

GDIS-Bio framework for localizing transcriptional state transitions in single-cell trajectories

Hamid Ismail

North Carolina A&T State University

Ahmad Harb

amharb@ncat.edu

North Carolina A&T State University

Basem William

OhioHealth Cancer Institute

Marwan Bikdash

North Carolina A&T State University

Article

Keywords: single-cell RNA sequencing, pseudotime, transcriptional state transitions, systems biology, dynamical instability, cell-fate transitions, state-space dynamics, GDIS

Posted Date: October 14th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-11191812/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Additional Declarations: No competing interests reported.

GDIS-Bio framework for localizing transcriptional state transitions in single-cell trajectories

Hamid Ismail¹, Ahmed Harb^{1,2,*}, Basem William³, and Marwan Bikdash¹

¹ Department of Computational Data Science and Engineering, College of Engineering, North Carolina A&T State University, Greensboro, NC, 27411, USA; hdismail@ncat.edu (H.I.); bikdash@ncat.edu (M.B.)

² School of Sustainable Systems Engineering (SSSE), German Jordanian University, Amman, 11180, Jordan

³ Blood and Marrow Transplant Program, Cell Therapy Laboratory, OhioHealth Cancer Institute, Columbus, OH 43214, USA; william.21@osu.edu

* Correspondence: amharb@ncat.edu

Abstract

Single-cell RNA sequencing reconstructs developmental trajectories at high resolution; however, identifying where transcriptional state spaces undergo major reorganization remains difficult without an explicit dynamical model. We introduce GDIS-Bio, a model-independent framework that converts pseudotemporally ordered single-cell state spaces into profiles of transition-associated dynamical instability by integrating local sensitivity, trajectory stretching, state-space expansion, nonlinear complexity, and persistence. Biological landmarks are defined independently of the score. In pancreatic endocrine differentiation, GDIS-Bio reproducibly localized the NEUROG3-associated transition across independent differentiations and lineage branches. Transfer of the unchanged framework to human iPSC-derived cardiac differentiation identified significant regions associated with cardiac mesoderm specification and terminal cardiomyocyte and fibroblast differentiation without dataset-specific retuning. Localization remained supported by prespecified sensitivity analyses and structure-preserving null models. GDIS-Bio provides a transferable systems-biology framework for mapping transcriptional state-space reorganization and prioritizing candidate transition regions for downstream investigation of regulatory programs, lineage commitment, and cell-fate change.

Keywords: single-cell RNA sequencing; pseudotime; transcriptional state transitions; systems biology; dynamical instability; cell-fate transitions; state-space dynamics; GDIS

1. Introduction

Single-cell RNA sequencing (scRNA-seq) has transformed the study of developmental and disease-associated cellular processes by enabling transcriptional states to be resolved at the level of individual cells. Because most single-cell experiments provide cross-sectional snapshots rather than continuous observations of the same cells, computational trajectory-inference methods have become essential for reconstructing developmental progressions from cellular heterogeneity. Approaches such as pseudotemporal ordering and diffusion pseudotime arrange cells along inferred developmental paths and can reveal intermediate states, lineage decisions, and transcriptional programs associated with differentiation [1, 2]. The rapid expansion of trajectory-inference methods has substantially increased the ability to study complex lineage structures, although their performance and interpretation depend on trajectory topology, sampling, and underlying methodological assumptions [3, 4].

More recent approaches have attempted to move beyond static ordering toward explicit characterization of cellular dynamics. RNA velocity estimates local directions of transcriptional change from spliced and unspliced RNA abundances, while dynamical extensions such as scVelo model transient transcriptional states [5, 6]. Optimal-transport approaches use population

measurements collected across experimental time points to reconstruct probabilistic relationships between earlier and later cellular populations [7], and lineage-tracing studies have provided direct evidence that transcriptional state and eventual cellular fate are related but not interchangeable [8]. Collectively, these methods have greatly improved reconstruction of developmental trajectories. Nevertheless, identifying where along a high-dimensional trajectory the organization of the cellular state space undergoes a major transition remains a distinct analytical problem.

The concept of a critical transition provides a useful theoretical perspective for this problem. In nonlinear dynamical systems, substantial changes in system behavior may be preceded by changes in statistical or dynamical properties, including increased variance, autocorrelation, loss of resilience, or altered fluctuations [9]. Related ideas have been extended to high-dimensional biological systems through dynamical network biomarkers, in which changes in variability and correlation structure are used to identify states preceding abrupt transitions [10]. At the single-cell level, Mojtahedi et al. demonstrated that cell-fate commitment can be accompanied by pronounced changes in high-dimensional population structure and proposed that such changes may characterize a critical state associated with differentiation [11]. More recently, nonlinear dynamical-systems analysis has been applied directly to transcriptomic state-transition dynamics in cancer, demonstrating the potential of recurrence-based, Lyapunov-based, and composite instability measures for characterizing transcriptional transitions along single-cell trajectories [12]. These observations have helped establish a connection between cell-fate transitions and dynamical-systems concepts, although the precise applicability of classical bifurcation and critical-transition theory to cross-sectional single-cell data requires careful interpretation.

Cell differentiation is particularly well suited to such analysis because it involves coordinated reorganization across many genes and regulatory programs rather than changes in a single molecular marker. Conceptual models such as Waddington's landscape have long represented differentiation as movement between cellular states, and modern single-cell measurements now permit aspects of this state-space organization to be examined quantitatively [13]. However, pseudotime is an inferred developmental coordinate rather than physical time, and cells positioned consecutively along a pseudotemporal trajectory are generally independent observations rather than repeated measurements of the same biological unit [4]. Consequently, dynamical quantities computed from such trajectories should be interpreted as properties of ordered transcriptional state-space geometry, rather than as direct measurements of physical-time dynamics. This distinction is particularly important when applying concepts such as instability, autocorrelation, attractors, or critical transitions to single-cell data.

Current approaches also tend to emphasize individual manifestations of transition-associated change. Variance quantifies local dispersion, entropy summarizes aspects of state-space complexity, pseudotemporal displacement characterizes local trajectory movement, and autocorrelation measures similarity between neighboring observations. Each can be informative, but biological transitions may involve several such properties simultaneously. A multidimensional transition may therefore be difficult to characterize fully using any single statistic. Moreover, different indicators may respond differently depending on the geometry, lineage structure, and biological context of the transition. This motivates an integrative framework that can summarize multiple complementary aspects of local dynamical reorganization while remaining applicable when an explicit mechanistic model or system Jacobian is unavailable.

The Generalized Dynamical Instability Score (GDIS) was developed as a model-independent framework for integrating multiple characteristics of instability in nonlinear state trajectories and

is implemented in the open-source pyGDIS software package [14]. Rather than relying on a single early-warning indicator, the reference formulation integrates descriptors related to local dynamical sensitivity, trajectory stretching, local state-space expansion, nonlinear complexity, and persistence into a bounded instability score. For application to single-cell biology, however, such a framework requires additional safeguards: the biological transition must be defined independently of the score, pseudotime must not be equated with physical time, overlapping trajectory windows cannot be treated as independent inferential observations, and transition localization must be distinguished from prospective prediction of an exact transition time.

In the present study, we introduce **GDIS-Bio**, an analytical framework that adapts GDIS to high-dimensional single-cell transcriptional trajectories while explicitly incorporating these constraints. We first evaluate the approach using the GSE114412 human stem-cell pancreatic differentiation dataset of Veres et al., which provides high-resolution sampling of pancreatic endocrine differentiation and independently annotated developmental states [15]. We then perform external validation using GSE175634, an independent human induced-pluripotent-stem-cell cardiac differentiation dataset containing multiple individuals, experimental time points, and bifurcating cardiomyocyte and cardiac-fibroblast trajectories [16].

Our study was designed to test three related questions. First, can GDIS localize independently defined transcriptional state transitions reproducibly across biological replicates and lineage branches? Second, are these localization results robust to prespecified changes in state-space dimensionality and trajectory-window construction and unlikely under structure-preserving null models? Third, how does GDIS compare with simpler transition-associated measures, including total variance, Gaussian differential entropy, pseudotemporal step distance, and lag-1 pseudotemporal autocorrelation? Importantly, the analytical settings and biological landmarks were defined independently of the GDIS localization results, and the framework was transferred to the external dataset without dataset-specific parameter retuning. Through this design, we evaluate GDIS not as a universal replacement for established transition statistics, but as a complementary integrative framework for localizing and characterizing transition-associated dynamical instability in single-cell omics trajectories.

2. Results

2.1. Discovery dataset trajectory structure and state-space validation

The GSE114412 discovery dataset provided a well-resolved endocrine differentiation trajectory spanning early pancreatic progenitor states, intermediate NEUROG3-positive states, and terminal endocrine fates. After restriction to the authors’ deposited endocrine trajectory, 18,099 cells were retained for analysis. The deposited pseudotime ordering showed the expected progression from *prog_nkx6l* through *neurog3_early*, *neurog3_mid*, and *neurog3_late* states toward the terminal *SC-β* and *SC-EC* populations. The two deposited branches corresponded to distinct terminal trajectories, with Branch 1 leading toward *SC-β* cells and Branch 0 toward *SC-EC* cells.

Deposited endocrine pseudotime was also consistent with the independent experimental sampling structure. Across the endocrine population, experimental day and deposited pseudotime were positively associated, with a Spearman correlation of $\rho = 0.767$. Cells from earlier experimental days were concentrated toward the beginning of the pseudotemporal trajectory, whereas later-day cells occupied progressively more advanced positions. Experimental day was nevertheless retained as an independent biological covariate rather than used to construct or modify the deposited

pseudotime. Accordingly, pseudotime was interpreted as a developmental ordering coordinate rather than as continuous physical time.

Expression preprocessing retained 16,178 genes across the 18,099 endocrine cells, from which 2,000 highly variable genes were selected while accounting for differentiation replicate during variable-gene identification. Principal-component representations containing 5, 10, 20, 30, and 50 components were evaluated before GDIS analysis. Among the candidate representations, the 50-PC state space was the only dimensionality satisfying all six prespecified representation-stability criteria and was therefore selected as the primary state space (**Supplementary Table S1**). Lower-dimensional representations satisfied five of six criteria but did not reach the prespecified threshold for local-neighborhood preservation and were retained for sensitivity analyses.

The two independent differentiation experiments also showed sufficient consistency in trajectory structure to support replicate-resolved analysis. Both differentiations contained the expected endocrine progression and provided adequate cell coverage across the two terminal branches. Consequently, the primary discovery analysis comprised four branch $\times$ differentiation trajectories, corresponding to the SC- β and SC-EC branches within each of the two independent differentiations. This design enabled evaluation of both cross-branch and cross-replicate reproducibility while preserving the deposited biological structure of the dataset.

Together, these results established a reproducible and sufficiently resolved transcriptional state space for subsequent sliding-window and GDIS analyses. The 50-PC representation was therefore used for the primary discovery analysis, with the lower-dimensional state spaces reserved for prespecified robustness testing.

2.2. GDIS identifies the pancreatic endocrine transition region in GSE114412

Application of GDIS to the four prespecified discovery trajectories revealed a consistent increase in dynamical instability during progression from the pancreatic progenitor state toward the early NEUROG3-positive endocrine state. In all four branch $\times$ differentiation trajectories, the global GDIS maximum occurred within the NEUROG3-early region, although the trajectories subsequently extended through NEUROG3-mid, NEUROG3-late, and the terminal SC-EC or SC- β states. The resulting GDIS profiles showed a prominent early maximum in both endocrine lineages and in both independent differentiation replicates (**Figure 1**).

The locations of the global GDIS maxima were highly consistent across the four discovery trajectories. In the SC-EC lineage, the GDIS peak occurred at pseudotime 0.1924 in Differentiation 1 and 0.1726 in Differentiation 2, with maximum GDIS values of 0.8528 and 0.8639, respectively. In the SC- β lineage, the corresponding peaks occurred at pseudotime 0.1897 and 0.1706, with maximum GDIS values of 0.8302 and 0.8636. The independently frozen median NEUROG3-early landmarks ranged from approximately 0.1705 to 0.1750, giving absolute GDIS peak-to-landmark distances of approximately 0.0022–0.0210 pseudotime units (**Supplementary Table S2**). Thus, the major GDIS maxima localized closely to an independently defined biological transition region without the NEUROG3-early annotation being supplied to the score calculation.

Evaluation of GDIS at the frozen biological landmarks further supported localization of increased instability to the early endocrine transition. Relative to the progenitor landmark, GDIS increased at the NEUROG3-early landmark in all four trajectories, with increases of 0.303 and 0.579 in the two SC-EC trajectories and 0.351 and 0.584 in the two SC- β trajectories. Following NEUROG3-early, GDIS generally decreased through the NEUROG3-mid and NEUROG3-late regions, although moderate increases were observed again toward the terminal endocrine states.

Importantly, these later increases did not exceed the earlier global maxima (**Figure 1**). The primary discovery analysis therefore did not identify a distinct second global GDIS maximum associated with terminal SC-EC or SC- β differentiation.

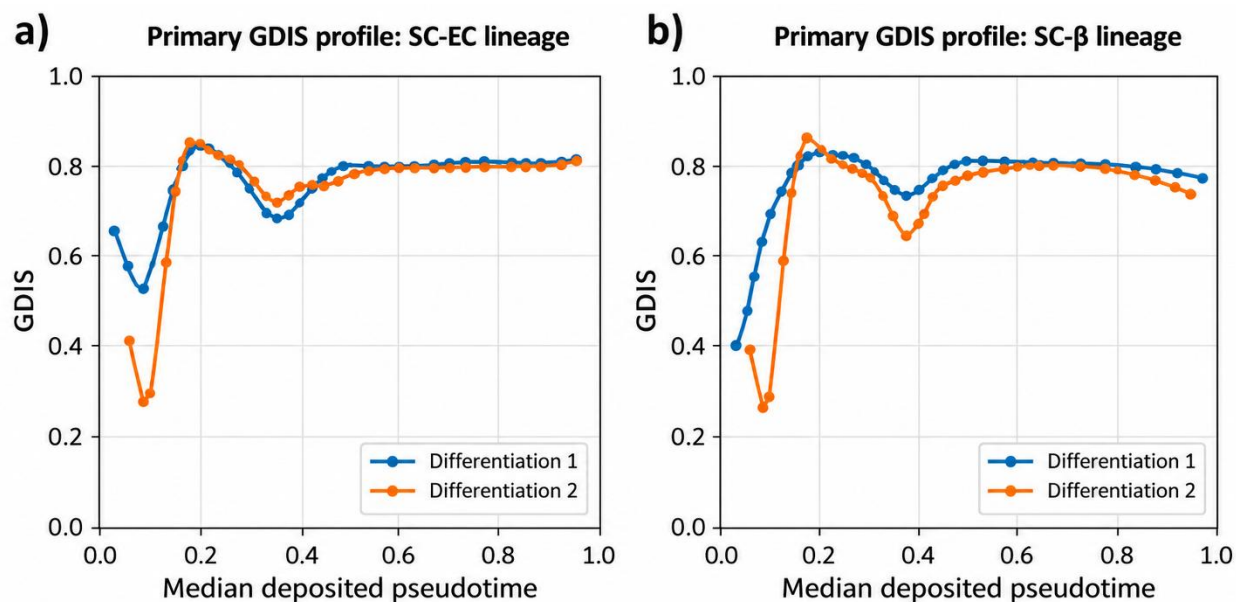


Figure 1. Primary GDIS profiles across pancreatic endocrine differentiation trajectories in GSE114412. GDIS profiles are shown for the **SC-EC lineage (a)** and **SC- β lineage (b)** across the two independent differentiation replicates. In both lineages, the profiles exhibit a prominent early maximum near the NEUROG3-early transition region, followed by lower or relatively stable GDIS values across later pseudotemporal states.

Decomposition of the GDIS profiles provided additional insight into the structure of the early signal. Across all four trajectories, the transition-instability component was concentrated within an early, relatively narrow pseudotemporal region, whereas sustained instability contributed more broadly across later portions of the trajectories (**Supplementary Figure S1**). The internally derived transition-energy maximum occurred at pseudotimes 0.1207, 0.1405, 0.1542, and 0.1385 for the four branch $\times$ differentiation trajectories, respectively. These positions preceded the corresponding frozen NEUROG3-early median landmarks by 0.0507, 0.0344, 0.0163, and 0.0344 pseudotime units, with a mean separation of approximately 0.0340 pseudotime units (**Supplementary Table S2**).

This earlier positioning of the transition-energy maximum should not be interpreted as prospective prediction of the true biological onset of endocrine commitment. The deposited NEUROG3-early median represents an independently defined cell-state landmark rather than a directly observed onset time, and pseudotime itself represents an ordering of independent cells rather than longitudinal physical time. Accordingly, the transition-energy result indicates relative localization within the pseudotemporal transition region rather than demonstrated temporal anticipation of a biological event.

Taken together, the discovery analysis identified a reproducible region of elevated GDIS near the independently defined **NEUROG3-early transition phase** in both endocrine branches and across both differentiation replicates. The close correspondence between the global GDIS maxima and the frozen biological landmark supports the ability of GDIS to localize a major transcriptional

state-transition region without using the biological annotation during score construction (**Figure 1; Supplementary Table S2**). The corresponding component profiles further show that this early transition-associated signal arises from the combined contribution of sustained instability and a temporally localized transition component (**Supplementary Figure S1**).

2.3. Reproducibility across differentiation replicates and endocrine branches

The primary GDIS profiles were highly reproducible across the two independent pancreatic differentiation experiments. Within the SC-EC lineage, the profiles from Differentiations 1 and 2 showed a Pearson correlation of 0.9278 and a Spearman correlation of 0.8499, with a mean absolute profile difference of 0.0313 across their shared pseudotime range. Similarly, the SC- β replicate profiles showed a Pearson correlation of 0.9123, a Spearman correlation of 0.8534, and a mean absolute profile difference of 0.0474. The close correspondence between differentiation replicates is apparent in both lineage-specific profiles in **Figure 1**, with the quantitative measures of replicate agreement summarized in **Supplementary Table S3**. These values represent the primary replicate-profile statistics calculated over the shared pseudotime ranges used for the reproducibility analysis. The slightly different replicate-correlation values reported in **Section 2.6** were calculated on the benchmark interpolation grid used for the circular-shift null analysis and therefore should not be interpreted as conflicting estimates.

