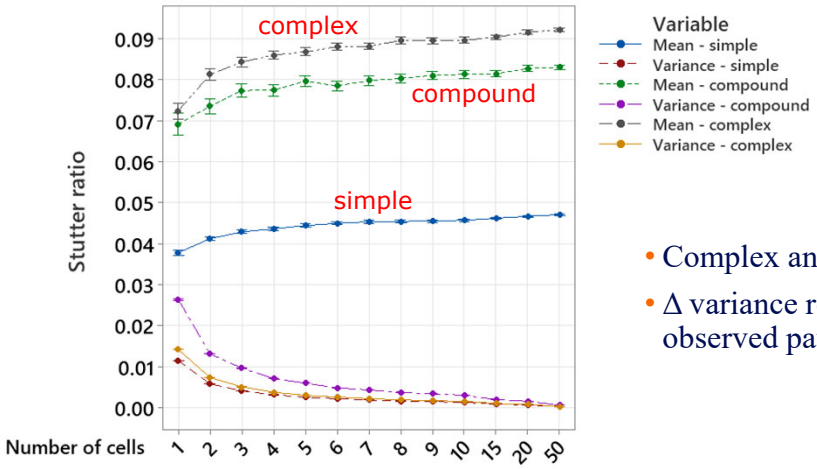
n-1 (29 cycle): by repeat type



- Complex and compound >>> simple
- Δ variance remains consistent to observed pattern

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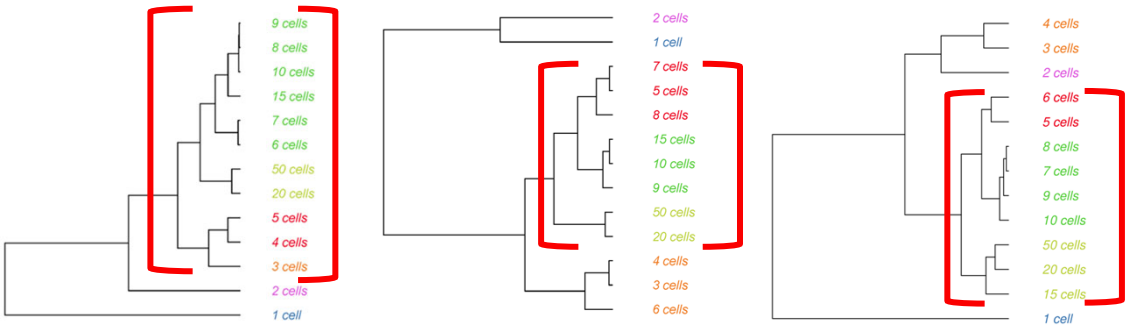
n-1 (29 cycle): hierarchical clustering by repeat type



complex compound simple

- Summary of ANOVA, Tukey's test result

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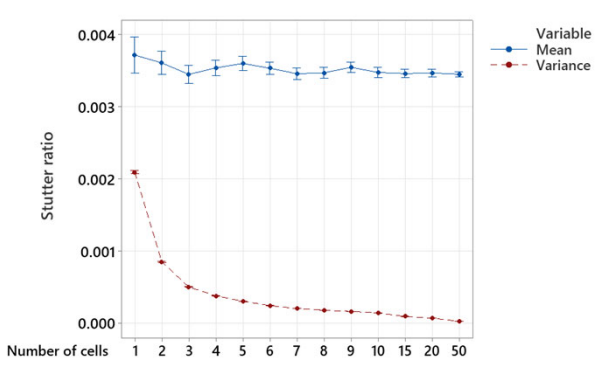
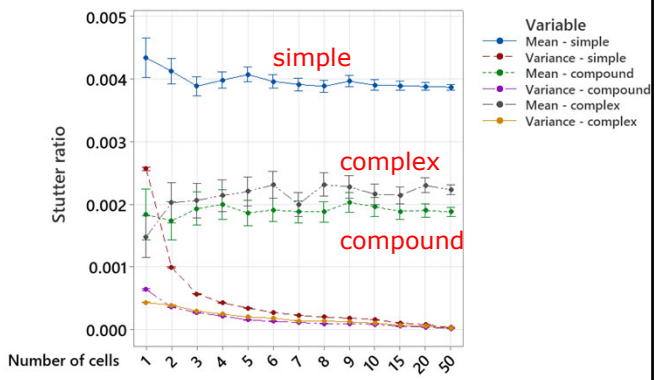
n+1 (29 cycle)

- Absent (generally) – asymptotic incline
- n+1 S_R higher in simple than complex and compound
- Generally → stabilization of variance at 3 cells





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Discussion

- Horwitz curve→ stochasticity is expected, and we do see high levels of stutter
- Stutter can be high *when it occurs*
  - n-1 stutter in sc does not match the expectations according to developmental validation
- Generally, as locus size increases stutter decreases
- n-1 stutter in sc does not match the expectations according to developmental validation
- Over 23% increase in frequency of stutter 29 to 30 cycles
- Single cell to multi-cell
  - n-1 Asymptotic incline with mean and variance stabilizing between 5 and 7 cells
  - n+1 and n-2: no asymptotic incline
  - n-1: simple repeats stutter ratios much lower than compound or complex, opposite for n+1
- Areas for further work: GC content, and LUS based analyses, modeling

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