● Great question! Understanding what influences your results is crucial for interpreting

them correctly. Here are the key factors that affect your neighborhood enrichment

analysis:

Critical Input Parameters

1. Radius Parameter (Most Important!)

Location: spatial\_analysis\_wsi.py:461 - radius=50

What it does:

- Defines what counts as a "neighbor"

- Cells within this radius (in pixels) are considered spatial neighbors

How it affects results:

- Too small (e.g., 10-20 pixels): Only captures direct cell-cell contact, might miss

broader tissue organization patterns

- Too large (e.g., 200+ pixels): Dilutes local interactions with distant cells, creates

noise

- Just right: Captures biologically meaningful neighborhoods

How to validate:

- Check "Average neighbors per cell" in your console output

- Typical range: 5-15 neighbors per cell is reasonable

- <5 neighbors: radius too small

30 neighbors: radius too large

- Compare with cell diameter: radius should be 2-5× cell diameter

2. Subsampling (n\_cells\_analysis)

Location: spatial\_analysis\_wsi.py:459 - n\_cells\_analysis=50000

What it does:

- Randomly selects 50k cells from your 1M+ cell WSI for analysis

How it affects results:

- Pros: Makes computation feasible

- Cons: May miss rare spatial patterns or region-specific interactions

How to validate:

- Check validation output: "Cell type proportions" - mean difference should be <2%

- Check "Spatial coverage" - should be >90% in X and Y directions

- Key validation: Run the robustness test (lines 482-494) to see if different random

samples give consistent results

- If z-score std < 0.5: Results are robust

- If z-score std > 1.0: Need larger subsample

3. Number of Permutations (n\_perms)

Location: spatial\_analysis\_wsi.py:462 - n\_perms=1000

What it does:

- Statistical test: randomly shuffles cell labels 1000 times to generate null

distribution

- Determines if observed enrichment is statistically significant

How it affects results:

- Higher n\_perms (5000-10000): More stable p-values, longer computation

- Lower n\_perms (<500): Faster but less reliable statistics

How to validate:

- 1000 is standard and usually sufficient

- If you see borderline significant interactions (z-score ≈ 2), consider increasing to

5000

Biological/Technical Factors

4. Cell Type Classification Accuracy

Source: Your cell type annotations in the h5ad file

How it affects results:

- Misclassified cells create false positive OR false negative interactions

- Example: If some tumor cells are mislabeled as fibroblasts, you'll see false

tumor-fibroblast enrichment

How to validate:

- Visually inspect spatial\_distribution.png - do cell type spatial patterns make sense?

- Check if known biological interactions appear (e.g., T cells near tumor cells)

- If interactions seem random/noisy, classification might be poor

5. Tissue Architecture/Heterogeneity

Source: Intrinsic to your tissue

How it affects results:

- WSI often contains multiple tissue regions with different microenvironments

- Subsampling might over/under-represent certain regions

How to validate:

- Visual inspection: Does your WSI have distinct regions (tumor core, invasive margin,

stroma)?

- If yes, consider regional analysis instead of whole-slide

- Check if rare regions (e.g., tertiary lymphoid structures) are captured in subsample

6. Spatial Coordinate Quality

Source: Your segmentation/cell detection pipeline

How it affects results:

- Inaccurate coordinates → wrong neighbor assignments

- Missing cells → biased neighborhoods

How to validate:

- Overlay cell positions on original image - do they match?

- Check if cell density varies unnaturally (might indicate detection artifacts)

How to Interpret Your Results

Z-scores in neighborhood\_enrichment.png:

- Positive (red): Cell types prefer each other (attraction/co-localization)

- Negative (blue): Cell types avoid each other (segregation)

- Near zero (white): Random spatial distribution

Significance thresholds:

- |z| > 2.0: Statistically significant (default threshold)

- |z| > 3.0: Highly significant

- |z| < 2.0: Not significant - could be random

Validation Checklist

To verify your results are accurate, check:

1. ✅ Validation metrics (automatically generated):

- Cell type proportion difference < 2%

- Spatial coverage > 90%

2. ✅ Biological plausibility:

- Do enriched interactions make biological sense?

- Example expected patterns:

- Immune cells cluster together (positive z-scores)

- Tumor cells often segregate from certain immune types

- Fibroblasts often associate with tumor cells

3. ✅ Technical checks:

- Average neighbors per cell: 5-15 range

- Clear patterns in heatmap (not all noise)

- Diagonal should be positive (cells near same type)

4. ✅ Robustness (optional but recommended):

- Uncomment lines 482-494 and run robustness test

- Check if different random subsamples give similar results

5. ✅ Consistency with literature:

- Compare your findings with published spatial studies

- Do key interactions align with known biology?

Red Flags (Results May Be Inaccurate)

⚠️ Warning signs:

- Mean cell type difference > 5% in validation

- Spatial coverage < 80%

- No clear patterns in enrichment heatmap (all values near zero)

- Contradicts well-established biology without explanation

- Very few significant interactions with 1M+ cells

- Average neighbors <3 or >50

What to Try If Results Seem Off

1. Adjust radius - Most common issue. Try radius = 30, 50, 100 and compare

2. Increase n\_cells\_analysis to 100k if you have memory

3. Check cell type annotations - Visualize uncertain cell types

4. Run robustness test - See if patterns are stable

5. Stratify by region - Analyze tumor vs stroma separately if tissue is heterogeneous

The validation you've already built in (cell type proportions, spatial coverage) is a

great start! The robustness test is your best tool to verify the analysis subsample

accurately represents the full WSI.