Reproducibility also extended to the locations of the principal GDIS events. The global GDIS peak positions differed between differentiation replicates by only 0.0197 pseudotime units for the SC-EC lineage and 0.0190 pseudotime units for the SC- β lineage. The corresponding differences in transition-energy peak position were 0.0198 and 0.0157 pseudotime units, respectively (**Supplementary Table S3**). Replicate-consensus analysis placed the SC-EC GDIS maximum at pseudotime 0.1846, compared with a mean frozen NEUROG3-early landmark of 0.1732. For the SC- β lineage, the consensus GDIS maximum occurred at pseudotime 0.1717, essentially coinciding with the mean NEUROG3-early landmark of 0.1717. These consensus results were consistent with the peak localization observed in the four individual trajectories (**Supplementary Table S2**).

The underlying biological trajectory structure was also reproducible independently of the GDIS calculation. Daily median pseudotime increased monotonically across experimental differentiation in every branch $\times$ differentiation group, and cross-replicate correlations of the daily median pseudotime profiles were 0.9960 for SC-EC and 0.9991 for SC- β . The deposited biological-state landmarks were similarly consistent between differentiation replicates. For example, the median NEUROG3-early pseudotimes differed by only 0.0036 pseudotime units in the SC-EC lineage and 0.0024 pseudotime units in the SC- β lineage. All seven prespecified trajectory-reproducibility checks were satisfied before downstream GDIS analysis.

Agreement was also strong between the two endocrine branches during their common developmental phase, before divergence toward the terminal SC-EC and SC- β fates. Over the shared pseudotime interval from approximately 0.061 to 0.418, the branch-level consensus GDIS profiles showed a Pearson correlation of 0.9757, a Spearman correlation of 0.9438, and a mean absolute difference of 0.0269. These findings indicate that the early GDIS pattern was not restricted to a single terminal lineage but was shared across the common NEUROG3-associated endocrine differentiation program.

The internal GDIS components also showed substantial replicate concordance. Transition-instability profiles had Pearson correlations of 0.9387 for SC-EC and 0.9495 for SC- β , whereas

sustained-instability profiles showed correlations of 0.9452 and 0.9064, respectively. The corresponding component profiles are shown in **Supplementary Figure S1**. Transition-energy profiles were less reproducible than the overall GDIS profiles, particularly in the SC- β lineage, indicating greater replicate variability in the detailed shape and magnitude of this localized component despite the consistent positioning of the principal GDIS signal.

Overall, the principal GDIS pattern was reproducible both between independent differentiation replicates and across the two endocrine branches during their shared developmental phase. The consistency of profile shapes, peak locations, and the early transition-associated signal indicates that the observed GDIS pattern was not attributable to a single differentiation replicate or terminal lineage (**Figure 1**; **Supplementary Tables S2 and S3**; **Supplementary Figure S1**).

2.4. Sensitivity to state-space dimensionality and sliding-window configuration

The primary GDIS analysis used the prespecified 50-PC state space together with 400-cell windows advanced in 100-cell steps. To assess whether the discovery result depended strongly on these analytical choices, GDIS was recalculated using the frozen lower-dimensional state spaces and the two prespecified alternative sliding-window configurations. The 50-PC representation had been selected before GDIS calculation because it was the smallest dimensionality satisfying all six state-space stability criteria, whereas the 5-, 10-, 20-, and 30-PC representations were retained for sensitivity analysis (**Supplementary Table S1**).

GDIS profiles were particularly stable in the 30-PC representation. Across the four branch $\times$ differentiation trajectories, Pearson correlations between the 30-PC and primary 50-PC GDIS profiles ranged from 0.9875 to 0.9973, with a median of 0.9928. Spearman correlations ranged from 0.9014 to 0.9767, and the median absolute profile difference was 0.0109. Most notably, the global GDIS peak position was unchanged in all four trajectories when the state space was reduced from 50 to 30 PCs (**Supplementary Figure S2A,B**; **Supplementary Table S4**). This close agreement indicates that the principal discovery result was not dependent on the full 50-dimensional representation.

Greater variability emerged as state-space dimensionality was reduced further. The 20-PC representation generally retained good agreement with the primary profiles, but the SC- β Differentiation 1 trajectory showed a global-peak displacement of 0.0723 pseudotime units. The 5- and 10-PC representations showed larger profile deviations overall and less consistent rank-order agreement with the 50-PC profiles. These results were consistent with the pre-GDIS state-space validation, in which lower-dimensional representations preserved broad biological geometry but progressively lost local-neighborhood structure relative to the 50-PC reference (**Supplementary Table S1**; **Supplementary Figure S2A,B**; **Supplementary Table S4**).

The discovery result was also broadly stable to the prespecified changes in sliding-window configuration. With 500-cell windows and a 125-cell step, Pearson correlations with the primary 400/100 profiles ranged from 0.9097 to 0.9844, with a median of 0.9323. Global-peak displacements ranged from only 0.0033 to 0.0124 pseudotime units. The 300-cell/75-step configuration also retained substantial profile agreement overall, with a median Pearson correlation of 0.8690 and a median Spearman correlation of 0.9625. Three of the four trajectories showed little or no change in global GDIS peak position; however, the SC- β Differentiation 1 trajectory showed a larger displacement of 0.0947 pseudotime units, indicating greater sensitivity to the smaller-window configuration in that trajectory (**Supplementary Figure S2C,D**; **Supplementary Table S4**).

Despite this localized sensitivity, the analyses consistently preserved an early transition-associated GDIS signal across the discovery dataset. The strongest agreement was observed between the primary 50-PC analysis and the prespecified 30-PC sensitivity representation, while the 500-cell/125-step configuration also reproduced the primary profiles with only small shifts in peak location. Thus, the principal conclusion that elevated GDIS localizes near the early endocrine transition was not dependent on a single state-space dimensionality or sliding-window configuration, although lower-dimensional representations and the smaller-window configuration introduced greater variability in selected trajectories.

2.5. Comparison with conventional transition-associated metrics

To determine whether the transition localization observed with GDIS was also captured by conventional trajectory statistics, the four frozen discovery trajectories were benchmarked against total variance, mean lag-1 pseudotemporal autocorrelation, Gaussian differential entropy, and mean pseudotemporal step distance. The internal GDIS transition-energy component was also included as a reference. All metrics were calculated using the same frozen 50-PC state space and 400-cell/100-step sliding-window configuration, without modification of the biological landmarks or GDIS parameters. Because the trajectories represent pseudotemporally ordered independent cells, lag-1 autocorrelation and step distance are interpreted as pseudotemporal, rather than physical-time, quantities.

GDIS showed the smallest mean absolute distance between the global metric maximum and the independently frozen NEUROG3-early landmark among the evaluated measures. Across the four branch $\times$ differentiation trajectories, the mean absolute peak-to-landmark distance was 0.0112 pseudotime units for GDIS, compared with 0.0269 for total variance, 0.0340 for GDIS transition energy, 0.0587 for mean pseudotemporal step distance, 0.0739 for mean lag-1 pseudotemporal autocorrelation, and 0.1845 for Gaussian differential entropy. Median absolute distances showed the same overall ordering, with corresponding values of 0.0107, 0.0229, 0.0344, 0.0487, 0.0728, and 0.1774 pseudotime units (**Figure 2a; Supplementary Table S5**).

The biological locations of the metric maxima also differed substantially. The global GDIS maximum occurred within a NEUROG3-early-dominated window in all four trajectories, whereas total variance did so in three of four, GDIS transition energy in two of four, and mean lag-1 pseudotemporal autocorrelation in one of four. Neither Gaussian differential entropy nor mean pseudotemporal step distance placed its global maximum within a NEUROG3-early-dominated window (**Figure 2b; Supplementary Table S5**). Total variance nevertheless emerged as a strong conventional comparator: in two trajectories its global maximum coincided with the GDIS maximum, while in the remaining trajectories it localized only modestly later along pseudotime. The complete standardized metric profiles demonstrate these differences in profile shape and peak position across the individual trajectories (**Supplementary Figure S3**).

Changes between the frozen progenitor and NEUROG3-early landmarks further showed that the transition was detectable through several aspects of local trajectory structure. GDIS increased from the progenitor to the NEUROG3-early landmark in all four trajectories, as did total variance, Gaussian differential entropy, mean pseudotemporal step distance, and GDIS transition energy. In contrast, mean lag-1 pseudotemporal autocorrelation increased in only two of four trajectories. Thus, the early endocrine transition was associated with coordinated changes in several trajectory-derived measures rather than with a signal unique to GDIS (**Supplementary Table S5**).

Replicate agreement likewise demonstrated that several conventional metrics were highly reproducible. In the benchmark analysis, Gaussian differential entropy showed cross-replicate Pearson correlations of 0.9915 for SC-EC and 0.9943 for SC- β , while total variance showed corresponding correlations of 0.9560 and 0.9796. Mean pseudotemporal step distance was also strongly reproducible across replicates. These correlations were comparable to or higher than those observed for GDIS, despite the larger peak-to-landmark distances of these conventional metrics (**Supplementary Table S5**). Thus, high replicate reproducibility alone did not imply accurate localization of the independently defined biological transition.

Taken together, the descriptive benchmark showed that GDIS localized most closely to the frozen NEUROG3-early landmark across the four discovery trajectories, while total variance represented the strongest conventional comparator (**Figure 2**; **Supplementary Figure S3**; **Supplementary Table S5**). At the same time, several conventional metrics showed strong biological responses and high replicate reproducibility. These results therefore support GDIS as a complementary integrative measure of transition-associated dynamical instability rather than establishing universal superiority over conventional trajectory statistics. Formal structure-preserving null validation and paired statistical comparisons are evaluated separately in the following section.

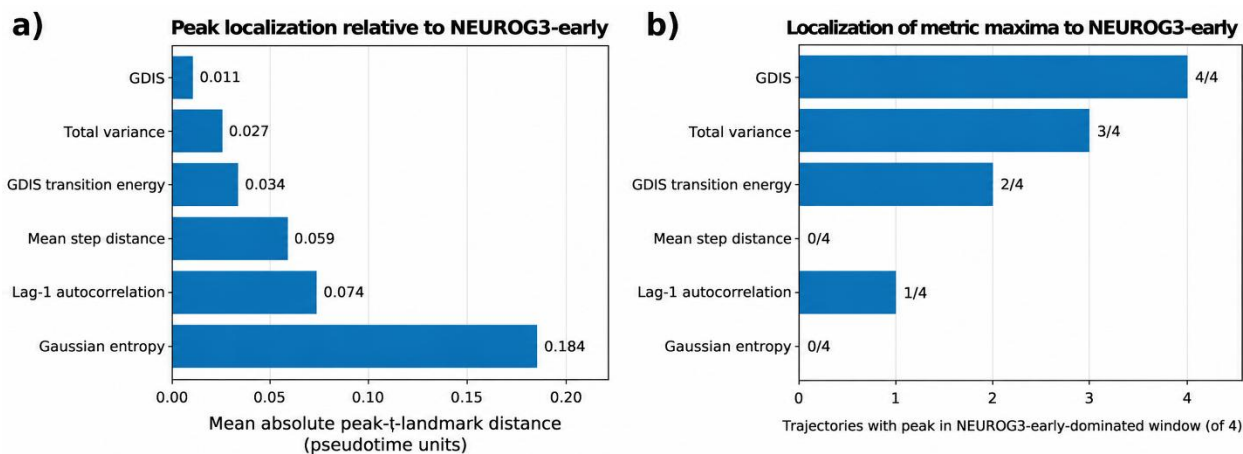


Figure 2. Comparison of GDIS with conventional transition-associated metrics in GSE114412. **(a)** Mean absolute distance between the global maximum of each metric and the independently frozen NEUROG3-early median landmark across the four discovery trajectories; smaller values indicate closer localization to the independently defined transition region. **(b)** Number of trajectories in which the global metric maximum occurred within a NEUROG3-early-dominated window. GDIS showed the smallest mean peak-to-landmark distance and localized to a NEUROG3-early-dominated window in all four trajectories, while total variance was the strongest conventional comparator. GDIS transition energy denotes the internally derived transition-localization component of the GDIS framework. Formal statistical comparisons are reported separately.

2.6. Structure-preserving null validation and paired statistical comparison

To determine whether the observed alignment of GDIS with the independently defined NEUROG3-early transition region exceeded that expected from the intrinsic structure of the trajectory profiles, structure-preserving circular-shift null tests were applied to each metric. For each test, 20,000 circular shifts were generated. This procedure preserved the observed profile values, local sequential structure, and trajectory length while disrupting their alignment with the biological pseudotime landmarks. Because adjacent sliding windows overlap extensively, individual windows were not treated as statistically independent observations.

GDIS showed strong evidence of non-random localization relative to the frozen NEUROG3-early landmark. The observed mean absolute peak-to-landmark distance was 0.0112 pseudotime units, with a circular-shift $p = 0.000050$ and a Benjamini–Hochberg-adjusted $q = 0.000300$. Moreover, the global GDIS maximum occurred within a NEUROG3-early-dominated window in all four discovery trajectories, an enrichment that was also significant relative to the circular-shift null ($p = 0.000050$, $q = 0.000300$; **Figure 3; Supplementary Table S6**).

GDIS also increased from the frozen progenitor landmark to the NEUROG3-early landmark in all four trajectories. However, the null test based solely on the number of trajectories showing a positive directional change was not significant after multiple-testing correction ($p = 0.083096$, $q = 0.099715$). In contrast, the mean magnitude of the progenitor-to-NEUROG3-early GDIS increase was substantially greater than expected under the circular-shift null ($p = 0.000050$, $q = 0.000150$; **Supplementary Table S6**). Thus, the statistical evidence supported the magnitude of the transition-associated GDIS increase rather than merely the occurrence of four concordant positive changes.

The conventional metrics showed heterogeneous evidence of transition alignment. Total variance exhibited significant peak localization after multiple-testing correction ($q = 0.000900$), as did GDIS transition energy ($q = 0.001900$), mean pseudotemporal step distance ($q = 0.012299$), and mean lag-1 pseudotemporal autocorrelation ($q = 0.020519$). In contrast, Gaussian differential entropy did not show significant peak localization relative to the circular-shift null ($q = 0.261437$). These results demonstrate that transition-associated localization was not unique to GDIS, although GDIS showed the smallest observed mean peak-to-landmark distance among the evaluated metrics (**Figure 3; Supplementary Table S6**).

Cross-replicate GDIS agreement was likewise substantially greater than expected under profile misalignment. For the null-validation analysis, replicate profiles were evaluated on the common interpolation grid used by the benchmark and circular-shift procedure. On this grid, the observed Pearson correlations between the two independent differentiations were 0.9275 for SC-EC and 0.9134 for SC- β , and both were highly significant relative to their corresponding circular-shift null distributions ($p = 0.000050$, $q = 0.000075$ for each lineage; **Supplementary Table S7**). These values differ slightly from the primary shared-pseudotime-range correlations reported in **Section 2.3** (0.9278 for SC-EC and 0.9123 for SC- β) because the two analyses used different interpolation grids for their respective purposes. Strong null-adjusted replicate agreement was also observed for several conventional metrics, including total variance, Gaussian differential entropy, and mean pseudotemporal step distance (**Supplementary Table S7**). These findings reinforce the distinction between profile reproducibility and biological transition localization: a metric can be highly reproducible across replicates without necessarily placing its global maximum close to the independently defined transition landmark.

Direct comparisons between GDIS and the conventional metrics were performed at the level of the four branch $\times$ differentiation trajectories, rather than by treating overlapping windows as independent samples. GDIS had a smaller peak-to-landmark distance than total variance in two trajectories, while the remaining two were tied; the exact one-sided paired comparison was not significant ($p = 0.2500$, $q = 0.2500$). GDIS showed a smaller peak-to-landmark distance than mean lag-1 pseudotemporal autocorrelation, Gaussian differential entropy, and mean pseudotemporal step distance in all four trajectories. Nevertheless, with only four trajectory-level comparisons, the smallest attainable one-sided exact p -value for four concordant nonzero differences was 0.0625, and none of these comparisons remained significant after FDR correction ($q = 0.083333$;

Supplementary Table S6). The discovery dataset therefore does not provide formal evidence that GDIS is statistically superior to these conventional metrics.

The signed position of each peak relative to the NEUROG3-early median landmark was also evaluated. The mean signed GDIS peak offset was +0.0089 pseudotime units, indicating a slight average displacement after the NEUROG3-early median; this directional offset did not remain significant after multiple-testing correction ($p = 0.028299$, $q = 0.056597$). By contrast, the GDIS transition-energy maximum occurred earlier than the NEUROG3-early median on average, with a mean signed offset of -0.0340 pseudotime units ($p = 0.007750$, $q = 0.023249$; **Supplementary Table S6**). Because the NEUROG3-early median represents a cell-state landmark rather than a directly observed transition-onset time, this earlier positioning should be interpreted only as relative localization along pseudotime and not as prospective prediction of biological transition onset.

Overall, the structure-preserving null analyses indicate that the close localization of GDIS to the NEUROG3-early transition region was unlikely to result from arbitrary alignment between the observed GDIS profile and the biological trajectory. At the same time, several conventional metrics also showed significant transition-associated structure, and the limited number of independent discovery trajectories did not support a claim of universal statistical superiority for GDIS. The discovery analysis therefore supports GDIS as a reproducible and significantly transition-aligned integrative instability measure, while motivating independent external validation to determine whether this behavior generalizes to a distinct biological differentiation system.

Observed transition localization versus circular-shift null distributions

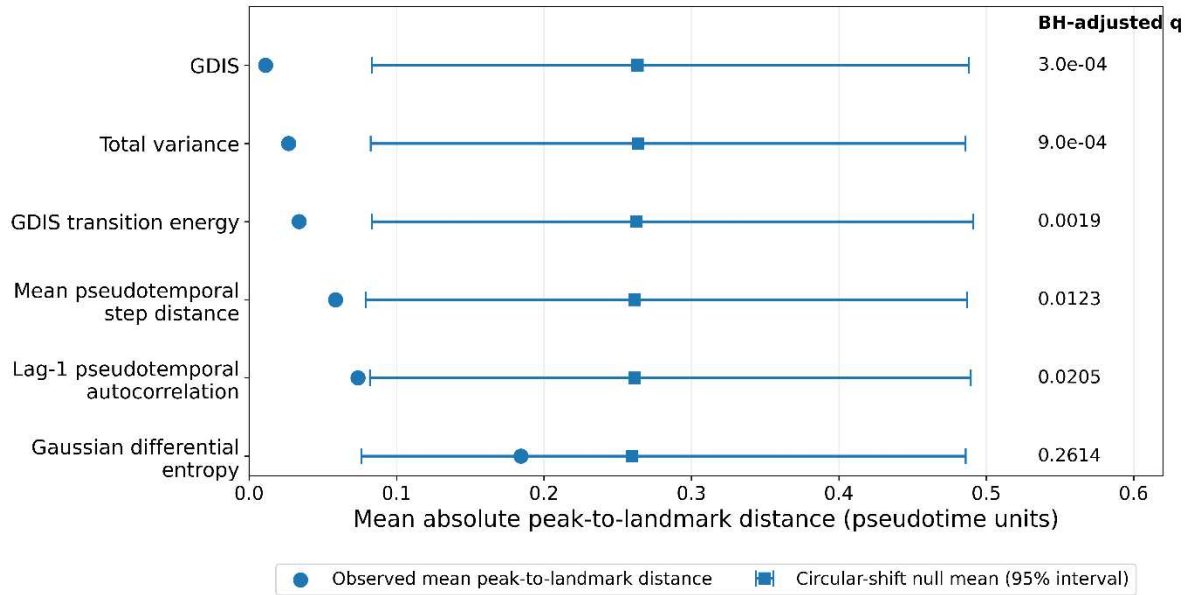


Figure 3. Structure-preserving null validation of transition localization in GSE114412. Observed mean absolute peak-to-landmark distances are compared with circular-shift null distributions for GDIS and the conventional transition-associated metrics. Circles indicate the observed mean distance between each metric's global maximum and the independently frozen NEUROG3-early landmark; squares indicate the corresponding null mean, and horizontal error bars represent the 2.5th–97.5th percentile interval of the null distribution obtained from 20,000 circular shifts. Benjamini–Hochberg-adjusted q -values are shown for the localization tests. Smaller observed distances indicate closer localization to the independently defined transition region.

2.7. Independent validation in cardiac differentiation

To determine whether the transition-localization behavior observed in the pancreatic discovery system generalized to a biologically distinct developmental process, the frozen GDIS-Bio framework was applied without parameter retuning to the independent GSE175634 human iPSC-to-cardiac differentiation dataset. The external-validation design comprised a shared developmental backbone, IPSC→MES→CMES→PROG, together with terminal extensions from PROG toward cardiomyocytes (CM) and cardiac fibroblasts (CF). Biological transition definitions, state-space dimensionality, trajectory construction, sliding-window parameters, and GDIS settings were fixed before examination of the external GDIS results. The prespecified transitions evaluated were the shared-backbone MES→CMES transition (T2) and the terminal PROG→CM (T3-CM) and PROG→CF (T3-CF) transitions (**Figure 4**).

After transition-evaluability filtering, the external-validation analysis comprised 19 shared-backbone profiles, 15 CM-extension profiles, and 14 CF-extension profiles, yielding 48 scope × individual GDIS profiles. These 48 profiles were not treated as mutually independent biological replicates because individuals could contribute to more than one analysis scope and the terminal extensions shared portions of the developmental backbone. The independent biological unit was therefore the individual within each analysis scope. Individual 19108 remained eligible for the shared-backbone analysis but was excluded prospectively from the CM and CF terminal-transition analyses because its complete-window pseudotime range did not extend to the corresponding frozen terminal-state landmarks. No landmark, window configuration, or GDIS parameter was altered to restore evaluability (**Supplementary Table S8**).

Application of the unchanged pyGDIS configuration produced significant transition-associated localization across all three validation scopes. For the primary MES→CMES transition (T2), the median absolute distance between the global GDIS maximum and the independently frozen CMES median diffusion-pseudotime landmark was 0.0376 pseudotime units across 19 individuals. The corresponding median distances were 0.0954 for T3-CM and 0.0405 for T3-CF (**Figure 4; Table 1; Supplementary Tables S8 and S9**). Thus, GDIS localized most closely to the shared MES→CMES and CF transition landmarks, whereas localization around the CM terminal transition was more dispersed.

Structure-preserving circular-shift tests nevertheless demonstrated significant transition localization in all three scopes. After Benjamini–Hochberg correction within the external benchmark localization family, the adjusted values were $q = 0.0040$ for T2, $q = 0.00252$ for T3-CM, and $q = 0.02797$ for T3-CF (**Figure 4; Supplementary Table S9**). These results indicate that the observed proximity of the GDIS maxima to the prespecified cardiac transition landmarks was unlikely to arise solely from arbitrary alignment of structured GDIS profiles with deposited diffusion pseudotime.

The direction of the source-to-destination GDIS change differed substantially among the three transitions. For T2, GDIS increased from MES to CMES in 15 of 19 individuals, with a median change of +0.0534; the paired source-to-destination increase remained significant after multiple-testing correction ($q = 0.0371$). For T3-CM, 9 of 15 individuals showed an increase and 6 showed a decrease, yielding a small median change of +0.0084 that was not significant after correction ($q = 0.2031$). In contrast, GDIS decreased from PROG to CF in all 14 evaluable T3-CF profiles, with a median change of −0.0510. Because the prespecified paired test evaluated an increase toward the destination state, this opposite-direction pattern yielded $q = 1.0$ for that test (**Supplementary Table S9**).

The T3-CF result highlights an important distinction between transition localization and monotonic source-to-destination change. Although GDIS decreased consistently from the PROG landmark to the CF landmark, the location of the GDIS maximum remained significantly associated with the independently frozen CF transition region under the circular-shift null ($q = 0.02797$). Likewise, significant localization at T3-CM occurred despite the absence of a statistically significant source-to-destination increase. Thus, successful transition localization by GDIS does not require GDIS to increase monotonically toward a mature destination state (**Figure 4; Supplementary Table S9**).

The external-validation analysis therefore reproduced the principal discovery finding in a distinct developmental system: GDIS maxima localized significantly relative to independently defined biological transition regions without dataset-specific parameter retuning. At the same time, the magnitude and direction of the GDIS response varied across developmental contexts. These findings support the transferability of GDIS as a transition-localization framework, rather than implying that all biological state transitions must exhibit the same directional change or magnitude of GDIS.

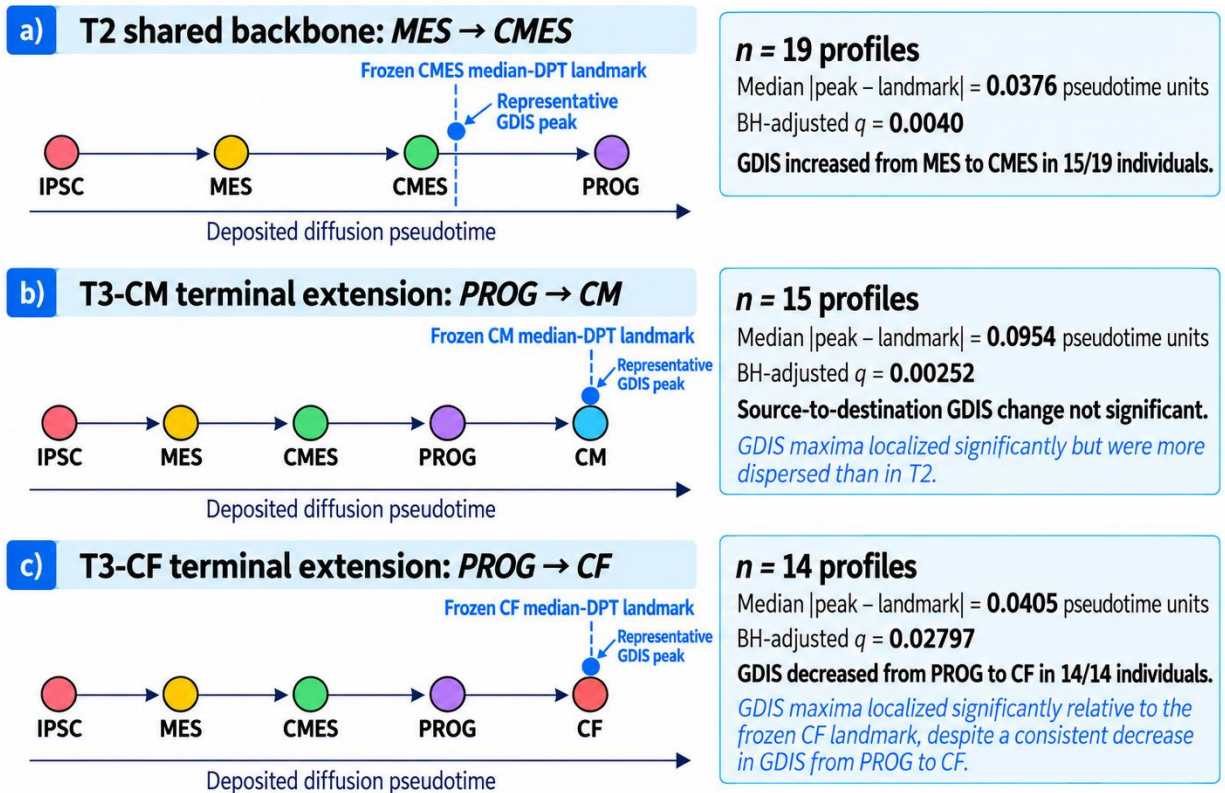


Figure 4. External validation of GDIS transition localization in GSE175634. GDIS localization is summarized for (a) MES→CMES (T2), (b) PROG→CM (T3-CM), and (c) PROG→CF (T3-CF). All transition landmarks and analysis settings were defined before external GDIS calculation, and the framework was applied without dataset-specific parameter retuning.

The principal discovery and external-validation transition-localization results are summarized in **Table 1**.

Table 1. Principal GDIS transition-localization results

Dataset / transition	<i>n</i>	Peak-to-landmark distance	Null-adjusted significance	Main interpretation
GSE114412 NEUROG3-early	4	Mean 0.0112	$q = 0.000300$	Reproducible localization near early endocrine transition
GSE175634 T2 MES→CMES	19	Median 0.0376	$q = 0.0040$	Significant external localization
GSE175634 T3-CM PROG→CM	15	Median 0.0954	$q = 0.00252$	Significant but more dispersed localization
GSE175634 T3-CF PROG→CF	14	Median 0.0405	$q = 0.02797$	Significant localization despite decreasing source-to-destination GDIS

2.8. Robustness and benchmark performance in the external-validation dataset

The external-validation results were further evaluated for robustness to the prespecified analytical choices and for comparative performance against conventional transition-associated metrics. As in the discovery analysis, all sensitivity analyses were performed without altering the biological landmarks or retuning the GDIS formulation. Alternative state-space dimensionalities and sliding-window configurations were therefore used only to assess the stability of the frozen primary analysis.

GDIS profiles were generally robust to the prespecified changes in sliding-window configuration. Across T2, T3-CM, and T3-CF, profiles generated using the 300-cell/75-step and 500-cell/125-step configurations remained strongly correlated with those from the primary 400-cell/100-step analysis, with median Pearson correlations ranging from approximately 0.936 to 0.988. Median absolute GDIS peak shifts were small across all three transition scopes, ranging from approximately 0.0018 to 0.0092 pseudotime units, although larger shifts occurred in selected individual profiles. State-space sensitivity showed a similar pattern. The 30-PC representation closely reproduced the primary 50-PC analysis, with median Pearson correlations of 0.9850 for T2, 0.9818 for T3-CM, and 0.9359 for T3-CF, and a median global-peak displacement of zero in all three scopes. In contrast, lower-dimensional representations were less stable. This was particularly evident for T2, where the 5-PC representation produced a median absolute peak-to-landmark distance of 0.1808 pseudotime units, compared with 0.0376 in the primary 50-PC analysis. These results indicate substantial robustness of the external-validation findings while also emphasizing the importance of retaining sufficient state-space dimensionality to preserve local trajectory geometry (**Supplementary Figure S4; Supplementary Table S10**).

Benchmarking against conventional transition-associated metrics showed that GDIS was not uniformly the closest-localizing measure in the external dataset. For the primary T2 MES→CMES transition, the median absolute peak-to-landmark distances were 0.0331 for mean pseudotemporal step distance, 0.0357 for total variance, 0.0363 for Gaussian differential entropy, 0.0376 for GDIS, and 0.0991 for mean lag-1 pseudotemporal autocorrelation. Thus, GDIS performed similarly to variance-, entropy-, and step-distance-based measures at T2, while localizing substantially more closely than lag-1 pseudotemporal autocorrelation (**Figure 5a; Supplementary Table S11**).

For the terminal T3-CM transition, total variance showed the smallest median absolute peak-to-landmark distance (0.0475), followed by Gaussian differential entropy and mean pseudotemporal

step distance (0.0676 each), GDIS (0.0954), and lag-1 pseudotemporal autocorrelation (0.2040). For T3-CF, Gaussian differential entropy showed the smallest median distance (0.0238), followed by total variance and mean pseudotemporal step distance (0.0286 each), GDIS (0.0405), and lag-1 pseudotemporal autocorrelation (0.2224) (**Figure 5b, c; Supplementary Table S11**). Individual-level distributions showed substantial heterogeneity among profiles, particularly for the terminal transitions, while preserving the same overall ranking patterns observed in the transition-level summaries (**Supplementary Figure S5**). These results demonstrate that variance, entropy, and pseudotemporal step distance were strong transition-localization measures in the external dataset and, in several settings, localized as closely as or more closely than GDIS.

Paired individual-level comparisons reinforced this more nuanced interpretation. GDIS showed its clearest comparative advantage over mean lag-1 pseudotemporal autocorrelation in the terminal transitions. At T3-CM, GDIS had a smaller absolute peak-to-landmark distance in 12 of 15 individuals, with one tie and two losses. This advantage remained significant after multiple-testing correction by both the sign test ($q = 0.0388$) and the paired Wilcoxon test ($q = 0.0190$). At T3-CF, GDIS localized more closely in 13 of 14 individuals, with no ties and one loss, and again remained significant after correction ($q = 0.0110$ for the sign test and $q = 0.00366$ for the paired Wilcoxon test). At T2, GDIS localized more closely than lag-1 autocorrelation in 14 of 19 individuals, with one tie and four losses; however, this comparison did not remain significant after FDR correction ($q = 0.0618$ for the sign test and $q = 0.0693$ for the Wilcoxon test; **Supplementary Table S11**).

In contrast, the paired analyses provided no evidence that GDIS was superior to total variance, Gaussian differential entropy, or mean pseudotemporal step distance. At T3-CM and T3-CF, these conventional measures frequently produced smaller individual peak-to-landmark distances than GDIS, consistent with their lower median localization distances in **Figure 5**. The external benchmark therefore does not support a claim of universal superiority of GDIS over conventional transition-associated statistics (**Figure 5; Supplementary Figure S5; Supplementary Table S11**).

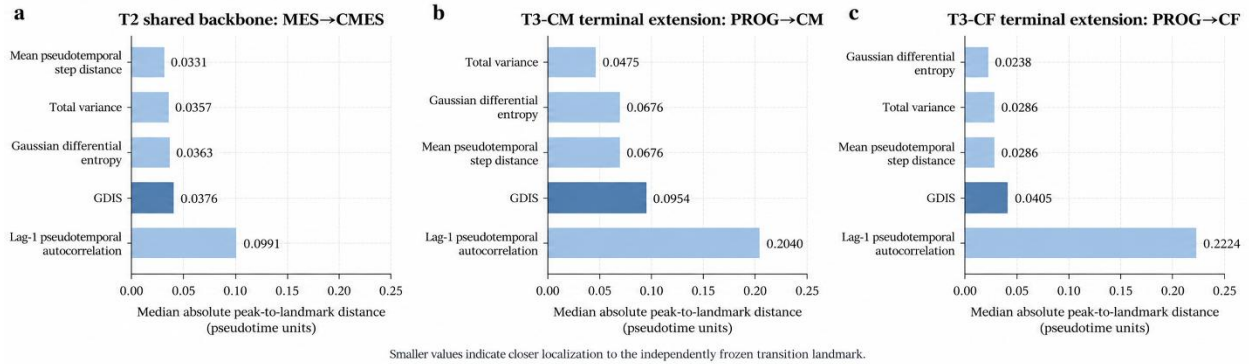


Figure 5. External benchmark comparison of GDIS and conventional transition-associated metrics in GSE175634. Median absolute peak-to-landmark distances are shown for **(a)** the shared-backbone MES→CMES transition (T2), **(b)** the terminal PROG→CM transition (T3-CM), and **(c)** the terminal PROG→CF transition (T3-CF). Smaller values indicate closer localization to the independently frozen transition landmark. GDIS performed comparably to variance-, entropy-, and step-distance-based metrics at T2, while these conventional metrics localized more closely than GDIS for the terminal transitions. Lag-1 pseudotemporal autocorrelation showed the largest median localization distances across all three scopes.

The integrated GDIS score nevertheless showed substantially smaller absolute peak-to-landmark distances than its internal transition-energy component. GDIS showed a smaller absolute peak-to-landmark distance than transition energy in 18 of 19 T2 profiles, 15 of 15 T3-CM profiles, and 9 of 14 T3-CF profiles, with three ties in T3-CF. The corresponding median transition-energy localization distances were 0.2587, 0.3982, and 0.3000 pseudotime units, respectively, compared with 0.0376, 0.0954, and 0.0405 for the integrated GDIS score (**Supplementary Table S11; Supplementary Figure S5**). This distinction indicates that the complete GDIS formulation contributes substantial localization information beyond the isolated transition-energy term, particularly for the later cardiac transitions.

Overall, the external benchmark refines the interpretation of GDIS-Bio. The framework transferred to a biologically distinct differentiation system without dataset-specific parameter retuning and retained significant localization relative to independently defined transition regions. However, conventional variance-, entropy-, and distance-based metrics were also highly informative and were equally competitive or better in several transition scopes. GDIS showed its clearest comparative advantage over lag-1 pseudotemporal autocorrelation in the terminal transitions and markedly outperformed its isolated transition-energy component. The external results therefore support GDIS as a complementary integrative measure of transition-associated dynamical instability rather than a universally superior transition-localization statistic.

3. Discussion

This study developed and biologically evaluated **GDIS-Bio**, a single-cell trajectory framework based on the Generalized Dynamical Instability Score (GDIS), for identifying regions of increased dynamical instability associated with transcriptional state transitions. A central feature of the study design was the separation of biological transition definition from GDIS calculation: cell-state landmarks, trajectory populations, state-space representations, and primary window configurations were defined or frozen independently of the GDIS profiles. This design allowed transition localization to be evaluated against biological information that had not been used to construct or optimize the score.

In the pancreatic endocrine discovery dataset, GSE114412, GDIS reproducibly localized the major early endocrine transition near the independently defined NEUROG3-early state. Global GDIS maxima occurred close to the frozen NEUROG3-early landmarks in both SC-EC and SC- β lineages and across both independent differentiation replicates (**Figure 1; Supplementary Table S2**). The profiles were highly concordant between replicates and across the common developmental portions of the two endocrine branches (**Supplementary Table S3**), and the principal localization result remained stable under prespecified changes in PCA dimensionality and sliding-window configuration (**Supplementary Figure S2; Supplementary Table S4**). Importantly, structure-preserving circular-shift analyses showed that the observed GDIS localization was significantly closer to the NEUROG3-early transition region than expected from arbitrary alignment of the structured profiles with pseudotime (**Figure 3; Supplementary Table S6**). These results indicate that the discovery signal was not attributable simply to one differentiation replicate, one endocrine branch, or a single analytical parameterization.

The external-validation analysis provided a stronger test of generalizability by transferring the frozen framework, without dataset-specific parameter retuning, to the biologically distinct GSE175634 cardiac differentiation system. GDIS maxima showed significant localization relative to independently defined transition landmarks for the shared MES $\rightarrow$ CMES transition (T2) and both terminal extensions, PROG $\rightarrow$ CM (T3-CM) and PROG $\rightarrow$ CF (T3-CF) (**Figure 4;**

Supplementary Tables S8 and S9). The direction of the GDIS response, however, was not uniform across transitions. GDIS increased significantly from MES to CMES at T2, whereas no significant source-to-destination increase was observed for T3-CM and GDIS decreased from PROG to CF in all evaluable T3-CF profiles. Thus, the externally validated property was localization of transition-associated instability, rather than a requirement for monotonic elevation of GDIS toward a differentiated endpoint.

The comparative analyses further refine the significance of these findings. In the discovery dataset, GDIS showed the smallest mean absolute peak-to-landmark distance among the evaluated metrics, although total variance was a strong conventional comparator (**Figure 2; Supplementary Table S5**). In the cardiac validation dataset, total variance, Gaussian differential entropy, and mean pseudotemporal step distance showed similar or smaller peak-to-landmark distances in several transition scopes, whereas GDIS showed its clearest statistical advantage over lag-1 pseudotemporal autocorrelation, particularly for T3-CM and T3-CF (**Figure 5; Supplementary Figure S5; Supplementary Table S11**). The complete GDIS score also showed substantially smaller peak-to-landmark distances than its isolated transition-energy component. These results argue against interpreting GDIS as a universally superior early-warning statistic and instead support its value as an integrative measure that combines complementary features of trajectory instability.

The principal significance of GDIS-Bio therefore lies not in replacing established transition metrics, but in providing a generalized, data-driven framework for interrogating high-dimensional single-cell trajectories when an analytical dynamical-system model or Jacobian is unavailable. Its ability to localize independently defined transition regions across two distinct differentiation systems, while retaining performance under prespecified sensitivity analyses and without dataset-specific retuning, supports the broader applicability of the approach. At the same time, the strong performance of conventional metrics emphasizes that biologically meaningful state transitions can manifest through multiple statistical and geometrical signatures. GDIS-Bio is therefore best viewed as a complementary framework for characterizing transition-associated dynamical instability, with particular value arising from the integration of several dynamical descriptors within a single bounded score.

To interpret these findings biologically, the elevated GDIS observed near major cell-state transitions is consistent with the broader view that differentiation involves transient reorganization of cellular state-space structure rather than a simple monotonic progression between fixed expression states. Previous work has described cell-fate commitment as involving changes in population heterogeneity, regulatory organization, and the stability of alternative cellular states, and has related these changes to concepts of critical-state transition and developmental landscape reconfiguration [11, 13]. In the pancreatic discovery system, the strongest GDIS signal was concentrated near the NEUROG3-early transition region, where progenitor-associated transcriptional programs begin to give way to endocrine differentiation programs described in the original GSE114412 study [15]. The reproducibility of this signal across both differentiation replicates and both endocrine branches suggests that the elevated instability reflects a shared developmental reorganization preceding divergence toward SC- β and SC-EC fates rather than a lineage-specific terminal event (**Figure 1; Supplementary Figure S1**).

Biologically, such transition-associated elevation of GDIS may reflect several concurrent features of cellular reprogramming, including changes in transcription-factor activity, regulatory-network organization, population heterogeneity, and the relative stability of alternative cellular programs.

Similar increases in variability, loss of local stability, and restructuring of high-dimensional state-space organization have been proposed as signatures of systems approaching or traversing critical transitions [9-11]. At the single-cell level, Richard et al. further demonstrated a transient surge in cell-to-cell molecular variability preceding irreversible commitment during differentiation [17]. Within the GDIS framework, these changes can appear through increased local sensitivity, trajectory stretching, state-space expansion, nonlinear complexity, and persistence of instability along the ordered transcriptional trajectory. Importantly, however, these quantities should be interpreted as geometrical and statistical properties of the observed transcriptional state space. They do not, by themselves, establish the presence of a literal biological attractor, deterministic chaos, or a formal bifurcation in the classical dynamical-systems sense. This distinction is particularly important for single-cell pseudotemporal analyses, where reconstructed trajectories represent ordered populations of independent cells rather than longitudinal measurements of the same cells [4].

The distinction between transition localization and monotonic score increase became particularly clear in the external cardiac validation. At the shared MES→CMES transition, GDIS increased from the source to the destination landmark in most individuals, consistent with increased transcriptional reorganization during cardiac mesoderm specification. In contrast, the terminal PROG→CM transition did not show a significant source-to-destination increase, and GDIS decreased from PROG to CF in all evaluable PROG→CF profiles, despite significant localization of the GDIS maxima relative to the corresponding transition landmarks (**Figure 4; Supplementary Table S9**). The underlying cardiac differentiation system itself contains temporally and lineage-resolved transitions among mesodermal, progenitor, cardiomyocyte, and fibroblast states [16]. The present results therefore indicate that GDIS is sensitive to the location of a transient region of state-space reorganization, rather than requiring the mature destination state itself to exhibit persistently elevated instability.

Such non-monotonic behavior is biologically plausible within developmental systems. Theoretical and empirical studies of critical transitions suggest that changes in variability, resilience, and state-space organization may be transient and need not remain elevated once a system has entered a new regime [9-11]. Likewise, developmental-landscape models emphasize that lineage commitment can involve transient destabilization or reconfiguration followed by consolidation into a more restricted differentiated state [13]. Under this interpretation, the largest GDIS signal would be expected near the region of reorganization and could subsequently decline as cells become more transcriptionally constrained within a downstream state. The T3-CF result is particularly informative in this regard: the consistent decrease from PROG to CF indicates that a biologically meaningful transition can be associated with a preceding instability maximum rather than with continued accumulation of instability throughout differentiation.

The pancreatic results show a related pattern. After the prominent early GDIS maximum near NEUROG3-early, GDIS generally declined across NEUROG3-mid and NEUROG3-late states, with later increases toward the terminal endocrine states remaining below the initial maximum (**Figure 1**). This pattern is consistent with the extensive transcriptional reorganization accompanying endocrine commitment documented during human β -cell differentiation [15]. It suggests that the early endocrine transition represents a particularly strong reorganization of the transcriptional trajectory, whereas subsequent maturation may involve additional but less pronounced changes in local state-space structure. The absence of a second global maximum at terminal differentiation also argues against interpreting GDIS simply as a measure of developmental progression or distance along pseudotime.

The behavior of the internal GDIS components further supports this interpretation. In the pancreatic trajectories, the transition-instability contribution was concentrated within a relatively narrow early pseudotemporal region, whereas sustained instability contributed more broadly across later portions of the trajectory (**Supplementary Figure S1**). Similarly, in the cardiac validation, the complete GDIS score localized the later transitions closer to the independently frozen landmarks than did the isolated transition-energy component (**Supplementary Table S11**). These findings suggest that biologically relevant transition localization may emerge from the combined contribution of transient and sustained changes in trajectory geometry, rather than from a single local peak-detection mechanism.

Accordingly, elevated GDIS should be interpreted as identifying a region in which the organization of the transcriptional trajectory is undergoing increased dynamical reconfiguration. The biological meaning of that reconfiguration is likely to be context dependent: it may accompany lineage commitment, branch formation, loss of progenitor identity, acquisition of a differentiated program, or combinations of these processes [11, 13]. The results across pancreatic and cardiac differentiation therefore support a general interpretation of GDIS as a marker of transition-associated transcriptional instability, while cautioning against assigning a single universal biological mechanism to every GDIS maximum.

The biological interpretation is complemented by comparison with conventional trajectory statistics, which provides important context for interpreting the contribution of GDIS. The benchmark analyses showed that biologically meaningful transition regions can be reflected through several complementary properties of pseudotemporal trajectories, including variance, entropy, local displacement, and autocorrelation. Changes in variance and autocorrelation have long been discussed as potential indicators of reduced resilience or approaching state transitions in dynamical systems [9], while related multivariate concepts have been extended to high-dimensional biological systems [10, 11]. GDIS should therefore not be viewed as replacing these established measures, but rather as integrating several aspects of trajectory instability into a single bounded score.

In the pancreatic discovery dataset, GDIS showed the smallest mean absolute peak-to-landmark distance among the evaluated metrics and localized to a NEUROG3-early-dominated window in all four branch $\times$ differentiation trajectories (**Figure 2; Supplementary Table S5**). Total variance, however, emerged as a particularly strong conventional comparator. Its peaks were close to the NEUROG3-early landmark and its replicate profiles were highly reproducible. Gaussian differential entropy and mean pseudotemporal step distance also showed clear progenitor-to-NEUROG3-early changes, indicating that the endocrine transition was accompanied by coordinated changes in transcriptional dispersion and local trajectory geometry rather than by a signal uniquely detectable by GDIS.

The cardiac external-validation dataset reinforced this interpretation. At the primary T2 transition, GDIS showed a median peak-to-landmark distance similar to those of total variance, Gaussian differential entropy, and mean pseudotemporal step distance. For the terminal T3-CM and T3-CF transitions, these conventional metrics showed smaller median peak-to-landmark distances than GDIS (**Figure 5; Supplementary Figure S5; Supplementary Table S11**). Thus, the external validation does not support a universal ordering in which GDIS is expected to outperform simpler statistics across every biological transition. Instead, the relative informativeness of individual metrics appears to depend on the geometry and biological context of the transition being examined.

The clearest comparative advantage of GDIS was observed relative to lag-1 pseudotemporal autocorrelation. In the external terminal transitions, GDIS localized closer to the frozen landmarks than lag-1 autocorrelation in 12 of 15 T3-CM profiles and 13 of 14 T3-CF profiles, with both comparisons remaining significant after multiple-testing correction (**Supplementary Table S11**). At T2, the same directional tendency was observed but did not remain significant after correction. This result should be interpreted carefully because classical lag-1 autocorrelation is fundamentally a temporal concept, whereas the present calculation was performed along an ordering of independent cells in pseudotime [4, 9]. Its weaker performance here therefore does not argue against temporal autocorrelation as a general early-warning indicator; rather, it highlights the limitations of transferring a single time-series statistic directly to a cross-sectional pseudotemporal setting.

The performance of total variance deserves particular attention. Increased variance is a classical feature associated with some systems approaching critical transitions, where reduced restoring forces or greater state-space dispersion can accompany loss of stability [9]. Related increases in variability and correlation structure have also been proposed as early indicators of disease-state transitions and cell-fate decisions in high-dimensional biological systems [10, 11]. In the present study, variance was a strong comparator in both biological systems and showed smaller median peak-to-landmark distances than GDIS for the two terminal cardiac transitions. Consequently, the contribution of GDIS should not be framed as arising from universal superiority over variance, but from providing a broader integrative characterization of instability that combines local sensitivity, trajectory stretching, state-space expansion, nonlinear complexity, and persistence within a single formulation.

A similar distinction applies to Gaussian differential entropy and pseudotemporal step distance. These metrics characterize specific aspects of local trajectory organization: entropy summarizes multivariate dispersion or complexity within a local state distribution, whereas step distance quantifies local geometric displacement between adjacent positions in the ordered trajectory. Their strong performance in the external dataset indicates that several cardiac transitions were accompanied by pronounced changes in local dispersion and trajectory geometry. Such multidimensional reorganization is consistent with broader views of differentiation as movement through a changing developmental state landscape rather than along a single scalar progression [11, 13]. GDIS incorporates related but non-identical information and may therefore be most informative when interpreted alongside these simpler measures rather than in isolation.

The comparison between the complete GDIS score and its internal transition-energy component further clarifies the value of integration. The complete GDIS score showed a smaller absolute peak-to-landmark distance than transition energy in 18 of 19 T2 profiles, 15 of 15 T3-CM profiles, and 9 of 14 T3-CF profiles, with three additional ties in T3-CF (**Supplementary Table S11**). Transition energy alone tended to peak substantially earlier along pseudotime, particularly for the later cardiac transitions. Thus, the localization behavior of the complete GDIS score cannot be reduced to its transition-localization term alone; the incorporation of sustained instability and other trajectory-derived descriptors contributes materially to the final profile.

More broadly, these comparisons suggest that no single scalar statistic is likely to capture every manifestation of a biological state transition. Some transitions may be dominated by increased dispersion, others by local trajectory displacement or changes in correlation structure, and others by combinations of several features. Theoretical and experimental work on critical transitions and cell-fate decisions similarly indicates that different systems can exhibit different combinations of

early-warning or instability-related signatures [9-11, 13]. GDIS provides one approach for integrating several such properties within a common bounded framework, whereas individual conventional metrics may retain advantages in simplicity and direct interpretability.

A practical implication is therefore to view GDIS and conventional metrics as complementary descriptions of transition-associated state-space reorganization. Concordance among independent measures may strengthen evidence that a particular trajectory region is undergoing substantial reorganization, whereas disagreement among metrics may reveal which geometrical or statistical aspects of the transition are most pronounced. This interpretation is consistent with the present results, in which GDIS, variance, entropy, and step distance each captured biologically relevant transition structure, but with different relative performance across the pancreatic and cardiac systems.

Accordingly, the benchmark analyses support a deliberately restrained interpretation: GDIS provides a generalized integrative measure of transition-associated dynamical instability, but it is not universally superior to simpler trajectory statistics. Its principal contribution lies in combining multiple dynamical descriptors within a unified framework that transferred across two distinct single-cell differentiation systems without dataset-specific retuning.

These comparisons must also be interpreted in light of the nature of pseudotime. An important consideration in interpreting GDIS-Bio is that the trajectories analyzed here are reconstructed from independent single cells ordered along pseudotime, rather than from longitudinal measurements of the same cells through physical time. Pseudotime methods are designed to infer relative developmental progression from cross-sectional cellular measurements and therefore provide an ordering of transcriptional states rather than a direct record of chronological dynamics [1-4]. Accordingly, the quantities derived from these trajectories should be interpreted as properties of an ordered transcriptional state manifold and not as direct measurements of temporal evolution at the level of individual cells. This distinction is fundamental to the biological interpretation of GDIS and of the conventional pseudotemporal metrics used for comparison.

In both GSE114412 and GSE175634, pseudotime showed substantial concordance with experimental sampling time, supporting its use as an ordered coordinate of differentiation. In the pancreatic dataset, deposited endocrine pseudotime was positively associated with differentiation day, whereas in the cardiac dataset deposited diffusion pseudotime similarly tracked progression across experimentally sampled developmental stages [15, 16]. Nevertheless, pseudotime is not equivalent to chronological time. Cells occupying neighboring positions in pseudotime may have been collected at the same experimental time point, while cells collected at different time points may occupy nearby positions along the reconstructed trajectory [4]. GDIS profiles should therefore be interpreted along a developmental ordering coordinate rather than on an absolute temporal scale.

This distinction also affects the interpretation of local trajectory windows. Each sliding window represents a neighborhood of cells that are adjacent in pseudotemporal rank and occupy nearby positions along the reconstructed differentiation trajectory. The local cell $\times$ PC matrices analyzed by GDIS therefore characterize how transcriptional state-space organization changes across consecutive portions of this ordering (**Figures 9 and 10**). Importantly, pyGDIS operates on the ordered sequence of trajectory windows rather than on the absolute numerical spacing between pseudotime values. Consequently, the resulting descriptors reflect relative trajectory order and local state-space geometry, not the physical rate at which cells traverse the developmental process.

The same caution applies to terminology derived from dynamical-systems theory. Measures such as local sensitivity, trajectory stretching, state-space expansion, nonlinear complexity, and persistence provide quantitative descriptions of changes in the geometry and organization of the reconstructed transcriptional trajectory. They do not establish that the underlying biological system is a deterministic low-dimensional dynamical system, nor do they demonstrate classical chaos, a formal bifurcation, or the existence of a mathematically defined attractor. Although developmental processes are often conceptualized using dynamical or landscape-based representations [11, 13], such interpretations require stronger mechanistic or longitudinal evidence than is available from cross-sectional single-cell measurements alone. In particular, the quantity termed “attractor expansion” in the reference pyGDIS formulation is interpreted here more conservatively as local state-space expansion, reflecting observable trajectory geometry without asserting the presence of a biological attractor.

The interpretation of lag-1 autocorrelation likewise requires qualification. In classical early-warning theory, increasing lag-1 temporal autocorrelation may arise from critical slowing down as a dynamical system approaches a transition [9]. In the present analysis, however, lag-1 autocorrelation was calculated along the pseudotemporal ordering of independent cells. It is therefore more accurately described as lag-1 pseudotemporal autocorrelation, representing similarity between adjacent positions along the ordered cellular trajectory rather than temporal memory within the same evolving biological unit. Its weaker performance in several external-validation settings should therefore not be interpreted as evidence against classical temporal autocorrelation as an early-warning signal. Instead, it illustrates the limitations of transferring a statistic developed for genuine time series directly to a cross-sectional pseudotemporal representation (**Figure 5; Supplementary Table S11**).

These considerations also constrain the interpretation of the biological landmarks used for validation. The frozen NEUROG3-early landmark in GSE114412 and the destination-state median diffusion-pseudotime landmarks in GSE175634 provide reproducible reference coordinates for evaluating localization, but they do not represent directly observed transition-onset times. A small peak-to-landmark distance therefore indicates that a metric identifies a region close to an independently defined biological state transition; it does not establish that the metric predicts the exact time at which an individual cell changes fate. This distinction is consistent with broader cautions regarding inference of temporal or causal dynamics from pseudotemporal orderings [4] and is why the present study uses the language of transition localization rather than prospective transition-time prediction.

The structure of the GSE175634 analysis further illustrates this distinction. Early cells on the shared IPSC→MES→CMES→PROG backbone were retained as a common developmental trajectory and were not retrospectively assigned to CM or CF fates before lineage-specific evidence became available. This is consistent with the broader observation that transcriptional state and eventual lineage fate are related but not equivalent [8]. Terminal analyses were therefore restricted to individuals in which sufficient trajectory coverage existed relative to the frozen CM or CF landmarks. This design reduced the risk of using future lineage information to define earlier trajectory segments and preserved the intended interpretation of GDIS as a measure of instability along an observed pseudotemporal path rather than as a fate-prediction model.

Accordingly, GDIS-Bio should be understood as operating on a pseudotemporally ordered representation of cellular state transitions, rather than on a direct longitudinal time series. The framework uses ordered high-dimensional cellular states to identify regions where transcriptional

trajectory geometry becomes unusually unstable or reorganized. This representation can capture biologically meaningful transition-associated structure, as demonstrated by the reproducible pancreatic signal and its external cardiac validation, while remaining conceptually distinct from physical-time dynamical measurement. Maintaining this distinction is essential for avoiding overinterpretation and for defining the appropriate scope of GDIS-Bio in single-cell studies.

Several features of the study design strengthen the interpretation of the GDIS-Bio results and reduce the likelihood that the observed transition localization arose from post hoc analytical optimization. Most importantly, the biological transition regions used for evaluation were defined independently of GDIS. In the pancreatic discovery analysis, the NEUROG3-early landmark was derived from the deposited endocrine-state annotations and pseudotemporal structure of GSE114412 [15], whereas in the cardiac validation analysis, the T2, T3-CM, and T3-CF landmarks were defined from the destination-state distributions in deposited diffusion pseudotime from GSE175634 [16]. These reference positions were frozen before examination of the corresponding GDIS localization results. Consequently, agreement between GDIS peaks and biological landmarks represents validation external to the score construction, rather than circular definition of a transition from the GDIS profile itself.

A second strength is the use of a frozen analytical configuration across discovery and external validation. The GDIS formulation, component weights, primary state-space dimensionality, sliding-window parameters, cohort definitions, and biological landmarks were not reoptimized to improve agreement with the cardiac transition regions. The external dataset therefore provided a genuine transfer test in a biologically distinct differentiation system. This design is particularly important in single-cell trajectory analysis because reconstructed trajectories and downstream quantitative results can depend on representation, topology, sampling, and analytical assumptions [3, 4]. Despite substantial differences between the pancreatic and cardiac systems in tissue context, lineage organization, sampling structure, and trajectory geometry, GDIS retained significant transition localization for T2, T3-CM, and T3-CF without dataset-specific retuning (**Figure 4; Supplementary Table S9**).

The analysis also incorporated prespecified sensitivity testing rather than relying on a single favorable parameterization. In the discovery dataset, alternative PCA dimensionalities and sliding-window configurations were evaluated systematically, and the primary signal remained highly stable for sufficiently expressive state spaces (**Supplementary Figure S2; Supplementary Table S4**). The same strategy was applied independently in the external dataset, where the 30-PC representation and alternative window configurations largely reproduced the primary 50-PC, 400-cell/100-step analysis (**Supplementary Figure S4; Supplementary Table S10**). Importantly, the sensitivity analyses also identified conditions under which localization became less stable, particularly at very low dimensionality and in selected individual trajectories. Reporting these deviations is important because robustness is more convincingly demonstrated by characterizing both stable and sensitive analytical regimes than by presenting only parameter choices that reproduce the primary result.

Another strength is the use of structure-preserving null models. Circular shifting retained the observed metric values, trajectory length, and local sequential structure of each profile while disrupting its alignment with the independently defined biological landmarks. This strategy was better matched to the dependence structure of the trajectory profiles than independently randomizing overlapping windows, which would destroy much of the intrinsic profile organization. In the discovery analysis, GDIS localization relative to NEUROG3-early was highly

unlikely under this null model (**Figure 3; Supplementary Table S6**). Corresponding analyses in the external dataset showed significant localization for all three evaluated cardiac transition scopes (**Supplementary Table S9**). These results provide evidence that the observed alignments were not simply consequences of structured profiles occurring at arbitrary positions along pseudotime.

The study further benefits from evaluating reproducibility at multiple biological levels. In GSE114412, the two independent differentiations showed strong agreement in GDIS profile shape and peak position within both the SC-EC and SC- β lineages (**Supplementary Table S3**). Cross-branch agreement was also high over the common developmental region, consistent with localization of a shared early endocrine reorganization before terminal lineage divergence. In GSE175634, analysis at the individual level allowed external localization to be evaluated across multiple biological individuals rather than from a single pooled trajectory. The individual-level distributions shown in **Supplementary Figure S5** make this heterogeneity explicit and reduce reliance on transition-level summary statistics alone.

The benchmark design provides an additional strength. GDIS was compared not only with its internal transition-energy component but also with several transparent conventional measures representing different aspects of trajectory organization, including total variance, Gaussian differential entropy, mean pseudotemporal step distance, and lag-1 pseudotemporal autocorrelation (**Figures 2 and 5; Supplementary Tables S5 and S11**). Variance and autocorrelation have long been considered in the broader literature on critical-transition indicators [9], while multivariate instability and variability measures have also been investigated in complex biological state transitions [10, 11]. Importantly, the present study retained results in which conventional metrics matched or exceeded GDIS rather than restricting interpretation to favorable comparisons. This benchmarking strategy provides a more informative assessment of where GDIS contributes complementary information and prevents the conclusions from depending solely on the performance of the proposed framework.

The statistical framework was also designed to respect the dependence inherent in overlapping sliding windows and pseudotemporally ordered single-cell data. The 75% overlap in the primary window configuration introduces strong local dependence among neighboring windows, and pseudotime itself represents an ordering of independent cells rather than repeated longitudinal measurements [4]. Accordingly, windows were used to construct continuous metric profiles but were not treated as independent inferential replicates. Statistical comparisons were instead performed at the branch $\times$ differentiation level in the discovery analysis and at the individual level in the external-validation analysis. Avoiding window-level pseudoreplication provides a more appropriate basis for statistical inference from these trajectory profiles.

A further strength is the explicit separation of localization, directionality, and mechanism. A metric could localize near a transition without necessarily increasing from the source-state landmark to the destination-state landmark, as demonstrated most clearly by T3-CF. Likewise, localization near a biological transition was not interpreted as evidence of a classical bifurcation, deterministic chaos, or a mathematically defined attractor transition. This distinction is particularly important when dynamical-systems concepts are applied to cross-sectional single-cell trajectories, where pseudotemporal organization does not by itself establish underlying physical-time dynamics [4, 11, 13]. By keeping these concepts separate, the framework avoids assigning mechanistic interpretations that are not directly supported by the available data.

Finally, the study incorporates a high level of computational reproducibility. The complete analytical workflow—from public-data acquisition through preprocessing, trajectory construction,

GDIS calculation, sensitivity analyses, conventional benchmarking, null-model testing, external validation, and generation of manuscript outputs—is publicly available through the GDIS-Bio repository. The exact version associated with this study has been permanently archived as GDIS-Bio v1.0.0 in Zenodo [18]. The repository includes sequential analysis scripts, environment specifications, frozen parameters, Linux and Windows execution workflows, reference outputs, and manuscript-to-code mappings. This versioned archive enables the computational analyses reported here to be traced to a fixed and citable implementation.

Together, these features provide a stringent validation framework for GDIS-Bio. Independent biological landmarks, frozen analytical settings, transfer to an external biological system without retuning, prespecified sensitivity analyses, dependence-preserving null models, replicate- and individual-level inference, direct comparison with strong conventional alternatives, explicit separation of localization from mechanism, and versioned computational reproducibility collectively strengthen the credibility of the observed transition-localization results while placing appropriate limits on their interpretation.

Despite these strengths, several considerations define the appropriate interpretation and current scope of the present results. The most fundamental is that the analysis is based on cross-sectional single-cell trajectories reconstructed in pseudotime rather than longitudinal measurements of the same cells. Pseudotime methods provide an inferred ordering of cellular states and can recover biologically meaningful developmental progression, but they do not represent physical time or directly establish the temporal dynamics experienced by individual cells [1-4]. Consequently, GDIS should be interpreted as quantifying changes in instability and state-space organization along an ordered transcriptional trajectory rather than as directly measuring within-cell dynamical evolution. This limitation applies equally to the lag-1 autocorrelation benchmark, which in the present study represents pseudotemporal rather than true temporal autocorrelation. Accordingly, its behavior should not be interpreted as a direct test of classical temporal early-warning theory [9].

A related limitation concerns the biological transition landmarks used for validation. In both datasets, these landmarks were defined independently of GDIS, which strengthens the evaluation, but they remain state-based reference positions rather than directly observed transition-onset times. In GSE114412, the median pseudotime of the NEUROG3-early state provides a reproducible reference for the endocrine transition region [15], whereas in GSE175634 the median diffusion pseudotime of the destination state provides the corresponding cardiac landmark [16]. These positions should therefore be regarded as biologically informed localization targets rather than exact moments of fate commitment. The reported absolute peak-to-landmark distances quantify proximity to these reference regions and should not be interpreted as prediction errors relative to a known transition time. This distinction is also consistent with the broader observation that transcriptional state and eventual cell fate are related but not equivalent [8].

The GDIS profiles also depend on the representation of the underlying transcriptional state space. Although the primary 50-PC representation was supported by prespecified representation-stability criteria and remained robust to moderate dimensionality reduction, the sensitivity analyses showed that lower-dimensional representations could alter peak localization substantially in selected trajectories. This was particularly evident for the external T2 transition under the 5-PC representation and for individual profiles under other reduced-dimensional settings (**Supplementary Figures S2 and S4; Supplementary Tables S4 and S10**). These findings indicate that preservation of sufficient local state-space geometry is important for GDIS analysis

and that dimensionality reduction should not be regarded as a biologically neutral preprocessing step.

Similarly, sliding-window construction introduces an additional scale dependence. The primary 400-cell/100-step configuration and the prespecified 300-cell/75-step and 500-cell/125-step alternatives generally produced concordant profiles, but non-negligible peak displacement occurred in selected trajectories (**Supplementary Figures S2 and S4; Supplementary Tables S4 and S10**). Because adjacent windows overlap substantially, neighboring GDIS values are strongly dependent. The present analysis addressed this issue by performing statistical inference at the trajectory or individual level rather than treating windows as independent observations. Nevertheless, the detailed profile shape and peak position remain partly dependent on the local neighborhood scale represented by the window size and step.

Another limitation is that the present validation spans only two biological differentiation systems, both representing in vitro developmental processes. The pancreatic endocrine and cardiac differentiation datasets provide useful contrasts in tissue context, lineage structure, sampling design, and trajectory organization [15, 16], but they cannot establish universal applicability across single-cell systems. GDIS behavior may differ in immune activation, tumor evolution, treatment response, regeneration, developmental systems with extensive branching, spatially organized tissues, or trajectories with cyclic, discontinuous, or poorly sampled structure. Broader evaluation across such biological settings will therefore be necessary before GDIS-Bio can be considered generally validated.

The comparative benchmark also places an important limitation on claims of methodological superiority. In the discovery dataset, GDIS showed the smallest mean absolute peak-to-landmark distance, but total variance was a strong conventional comparator. In the external dataset, total variance, Gaussian differential entropy, and mean pseudotemporal step distance were competitive with or showed smaller median peak-to-landmark distances than GDIS in several transition scopes (**Figures 2 and 5; Supplementary Table S11**). These findings indicate that GDIS does not universally outperform simpler transition-associated statistics. Its contribution should therefore be interpreted in terms of the integration of multiple trajectory descriptors, transferability across datasets, and complementary characterization of state-space reorganization, rather than universal dominance in transition localization. This conclusion is consistent with the broader literature showing that different transition phenomena may manifest through different combinations of variance, correlation, and multivariate state-space changes [9-11].

The statistical power of some comparisons is also limited by the number and structure of the independent biological units. In the discovery analysis, the principal paired comparison involved only four branch $\times$ differentiation trajectories. This sharply limits the resolution of exact trajectory-level tests and explains why even four concordant nonzero differences against several comparators could not produce a one-sided exact p -value smaller than 0.0625. The external cardiac analysis provided substantially greater individual-level replication, but the number of evaluable individuals differed among T2, T3-CM, and T3-CF because terminal analyses required complete-window coverage relative to the frozen landmarks. In addition, the three external-validation scopes should not be regarded as three fully independent replication cohorts: an individual could contribute to more than one scope, and the CM and CF extensions shared portions of the common developmental backbone. Inference was therefore performed separately within each prespecified transition scope rather than by treating the 48 scope $\times$ individual profiles as mutually independent observations.

A further limitation is that the present implementation does not define a universal biological threshold for GDIS. Although GDIS is bounded between 0 and 1, a particular numerical value should not be assumed to represent a universal critical state across datasets, tissues, or cell systems. Transition localization in this study was based on relative profile structure, peak position, independently defined biological landmarks, and null-model comparison rather than on a fixed numerical cutoff. Establishing whether transferable calibration ranges or context-specific reference distributions can be developed will require substantially broader empirical evaluation.

The biological interpretation of the individual GDIS components also warrants caution. Local sensitivity, trajectory stretching, state-space expansion, nonlinear complexity, and persistence provide quantitative descriptions of the reconstructed transcriptional trajectory, but they do not map uniquely onto specific molecular mechanisms. For example, increased local state-space expansion could reflect transcriptional heterogeneity, developmental branching, asynchronous differentiation, changes in regulatory programs, or combinations of these phenomena. Likewise, the present data do not establish classical bifurcations, deterministic chaos, or mathematically defined attractor transitions. Developmental-landscape and critical-state concepts provide useful theoretical perspectives [11, 13], but stronger mechanistic interpretation will require orthogonal evidence such as lineage tracing, perturbation experiments, regulatory-network measurements, RNA-velocity information, or genuine longitudinal observations [5-8].

Finally, GDIS localization is inherently dependent on the quality of the underlying trajectory reconstruction, cell-state annotation, and sampling of transitional populations. Trajectory-inference performance can depend on topology, sampling density, representation, and methodological assumptions [3, 4]. Errors in cell ordering, insufficient representation of transitional states, unequal sampling across developmental stages, or unresolved branching could therefore alter the apparent geometry of the trajectory and consequently the resulting GDIS profile. The present study reduced these risks by using deposited trajectory coordinates and biological annotations, evaluating concordance with independent experimental sampling time, validating state-space geometry before GDIS calculation, and restricting terminal analyses to individuals with sufficient coverage relative to the frozen landmarks. Nevertheless, uncertainty in the reconstructed trajectory itself was not propagated quantitatively into GDIS confidence intervals or peak-location uncertainty and remains an important target for future methodological development.

These limitations define the appropriate scope of the current conclusions. GDIS-Bio provides a reproducible and externally transferable framework for localizing regions of transition-associated transcriptional instability in high-dimensional single-cell trajectories, but it should not be interpreted as a direct measurement of physical-time dynamics, an exact predictor of transition onset, evidence of a specific classical dynamical mechanism, or a universally superior alternative to simpler statistics. Rather, the present study establishes a rigorously evaluated foundation for broader biological validation and for future integration with uncertainty-aware trajectory analysis, orthogonal molecular measurements, and more direct observations of cellular dynamics.

Within these boundaries, the present findings suggest that GDIS-Bio may provide a useful framework for identifying regions of pronounced state-space reorganization in high-dimensional single-cell trajectories. Its application to pancreatic endocrine differentiation and independent external validation in cardiac differentiation indicate that the framework is not restricted to a single tissue context, lineage organization, or transition pattern. At the same time, the differences observed among transition types emphasize that GDIS should be interpreted within the biological

and geometrical context of each trajectory rather than through a universal expectation of monotonic score increase or a fixed critical threshold.

One immediate implication is that GDIS may be useful in studies in which a developmental or disease-associated transition is suspected but its location along a reconstructed trajectory is uncertain. Pseudotime and related trajectory-inference methods provide ordered representations of cellular progression but do not, by themselves, identify a unique point at which substantial state-space reorganization occurs [1-4]. In such settings, a localized GDIS maximum could be used to prioritize candidate transition regions for closer biological investigation, including changes in transcription-factor activity, regulatory-network structure, pathway activation, lineage commitment, or cell-state heterogeneity. Because the present study evaluated GDIS against independently defined biological landmarks, the framework is most appropriately viewed as a tool for transition-region discovery and prioritization, followed by biological interpretation using orthogonal evidence.

Future evaluation should extend GDIS-Bio to a broader range of biological systems. Important targets include immune-cell activation and differentiation, tumor progression and treatment response, embryonic development, tissue regeneration, cellular reprogramming, and drug-induced state transitions. Such studies will be necessary to determine whether the localization behavior observed here generalizes to trajectories with more extensive branching, cyclic or discontinuous structure, highly heterogeneous intermediate states, or less complete sampling than the pancreatic and cardiac differentiation systems examined in the present work. Large-scale benchmarking across diverse trajectory topologies will also be important because trajectory-inference performance itself is known to depend on biological structure, sampling density, and methodological assumptions [3, 4].

A particularly important next step is prospective experimental validation. Rather than evaluating GDIS retrospectively against existing annotations, future studies could use high-GDIS regions to nominate candidate transition intervals for targeted experimental interrogation. Cells sampled before, within, and after such regions could then be examined using lineage tracing, perturbation experiments, transcription-factor profiling, chromatin accessibility, or other molecular measurements. Lineage-tracing studies have shown that transcriptional state and eventual fate are related but not interchangeable [8], making such orthogonal validation especially important. RNA velocity and its dynamical extensions could additionally provide local directional information [5, 6], while time-resolved optimal-transport approaches could help relate GDIS localization to population-level developmental movement across experimentally sampled time points [7]. Combining these methods could provide a stronger test of whether high-GDIS regions correspond to biologically increased susceptibility to state reorganization.

Integration with additional single-cell modalities represents another promising direction. The present implementation operates on transcriptomic state spaces, but the same general framework could be evaluated in representations derived from chromatin accessibility, proteomics, epigenomics, or multimodal single-cell measurements. Joint latent representations may provide a more complete view of transition-associated instability and could help distinguish transient transcriptional variability from coordinated regulatory changes occurring across multiple molecular layers. Such analyses would also provide an opportunity to determine whether GDIS maxima are conserved across molecular modalities or are specific to particular layers of cellular regulation.

The framework could also be extended to incorporate trajectory uncertainty and branch ambiguity explicitly. In the present analysis, trajectories and biological scopes were defined before GDIS calculation, and terminal cardiac analyses were restricted to individuals with sufficient coverage relative to the frozen landmarks. However, trajectory reconstruction itself can be uncertain, particularly in sparsely sampled, highly branched, or topologically ambiguous systems [3, 4]. Future implementations could therefore propagate uncertainty from alternative pseudotemporal orderings or branch assignments into GDIS profiles, quantify the stability of peak locations across plausible trajectory reconstructions, and report confidence intervals for transition localization rather than relying on a single inferred ordering.

Relatedly, future development should incorporate more direct uncertainty quantification for GDIS itself. The present study evaluated robustness through biological replication, sensitivity analyses, structure-preserving null models, and individual-level comparisons. Bootstrap or other resampling approaches could additionally quantify uncertainty in GDIS values, individual component contributions, and global-peak positions. Such methods would make it possible to distinguish sharply localized and highly reproducible transition signals from broader or less certain instability regions at the profile level.

Further work should also examine the relative contribution of individual GDIS components across biological contexts. The present external-validation results showed that the integrated GDIS score had smaller peak-to-landmark distances than the isolated transition-energy component in most profiles, indicating that transition localization is not determined by the transition term alone. Systematic component-ablation studies across larger collections of datasets could determine whether particular combinations of local sensitivity, trajectory stretching, state-space expansion, nonlinear complexity, and persistence are especially informative for specific classes of biological transitions. Such analyses could also clarify whether the relative importance of these descriptors changes across lineage commitment, terminal differentiation, disease progression, or perturbation-induced transitions.

Comparison with additional trajectory-aware and dynamical approaches will also be important. The present benchmark deliberately emphasized transparent conventional measures—variance, Gaussian differential entropy, pseudotemporal step distance, and lag-1 pseudotemporal autocorrelation—so that the contribution of GDIS could be evaluated against interpretable alternatives. Future studies could extend these comparisons to RNA-velocity-derived measures [5, 6], time-resolved optimal-transport approaches [7], lineage-aware analyses [8], dynamical-network biomarkers [10], landscape-based formulations [13], and nonlinear dynamical approaches previously applied to transcriptomic state-transition analysis [12]. Such comparisons would help define more precisely the biological and geometrical settings in which an integrated instability score contributes information beyond established trajectory-analysis methods.

The bounded structure of GDIS also raises the possibility of developing cross-study calibration, although the present findings do not support a universal instability threshold. Classical and biological critical-transition frameworks likewise emphasize that transition-associated signatures can depend strongly on system context and on the measured variables [9-11]. A substantially larger reference collection spanning multiple tissues, experimental technologies, trajectory topologies, perturbations, and biological processes would therefore be required before attempting to define transferable GDIS reference ranges. Until such evidence is available, relative profile structure and localization with respect to independently defined biological landmarks remain more appropriate than interpreting any absolute GDIS value as intrinsically “critical.”

Finally, GDIS-Bio could be extended beyond pseudotemporally ordered scRNA-seq data to other ordered high-dimensional biological systems, including spatially resolved developmental gradients, longitudinal multi-omics, perturbation series, and experimentally sampled temporal trajectories. Genuine longitudinal measurements would be particularly informative because they would allow more direct evaluation of temporal persistence, sensitivity, and autocorrelation and would provide a stronger basis for testing concepts derived from classical dynamical-systems and critical-transition theory [9-11, 13]. Such data could help determine which GDIS descriptors retain their meaning when the ordering coordinate corresponds to physical time rather than pseudotemporal rank.

Overall, GDIS-Bio provides a reproducible and externally transferable framework for localizing regions of pronounced transcriptional state-space reorganization in single-cell trajectories without requiring an explicit mechanistic dynamical model. Its principal contribution lies in integrating complementary descriptors of trajectory instability while maintaining strict separation between score construction and biological validation. The transfer of the same analytical configuration across pancreatic and cardiac differentiation systems supports its potential utility for prioritizing candidate transition regions for downstream systems-level investigation. Broader validation across additional biological systems, prospective experimental testing, explicit uncertainty quantification, multimodal integration, and comparison with complementary trajectory-aware methods will be important for defining the biological settings in which GDIS-Bio provides the greatest added insight.

4. Methods

4.1. Study design and analytical safeguards

This study evaluated whether the Generalized Dynamical Instability Score (GDIS) could identify regions of increased dynamical instability associated with biological state transitions in high-dimensional single-cell transcriptomic trajectories. The analysis followed a discovery-validation design. Human pancreatic endocrine differentiation data from GSE114412 [15] were used as the discovery system, and an independent human induced-pluripotent-stem-cell cardiac differentiation dataset, GSE175634 [16], was used for external validation. The external-validation analysis was performed using the reference GDIS formulation and primary analysis settings established before examination of the external GDIS results. The overall study design and GDIS-Bio analytical workflow are summarized in **Figure 6**.

A central design principle was the separation of biological transition definition from GDIS calculation. Cell-state annotations, experimental sampling times, deposited pseudotime coordinates, analysis populations, transition landmarks, state-space dimensionality, and window configurations were defined or frozen before the corresponding GDIS analyses. Biological landmarks were never supplied to GDIS as optimization targets or biological critical values, and no GDIS parameter was subsequently modified to improve agreement with those landmarks. As illustrated in **Figure 6**, biological information entered the workflow only during validation and did not contribute to GDIS construction. In the external dataset, the cohort and transition definitions were explicitly frozen before expression preprocessing and before any GDIS value was calculated.

Throughout the study, pseudotime was interpreted as an ordering coordinate for independent single cells rather than as continuous physical time or longitudinal observation of the same cell. Accordingly, terms such as *pseudotemporal autocorrelation* and *pseudotemporal trajectory* describe relationships along the ordered cell sequence and should not be interpreted as direct

measurements of within-cell temporal dynamics. Overlapping trajectory windows were also recognized as statistically dependent and were not treated as independent samples in inferential analyses.

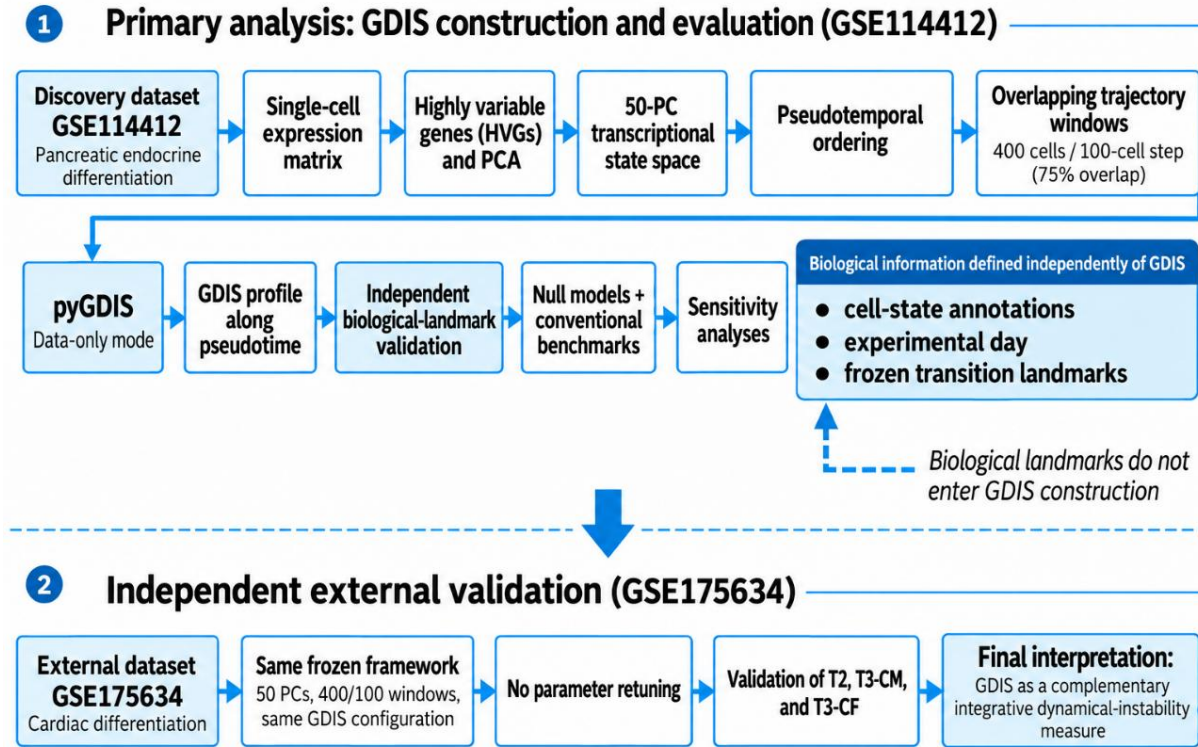


Figure 6. GDIS-Bio study design and analytical workflow. The discovery framework developed using GSE114412 was transferred without parameter retuning to the independent GSE175634 cardiac differentiation dataset, with biological transition landmarks defined independently of GDIS.

4.2. Discovery dataset: pancreatic endocrine differentiation

The discovery analysis used GSE114412, generated by Veres et al. during high-resolution characterization of human stem-cell differentiation toward pancreatic endocrine cell states [15]. The deposited Stage-5 data contained 51,274 cells and 16,224 genes, together with cell annotations, experimental differentiation information, and an endocrine-specific pseudotime dataset. The authors’ deposited endocrine trajectory contained 18,099 cells, all of which matched cells in the processed expression matrix. The endocrine pseudotime metadata included experimental day (*CellDay*), differentiation replicate, assigned biological state, deposited pseudotime value, and pseudotime branch. The original study and GEO accession are described by Veres et al. (2019).

The endocrine trajectory comprised the annotated states *prog_nkx61*, *neurog3_early*, *neurog3_mid*, *neurog3_late*, and the terminal *sc_beta* or *sc_ec* populations. Deposited *branch 0* terminated in the *SC-EC* population, whereas *branch 1* terminated in the *SC-β* population. Experimental days ranged from day 0 through day 7. Deposited pseudotime was positively associated with experimental day, and the ordering of the biological states was consistent with the expected endocrine differentiation sequence. This discovery design is summarized in **Figure 7**, which illustrates the pseudotemporal progression from *prog_nkx61* through NEUROG3-positive

intermediate states to the two terminal endocrine branches, together with the experimental-day sampling structure.

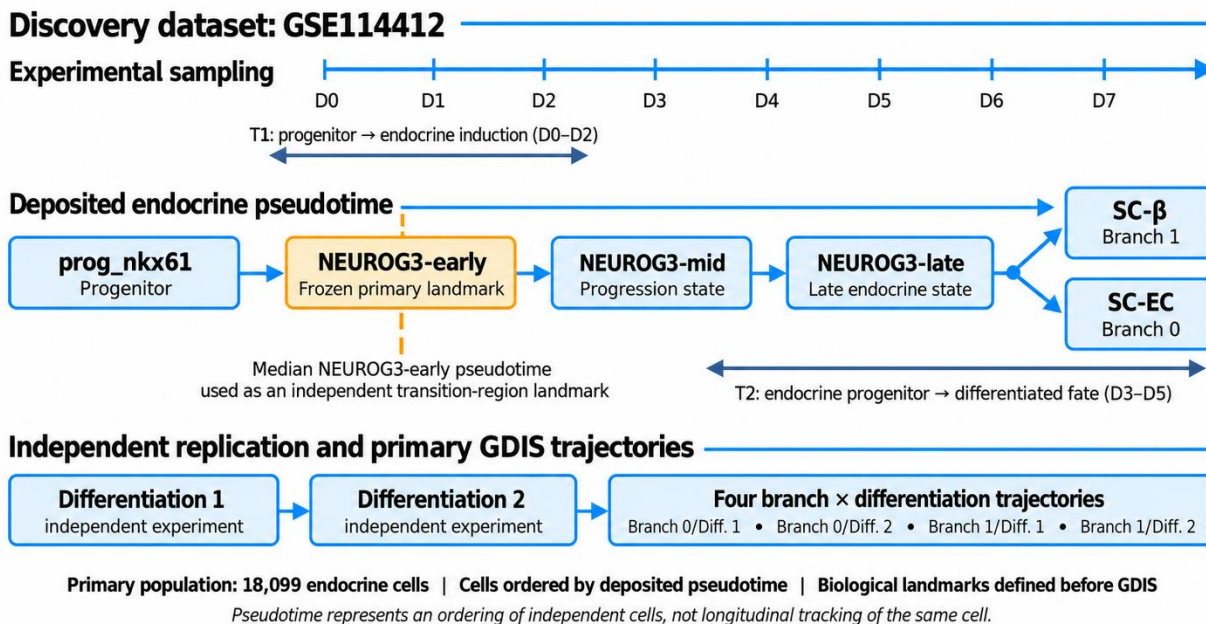


Figure 7. GSE114412 pancreatic endocrine differentiation discovery design. Endocrine cells were ordered by deposited pseudotime across two independent differentiations and two terminal branches, SC-β and SC-EC. The median pseudotime of the independently annotated **NEUROG3-early** state was prespecified as the primary transition-region landmark before GDIS analysis.

Two independent differentiations were retained separately. Thus, the primary discovery analysis consisted of four branch × differentiation trajectories: branch 0/differentiation 1, branch 0/differentiation 2, branch 1/differentiation 1, and branch 1/differentiation 2. Replicate trajectory validation was performed before GDIS analysis using day–pseudotime association, monotonicity of daily median pseudotime, agreement of biological-state ordering, and cross-replicate trajectory profiles. As also shown in **Figure 7**, these two independent differentiations provided the basis for the four primary GDIS discovery trajectories.

The median pseudotime of the independently annotated *NEUROG3-early* state within each branch × differentiation trajectory was frozen as the primary early endocrine transition-region landmark. Additional state medians were retained to describe progression from *prog_nkx61* through NEUROG3-positive states to the terminal fate. These landmarks were treated as reproducible biological state coordinates and not as measurements of the exact instant of biological transition onset. All discovery landmarks were frozen before GDIS was calculated.

4.3. Independent external-validation dataset: cardiac differentiation

External validation used GSE175634 from Elorbany et al. (2022), which profiled differentiation from human induced pluripotent stem cells toward cardiac cell states at seven experimental time points in 19 human cell lines. The deposited dataset contained 230,786 cells, 38,943 genes, and metadata describing individual, experimental collection, differentiation day, biological cell type, and diffusion pseudotime. The annotated cell states included induced pluripotent stem cells (IPSC), mesoderm (MES), cardiac mesoderm (CMES), cardiac progenitors (PROG), cardiomyocytes

(CM), cardiac fibroblasts (CF), and unclassified cells (UNK). The experimental dataset and its bifurcating cardiac differentiation design were described previously by Elorbany et al. (2022) [16]. Deposited diffusion pseudotime was available for 217,528 cells and for all cells belonging to the known developmental states. Experimental differentiation day was strongly positively associated with deposited pseudotime, although experimental-day median pseudotime was not perfectly monotonic at the final sampling point. Consequently, experimental day was retained as an independent biological covariate rather than used as the trajectory coordinate. Deposited diffusion pseudotime was used as the primary ordering coordinate.

Because the deposited metadata did not identify the eventual CM or CF fate of cells in the common early developmental states, early cells were not retrospectively assigned to a terminal lineage. The primary external-validation trajectory was therefore defined as the shared developmental backbone

$$\text{IPSC} \rightarrow \text{MES} \rightarrow \text{CMES} \rightarrow \text{PROG},$$

with secondary extensions from the shared backbone to either CM or CF. This external-validation design is summarized in **Figure 8**, which illustrates the common developmental backbone, the bifurcation into cardiomyocyte and cardiac fibroblast terminal branches, and the prespecified biological transitions evaluated in the external dataset. Common backbone cells could therefore contribute to both terminal-extension analyses, but they were not interpreted as prospectively CM-fated or CF-fated cells, and the CM and CF extension analyses were not regarded as statistically independent of one another. The independent replicate unit was the individual.

Eligibility criteria were fixed before preprocessing and GDIS calculation. Shared-backbone analysis required at least 100 cells from each of IPSC, MES, CMES, and PROG within an individual. Terminal-extension analysis additionally required at least 100 cells of the relevant terminal population. These rules yielded 19 initially eligible shared-backbone individuals, 16 CM-extension individuals, and 15 CF-extension individuals. As shown in **Figure 8**, the external design therefore consisted of one shared backbone analysis together with two terminal-extension analyses.

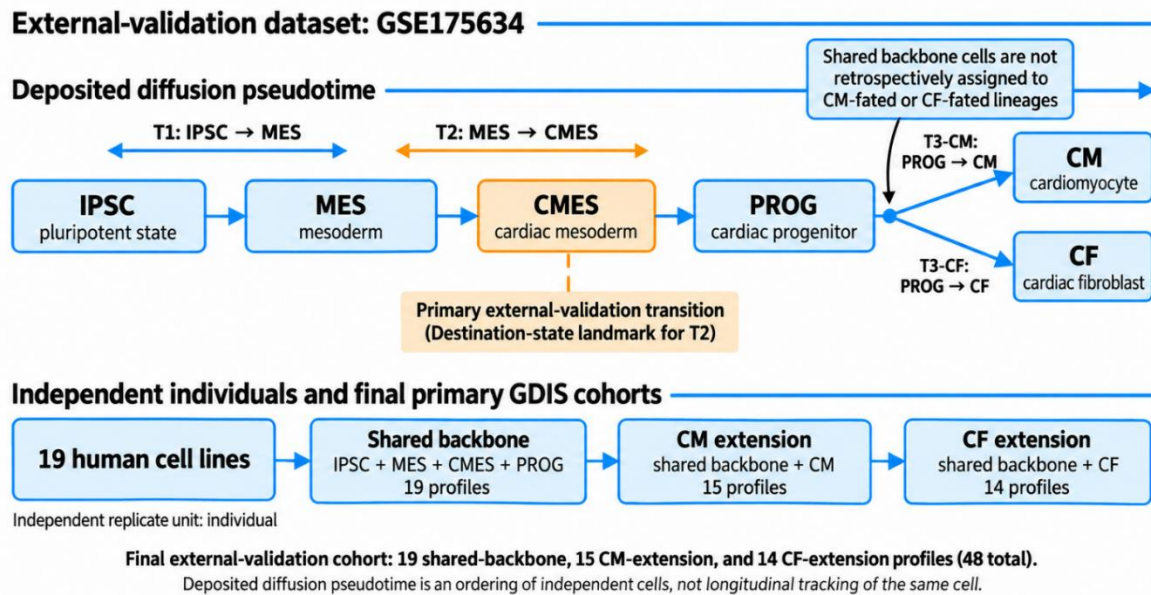


Figure 8. GSE175634 cardiac differentiation external-validation design. External validation was based on a shared developmental backbone (IPSC→MES→CMES→PROG) with terminal extensions to

cardiomyocytes (CM) and cardiac fibroblasts (CF). Prespecified transitions T1, T2, T3-CM, and T3-CF were defined independently of GDIS before analysis.

Four biological transitions were defined before GDIS calculation. T1 represented IPSC→MES mesoderm induction; T2 represented MES→CMES cardiac mesoderm specification and served as the primary external-validation transition; T3-CM represented PROG→CM terminal cardiomyocyte differentiation; and T3-CF represented PROG→CF terminal fibroblast differentiation. T1 was retained as a prespecified biological reference transition within the shared developmental backbone, whereas the primary external GDIS validation analyses were conducted for T2, T3-CM, and T3-CF. For these externally validated transitions, the deposited pseudotime median of the destination state was frozen as the reference landmark, with the destination-state interquartile range retained as a broader state-transition region. These landmarks were explicitly defined as state landmarks rather than estimates of true transition-onset times.

The principal characteristics of the discovery and external-validation datasets, together with the corresponding trajectory structures, biological replication, ordering coordinates, transition landmarks, primary state-space representations, sliding-window configurations, and final analysis scopes, are summarized in **Table 2**. This side-by-side summary highlights the transfer of the primary GDIS-Bio analytical configuration from the pancreatic discovery system to the independent cardiac validation system without dataset-specific parameter retuning.

Table 2. Summary of discovery and external-validation datasets and analysis design

Feature	Discovery: GSE114412	External validation: GSE175634
Biological system	Pancreatic endocrine differentiation	iPSC-to-cardiac differentiation
Original cells	51,274	230,786
Cells with deposited pseudotime	18,099 endocrine cells	217,528
Primary analyzed population	18,099 endocrine cells	217,283 selected cells
Primary ordering coordinate	Deposited endocrine pseudotime	Deposited diffusion pseudotime
Biological replicates	2 differentiations	19 individuals
Main trajectory structure	Two branches: SC-EC and SC-β	Shared backbone with CM and CF terminal extensions
Primary transition landmark	NEUROG3-early median pseudotime	Destination-state median diffusion pseudotime
Prespecified transitions	Progenitor → NEUROG3-early	T2: MES→CMES; T3-CM: PROG→CM; T3-CF: PROG→CF
Primary state space	50 PCs	50 PCs
Primary sliding window	400 cells / 100-cell step	400 cells / 100-cell step
Window overlap	75%	75%
Final GDIS profiles	4 branch × differentiation profiles	19 T2, 15 T3-CM, 14 T3-CF
Parameter retuning	—	None
Independent inferential unit	Branch × differentiation trajectory	Individual within transition scope

4.4. Expression preprocessing and construction of the transcriptional state space

Single-cell expression preprocessing was performed using Scanpy [19]. For GSE114412, the 18,099 validated endocrine cells were extracted from the deposited processed count matrix. Deposited values were nonnegative and effectively integer-like. Genes with no expression in the endocrine subset were removed, reducing the matrix from 16,224 to 16,178 expressed genes. Counts were normalized to a total of 10,000 counts per cell and log-transformed. Two thousand highly variable genes were selected using differentiation replicate as the batch key during highly

variable gene selection. No batch-correction transformation was applied to the expression matrix before PCA or primary GDIS analysis; differentiation replicate was used only during highly variable gene selection. Principal-component analysis was then performed on the highly variable genes, producing a common 50-dimensional PCA representation; coordinate subsets containing the first 5, 10, 20, and 30 PCs were also retained for prespecified dimensionality sensitivity analyses.

Before the first GDIS analysis, PCA dimensionality was evaluated using distance preservation, 30-nearest-neighbor overlap, biological-centroid geometry, replicate trajectory geometry, cross-replicate pseudotime generalization, and terminal-fate classification. The selection rule was to use the smallest dimensionality satisfying all prespecified representation-stability criteria. Fifty PCs were the only tested representation satisfying all six criteria and were therefore frozen as the primary discovery state space; lower-dimensional representations were retained as sensitivity analyses. An additional geometry audit confirmed that the 50-PC representation retained informative and reproducible local geometry.

For GSE175634, the expression state space was constructed from the union of the prespecified shared-backbone and terminal-extension populations, comprising 217,283 cells. Raw uncorrected UMI counts were used. Genes with zero expression across the selected cells were excluded, leaving 38,935 genes. Counts were normalized to 10,000 per cell and log-transformed. Two thousand highly variable genes were selected using the Seurat HVG formulation with individual as the batch key. HVG expression was zero-centered and scaled with a maximum value of 10, followed by PCA using the ARPACK solver and random seed 20260913. No batch-correction transformation was applied. The first 50 PCs formed the transferred primary external-validation state space, with 5-, 10-, 20-, and 30-PC subsets retained for sensitivity analysis.

The 50-PC dimensionality was transferred from the discovery analysis rather than reselected according to external GDIS behavior. Before external GDIS calculation, its geometry was independently checked using pairwise-distance concentration, local-neighborhood geometry, 30-to-50-PC distance and nearest-neighbor preservation, individual $\times$ state centroid structure, leave-one-individual-out state generalization, biological-state separation relative to inter-individual dispersion, and reproducibility of state-transition vectors. All prespecified geometry checks passed, and no dimensionality was modified on the basis of GDIS results.

4.5. Pseudotemporal trajectory and sliding-window construction

Within each discovery branch $\times$ differentiation group, cells were ordered by deposited endocrine pseudotime. The primary trajectory representation used sliding windows containing 400 cells with a step of 100 cells, corresponding to 75% overlap between adjacent windows. Each window therefore represented a locally ordered segment of the transcriptional trajectory in 50-dimensional PCA space. The parameter assigned to each window was its median deposited pseudotime. The four discovery trajectories produced 167 complete primary windows, covering 17,900 unique cells. Prespecified window-size sensitivity analyses used 300-cell windows with a 75-cell step and 500-cell windows with a 125-cell step, while maintaining 75% overlap. This procedure is illustrated in **Figure 9**, which shows how pseudotemporally ordered cells are segmented into overlapping local windows and how these windows are converted into a GDIS profile along pseudotime.

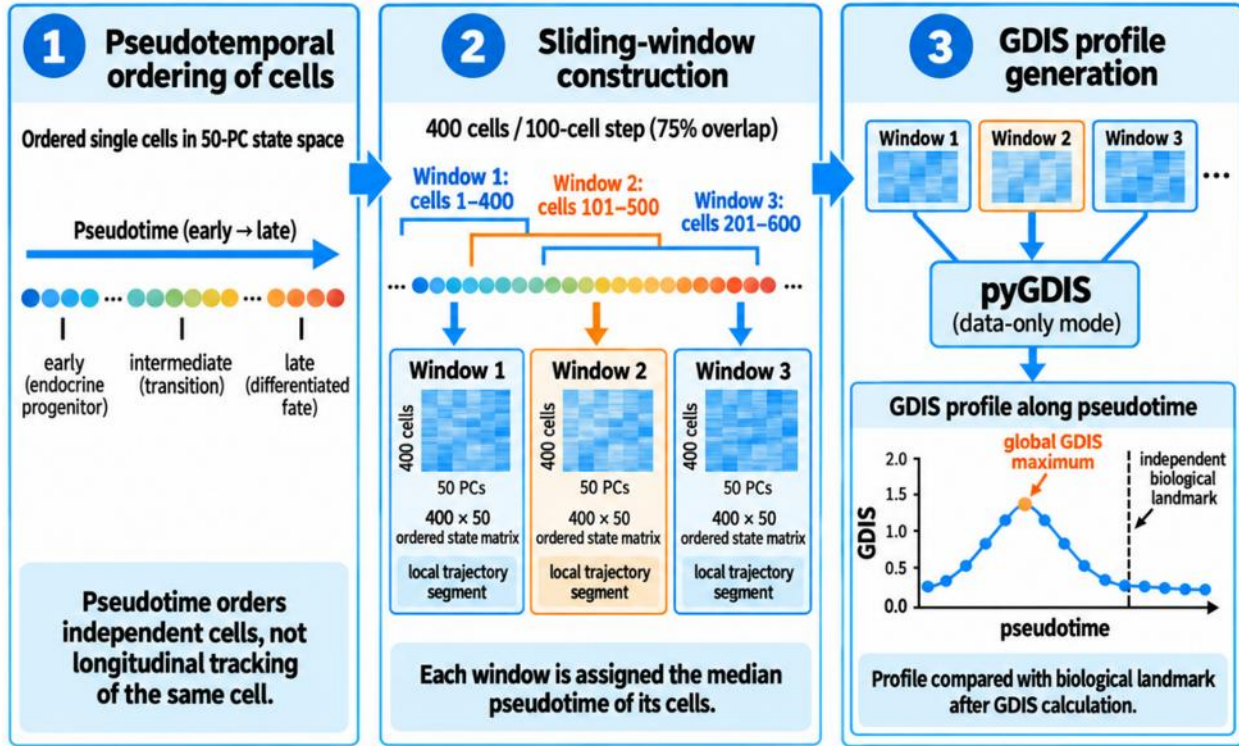


Figure 9. Sliding-window construction and generation of the GDIS profile along pseudotime. Pseudotemporally ordered cells were segmented into overlapping windows (400 cells, 100-cell step in the primary analysis), each represented as a local cell × PC matrix and assigned its median pseudotime. These ordered windows were analyzed by pyGDIS [14] to produce a GDIS instability profile along pseudotime.

The same primary window design was transferred unchanged to GSE175634. Cells were ordered by deposited diffusion pseudotime separately for each individual and analysis scope. Primary windows again contained 400 cells with a 100-cell step, and the same 300/75 and 500/125 configurations were retained for sensitivity analysis. Incomplete terminal windows were not padded. Each window was represented in the primary analysis as an ordered cell × PC matrix, and its median deposited diffusion pseudotime served as the window parameter. The ordering within these matrices represents progression across independent cells and not repeated observations from the same cell. As shown in **Figure 9**, the resulting ordered window series provides the direct input for pyGDIS [14], which returns a sequence of local instability values that together define the GDIS profile along pseudotime.

Before external GDIS analysis, the analyzable pseudotime range of every primary trajectory was compared with its previously frozen transition landmark. One individual (19108) did not have complete-window coverage extending to the frozen CM or CF terminal-state landmark and was therefore excluded from those two terminal-transition analyses without modifying window construction or moving the biological landmarks. The individual remained eligible for the shared-backbone analysis. This yielded a final external-validation cohort of 19 shared-backbone, 15 CM-

extension, and 14 CF-extension profiles, for a total of 48 primary GDIS profiles. This transition-evaluability decision was completed before external GDIS calculation.

4.6. GDIS-Bio analysis framework

The GDIS-Bio framework was developed to translate high-dimensional single-cell transcriptomic data into an ordered dynamical representation suitable for instability analysis while maintaining a strict separation between trajectory construction, GDIS calculation, and biological validation. The framework does not assume that single cells constitute longitudinal measurements of the same biological unit. Instead, cells are treated as independent observations sampled along a biological continuum, with deposited or independently defined pseudotime providing an ordering coordinate through transcriptional state space. Conceptually, GDIS-Bio consists of five sequential stages:

1. Construction of a transcriptional state space. After quality control and expression normalization, highly variable genes are identified and the high-dimensional transcriptomic data are projected into a common PCA representation. The resulting state vector for cell i is

$$\mathbf{x}_i = (x_{i1}, x_{i2}, \dots, x_{id}) \quad (1)$$

where $d = 50$ in the primary analyses. Lower-dimensional PCA representations are retained only for prespecified sensitivity analyses.

2. Biologically informed but GDIS-independent trajectory definition. Cells belonging to a prespecified developmental population are ordered according to deposited pseudotime. Biological states, experimental time points, branch definitions, replicate identities, and transition landmarks are determined before calculating GDIS. GDIS is therefore not used to construct the trajectory or identify the biological transition against which it is subsequently evaluated.
3. Transformation of the ordered trajectory into local state-space windows. For an ordered cell sequence

$$\mathbf{x}_1, \mathbf{x}_2, \dots, \mathbf{x}_N, \quad (2)$$

overlapping windows are extracted to represent local segments of transcriptional-state progression. For window w ,

$$\mathbf{X}^{(w)} = [\mathbf{x}_{s_w}, \mathbf{x}_{s_w+1}, \dots, \mathbf{x}_{s_w+L-1}]^T \quad (3)$$

where L denotes the number of cells in the window and s_w is its starting position. In the primary analyses, $L = 400$ and successive windows were separated by 100 cells, producing 75% overlap. Each window was assigned the median pseudotime of its constituent cells,

$$p_w = \text{median}(p_{s_w}, \dots, p_{s_w+L-1}). \quad (4)$$

The ordered family

$$\{\mathbf{X}^{(1)}, \mathbf{X}^{(2)}, \dots, \mathbf{X}^{(M)}\} \quad (5)$$

therefore represents successive local regions of the transcriptional trajectory.

4. Calculation of a GDIS instability profile. The ordered window family and its corresponding pseudotime parameters are supplied to pyGDIS in data-only mode. For every trajectory window, GDIS integrates complementary descriptors of local dynamical sensitivity, stretching,

state-space expansion, nonlinear complexity, and temporal persistence to produce a bounded instability score,

$$0 \leq \text{GDIS}(p_w) < 1. \quad (6)$$

The resulting sequence

$$\mathcal{G} = \{\text{GDIS}, (p_1), \text{GDIS}, (p_2) \dots \text{GDIS}(p_M)\} \quad (7)$$

defines the **GDIS profile** along the pseudotemporal trajectory. No biological transition landmark is supplied as a critical value during this calculation; transition localization therefore remains data-driven.

5. Independent biological validation of the GDIS profile. Only after the GDIS profile has been calculated is it compared with the previously frozen biological landmarks. Primary validation focuses on the distance between the GDIS maximum and the independently defined transition-region landmark, together with replicate consistency, source-to-destination score changes, sensitivity analyses, conventional benchmark statistics, and structure-preserving null models. Consequently, biological knowledge is used to evaluate GDIS rather than to optimize it.

The overall GDIS-Bio analytical workflow is summarized in **Figure 10**.

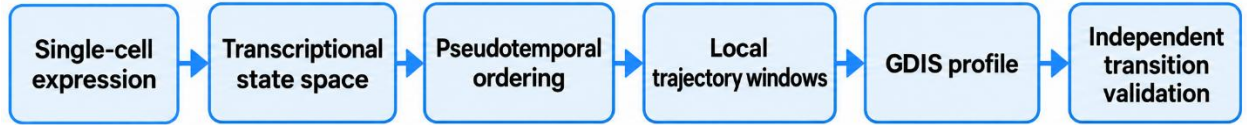


Figure 10. Overall GDIS-Bio analytical workflow. Single-cell expression profiles are transformed into a transcriptional state space, ordered along pseudotime, segmented into local trajectory windows, analyzed to generate a GDIS profile, and subsequently evaluated against independently defined biological transition landmarks.

This separation is central to GDIS-Bio. In particular, the biological landmark does not participate in score construction, and no window configuration, PCA dimensionality, trajectory cohort, or GDIS parameter is selected according to its eventual agreement with the biological transition. The same principle was retained during independent external validation, where the cohort, biological transitions, 50-PC primary state space, 400-cell windows, 100-cell step, and GDIS configuration were frozen before external GDIS values were examined.

Because adjacent trajectory windows overlap, neighboring GDIS values are not statistically independent. Likewise, because the pseudotemporal sequence is assembled from different cells, the resulting GDIS profile should be interpreted as describing changes in local transcriptional-state geometry along an ordered cellular continuum, rather than as a conventional longitudinal time series of a single biological system. Accordingly, GDIS-Bio is used here to identify and characterize transition-associated regions of dynamical instability, not to infer exact physical-time transition onset or to claim prospective prediction of a future state.

4.7. Generalized Dynamical Instability Score

GDIS was calculated using pyGDIS version 1.0.0, the reference Python implementation of the Generalized Dynamical Instability Score [14]. pyGDIS combines complementary dynamical descriptors into a bounded measure of instability and transition-associated behavior. The reference

implementation computes five descriptors: local dynamical sensitivity J , trajectory stretching S , attractor expansion A , nonlinear complexity H , and temporal persistence T .

The three principal descriptors J , S , and A characterize complementary aspects of local trajectory geometry and instability. Specifically, J represents local dynamical sensitivity, quantifying the tendency of nearby states to diverge along the ordered trajectory; S represents trajectory stretching, describing the extent to which the local state-space trajectory becomes elongated or dispersed; and A represents local state-space expansion, termed *attractor expansion* in the reference GDIS formulation, which quantifies expansion of the region occupied by the trajectory. In the present single-cell application, A is interpreted strictly as a geometric state-space expansion descriptor and does not by itself constitute evidence for the existence of a biological attractor. The additional descriptors H and T characterize nonlinear complexity and persistence of instability across neighboring trajectory regions, respectively.

Because an analytical dynamical-system Jacobian is unavailable for empirical single-cell trajectories, GDIS was used in its data-only mode, in which pyGDIS estimates local divergence directly from the ordered trajectory representation. Each principal descriptor is transformed and scaled according to the reference pyGDIS formulation, yielding the normalized quantities $\tilde{J}$, $\tilde{S}$, and $\tilde{A}$. These normalized descriptors are combined through the weighted geometric core

$$C = (\tilde{J}^{\alpha_J} \tilde{S}^{\alpha_S} \tilde{A}^{\alpha_A})^{1/(\alpha_J + \alpha_S + \alpha_A)} \quad (8)$$

where $\alpha_J = 0.42$, $\alpha_S = 0.33$, $\alpha_A = 0.25$. Thus, J : local dynamical sensitivity, S : trajectory stretching, and A : local state-space expansion.

The geometric core is subsequently modulated by the nonlinear-complexity and temporal-persistence terms and passed through the reference Hill-type saturation procedure to obtain the sustained-instability component. Sustained instability is then combined with the data-driven transition-localization term according to

$$\Phi = -\ln(1 - I_{\text{sustained}}) + \lambda_t I_{\text{transition,base}}, \quad (9)$$

where the reference transition weight is $\lambda_t = 0.18$.

The final GDIS value is

$$\text{GDIS} = 1 - \exp(-\Phi) \quad (10)$$

and is bounded by

$$0 \leq \text{GDIS} < 1. \quad (11)$$

These equations and descriptor definitions follow the reference pyGDIS formulation [14].

The complete default configuration was retained throughout the study: $\alpha_J = 0.42$, $\alpha_S = 0.33$, and $\alpha_A = 0.25$; $k_J = 3.0$, $k_S = 2.6$, and $k_A = 2.4$; Hill parameters $\gamma = 0.72$ and $c = 0.22$; complexity and temporal gains of 0.055; temporal threshold 0.60; transition weight 0.18; transition-width fraction 0.09; smoothing window 9; smoothing polynomial order 3; and maximum sustained-instability value 0.985. No biological *critical_value* was supplied. With *critical_value=None*, transition localization was resolved internally from the data-driven transition-energy profile rather than from an externally supplied biological annotation. The same configuration was used without retuning in both the discovery and external-validation datasets.

No universal numerical GDIS threshold was used to classify biological states as stable, unstable, or chaotic. Biological inference instead focused on the position, magnitude, and reproducibility of the GDIS profile relative to independently defined cell-state transition landmarks. Accordingly, elevated GDIS values were interpreted as evidence of increased local dynamical instability within the pseudotemporally ordered transcriptional state space rather than as proof of deterministic chaos or of a specific biological attractor.

4.8. Transition-localization endpoints

For each trajectory, the global GDIS maximum was identified and its pseudotime coordinate compared with the independently frozen biological landmark. The primary absolute peak-to-landmark distance was

$$E_i = |p_i^{\text{peak}} - p_i^{\text{landmark}}|, \quad (12)$$

where p_i^{peak} denotes the pseudotime parameter of the global score maximum for replicate i , and p_i^{landmark} denotes the corresponding frozen state landmark. Smaller values indicate closer transition-region localization.

Signed peak offset was additionally defined as

$$\Delta p_i = p_i^{\text{peak}} - p_i^{\text{landmark}}. \quad (13)$$

Thus, a negative value indicates that a score maximum occurs earlier in deposited pseudotime than the destination-state median, whereas a positive value indicates that it occurs later. A negative offset was not interpreted as prospective prediction of true biological transition onset because the state median is a landmark rather than an independently observed onset time.

For external validation, three complementary quantities were evaluated: absolute peak-to-landmark distance relative to the destination-state median, occurrence of the global peak within the destination state's independently defined interquartile pseudotime range, and the change in each metric from the source-state landmark to the destination-state landmark. The individual remained the independent inferential unit.

4.9. Conventional transition and early-warning benchmarks

GDIS was benchmarked against four conventional statistics calculated on exactly the same PCA windows: total variance, mean lag-1 pseudotemporal autocorrelation, Gaussian differential entropy, and mean pseudotemporal step distance. GDIS and its transition-energy component were reused from the corresponding GDIS analyses rather than recomputed during benchmarking. Autocorrelation and step-distance measurements were interpreted as pseudotemporal rather than physical-time quantities.

For a window $X \in \mathbb{R}^{N \times d}$, total variance summarized dispersion across the d state-space coordinates,

$$V = \sum_{j=1}^d \text{Var}(X_{.j}). \quad (14)$$

Mean lag-1 pseudotemporal autocorrelation summarized correlation between adjacent ordered cells across informative PC dimensions. Gaussian differential entropy summarized multivariate dispersion through the sample covariance matrix Σ ,

$$H_G = \frac{1}{2} [d(1 + \ln 2\pi) + \ln \det(\Sigma)] \quad (15)$$

Mean pseudotemporal step distance quantified successive movement through PCA space,

$$D = \frac{1}{N-1} \sum_{t=1}^{N-1} \|X_{t+1} - X_t\|_2. \quad (16)$$

The benchmark analysis used the frozen windows and was not used to modify GDIS parameters, landmarks, cohorts, dimensionality, or window size.

4.10. Structure-preserving null model and statistical inference

Statistical evidence for biological alignment was evaluated using structure-preserving circular-shift null models. For each metric profile, the ordered sequence of metric values was circularly shifted relative to the fixed pseudotime coordinates and biological landmarks. This procedure preserves the metric values, neighboring profile structure, and trajectory length while disrupting its specific alignment to the biological transition. Twenty thousand circular-shift permutations were generated using random seed 20260913. Overlapping windows were not treated as independent observations.

In the discovery analysis, inference was based on the four frozen branch $\times$ differentiation profiles. Null distributions were constructed for transition-landmark localization, peak placement, source-to-transition change, and replicate agreement. Pairwise comparisons of GDIS localization with conventional metrics used exact sign-flip testing over the four independent profiles; with four units, only $2^4 = 16$ possible sign patterns were available, and the resulting inferential resolution was therefore explicitly limited.

In the external-validation analysis, individual was the independent inferential unit. Twenty thousand circular-shift permutations were applied separately for each analysis scope and metric. Empirical permutation p -values were calculated using the standard +1 correction,

$$p = \frac{1 + n_{\text{extreme}}}{1 + N_{\text{perm}}}. \quad (17)$$

where n_{extreme} is the number of permuted statistics at least as extreme as the observed statistic.

Source-to-destination changes across individuals were assessed using exact sign tests and one-sample Wilcoxon signed-rank tests. For direct comparison of GDIS with a conventional metric, the paired localization difference for each individual was defined as

$$\Delta E_i = E_{i,\text{baseline}} - E_{i,\text{GDIS}}, \quad (18)$$

so that $\Delta E_i > 0$ indicated better localization by GDIS. One-sided exact sign tests and one-sided Wilcoxon signed-rank tests were applied to these paired differences. The four conventional comparators across the three prespecified external transitions produced 12 paired comparisons. The GDIS transition-energy component was analyzed separately because it is an internal component of the GDIS framework rather than an independent conventional baseline.

Multiple testing was controlled using the Benjamini–Hochberg false-discovery-rate procedure [20]. Corrections were performed separately for prespecified inferential families, including localization, destination-IQR enrichment, source-to-destination change, signed peak offset, and paired metric-superiority tests. Statistical significance was assessed at an FDR-adjusted $q < 0.05$.

4.11. Sensitivity analyses

Sensitivity analyses were prespecified to evaluate dependence on trajectory-window size and state-space dimensionality. In the discovery dataset, alternative window configurations of 300 cells with a 75-cell step and 500 cells with a 125-cell step were compared with the primary 400/100 configuration. PCA representations containing 5, 10, 20, and 30 components were evaluated relative to the primary 50-PC representation. The primary settings were selected before inspection of the final GDIS transition-localization result.

For external validation, the primary 50-PC, 400-cell/100-step configuration was transferred without retuning. Window sensitivity used 300/75 and 500/125 configurations in the 50-PC state space, while dimensionality sensitivity evaluated lower-dimensional projections of the same common PCA basis. The same frozen transition-evaluable individuals were retained to enable paired comparison with the primary profiles. Sensitivity was assessed using profile correlations, mean absolute score differences, shifts in global-peak location, and changes in absolute peak-to-landmark distance. No sensitivity result was used to replace the frozen primary configuration.

4.12. Software and reproducibility

All analyses were performed in Python 3.11. Single-cell data handling, preprocessing, dimensionality reduction, and principal-component analysis were performed using Scanpy and AnnData [19, 21]. The discovery workflow used Scanpy 1.11.5 and AnnData 0.12.19, while the external-validation workflow additionally used NumPy [22], pandas [23], and SciPy [24] for numerical computation, data manipulation, and statistical analysis. GDIS calculations were performed using pyGDIS version 1.0.0 in data-only mode [14]. Before external validation, the installed pyGDIS version and complete reference configuration were programmatically verified, and all final external GDIS profiles were generated using the same unchanged implementation and parameterization.

The analysis was implemented as a sequence of reproducible computational stages encompassing public-data acquisition, dataset qualification, cohort definition, preprocessing, state-space construction and validation, trajectory assembly, sliding-window generation, transition-evaluability assessment, GDIS computation, sensitivity analysis, conventional-metric benchmarking, structure-preserving null testing, external validation, and paired statistical comparisons. The external-validation design was frozen before the first external GDIS computation, and no subsequent result was used to modify the biological landmarks, cohort definitions, primary PCA dimensionality, sliding-window configuration, or GDIS parameters.

The reproducibility package includes environment and dependency specifications and automated execution workflows for Linux and Windows systems. GPU acceleration is not required. Additional installation instructions, computational-resource recommendations, and manuscript-to-code mappings are provided with the publicly archived software repository described in the Code availability section.

4.13. Ethics statement

This study involved secondary computational analysis of publicly available human single-cell datasets. No new participants were recruited and no new biological samples were collected. Ethical approval and informed-consent procedures for the original data collection are described in the source studies [15, 16].

5. Data availability

The single-cell transcriptomic datasets analyzed in this study are publicly available through the NCBI Gene Expression Omnibus under accession numbers GSE114412 and GSE175634. The datasets can be retrieved directly from GEO, and the accompanying computational workflow includes scripts for acquiring the required source data. Large raw and intermediate files generated during analysis are not duplicated in the software repository because they can be retrieved from the original public datasets or regenerated using the documented workflow.

6. Code availability

The complete source code and computational workflow supporting this study are openly available in the **GDIS-Bio GitHub repository** at <https://github.com/hamiddi/GDIS-Bio>. The exact **GDIS-Bio v1.0.0** reproducibility release used for the analyses reported in this manuscript has been permanently archived in **Zenodo** at <https://doi.org/10.5281/zenodo.22770645> [18]. The archived release contains the sequential analysis scripts, frozen analysis parameters, environment and dependency specifications, Linux and Windows execution scripts, dataset and results manifests, selected reference outputs, manuscript figures and tables, and documentation linking reported results to the corresponding computational stages. The repository provides `environment.yml` and `requirements.txt` files for software-environment reconstruction and `run_all.sh` and `run_all.ps1` scripts for sequential execution of the workflow.

Acknowledgements: The authors gratefully acknowledge the Department of Computational Data Science and Engineering at North Carolina A&T State University and the Department of Electrical and Energy Engineering at the German Jordanian University (GJU) for their support throughout this research. The authors also acknowledge the Visualization, Computing, and Applied Research (ViCAR) Laboratory at North Carolina A&T State University for providing a collaborative research environment and computational resources that supported this work.

Author contributions: Conceptualization, H.I., A.H., B.W. and M.B.; methodology, H.I., A.H., B.W. and M.B.; software, H.I.; validation, H.I., A.H., B.W. and M.B.; formal analysis, H.I.; investigation, H.I., A.H. and M.B.; resources, H.I.; data curation, H.I.; writing—original draft, H.I.; writing—review and editing, H.I., A.H., B.W. and M.B.; visualization, H.I.; supervision, A.H. and M.B.; project administration, H.I., A.H. and M.B.

Competing interests: The authors declare no competing interests.

References

1. Trapnell, C., et al., *The dynamics and regulators of cell fate decisions are revealed by pseudotemporal ordering of single cells*. Nat Biotechnol, 2014. **32**(4): p. 381-386.
2. Haghverdi, L., et al., *Diffusion pseudotime robustly reconstructs lineage branching*. Nat Methods, 2016. **13**(10): p. 845-8.
3. Saelens, W., et al., *A comparison of single-cell trajectory inference methods*. Nat Biotechnol, 2019. **37**(5): p. 547-554.
4. Tritschler, S., et al., *Concepts and limitations for learning developmental trajectories from single cell genomics*. Development, 2019. **146**(12).

5. La Manno, G., et al., *RNA velocity of single cells*. Nature, 2018. **560**(7719): p. 494-498.
6. Bergen, V., et al., *Generalizing RNA velocity to transient cell states through dynamical modeling*. Nat Biotechnol, 2020. **38**(12): p. 1408-1414.
7. Schiebinger, G., et al., *Optimal-Transport Analysis of Single-Cell Gene Expression Identifies Developmental Trajectories in Reprogramming*. Cell, 2019. **176**(4): p. 928-943.e22.
8. Weinreb, C., et al., *Lineage tracing on transcriptional landscapes links state to fate during differentiation*. Science, 2020. **367**(6479).
9. Scheffer, M., et al., *Early-warning signals for critical transitions*. Nature, 2009. **461**(7260): p. 53-9.
10. Chen, L., et al., *Detecting early-warning signals for sudden deterioration of complex diseases by dynamical network biomarkers*. Sci Rep, 2012. **2**: p. 342.
11. Mojtahedi, M., et al., *Cell Fate Decision as High-Dimensional Critical State Transition*. PLoS Biol, 2016. **14**(12): p. e2000640.
12. Ismail, H., et al., *Early Detection of Transcriptomic State-Transition Dynamics in Cancer Through Nonlinear Dynamical Systems Analysis*. BioMedInformatics, 2026. **6**(5): p. 73.
13. Moris, N., C. Pina, and A.M. Arias, *Transition states and cell fate decisions in epigenetic landscapes*. Nat Rev Genet, 2016. **17**(11): p. 693-703.
14. Ismail, H.D., A. Harb, and M. Bikdash, pyGDIS: Generalized Dynamical Instability Score. 2026, Zenodo. <https://doi.org/10.5281/zenodo.17171207>
15. Veres, A., et al., *Charting cellular identity during human in vitro β -cell differentiation*. Nature, 2019. **569**(7756): p. 368-373.
16. Elorbany, R., et al., *Single-cell sequencing reveals lineage-specific dynamic genetic regulation of gene expression during human cardiomyocyte differentiation*. PLoS Genet, 2022. **18**(1): p. e1009666.
17. Richard, A., et al., *Single-Cell-Based Analysis Highlights a Surge in Cell-to-Cell Molecular Variability Preceding Irreversible Commitment in a Differentiation Process*. PLOS Biology, 2016. **14**(12): p. e1002585.
18. Ismail, H., et al., GDIS-Bio: A Generalized Dynamical Instability Framework for Localizing Transcriptional State Transitions in Single-Cell Trajectories. 2026, Zenodo. <https://doi.org/10.5281/zenodo.22770645>
19. Wolf, F.A., P. Angerer, and F.J. Theis, *SCANPY: large-scale single-cell gene expression data analysis*. Genome Biol, 2018. **19**(1): p. 15.
20. Benjamini, Y. and Y. Hochberg, *Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing*. Journal of the Royal Statistical Society: Series B (Methodological), 1995. **57**(1): p. 289-300.
21. Virshup, I., et al., *anndata: Access and store annotated data matrices*. Journal of Open Source Software, 2024. **9**: p. 4371.
22. Harris, C.R., et al., *Array programming with NumPy*. Nature, 2020. **585**(7825): p. 357-362.
23. McKinney, W. Data structures for statistical computing in Python. In Proceedings of the 9th Python in Science Conference 56–61 (2010). <https://doi.org/10.25080/Majora-92bf1922-00a>
24. Virtanen, P., et al., *SciPy 1.0: fundamental algorithms for scientific computing in Python*. Nat Methods, 2020. **17**(3): p. 261-272.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [FigureS1.png](#)
- [FigureS2.png](#)
- [FigureS3.png](#)
- [FigureS4.png](#)
- [FigureS5.png](#)