Title
Single-cell approaches for molecular classification of endocrine tumors.

Journal
Current opinion in oncology, 28(1)

ISSN
1040-8746

Authors
Koh, James
Allbritton, Nancy L
Sosa, Julie A

Publication Date
2016

DOI
10.1097/cco.0000000000000246

Peer reviewed

HHS Public Access

Author manuscript

Curr Opin Oncol. Author manuscript; available in PMC 2016 January 01.

Published in final edited form as:

Curr Opin Oncol. 2016 January ; 28(1): 43–49. doi:10.1097/CCO.0000000000000246.

Single-cell approaches for molecular classification of endocrine tumors

James Koh[a], **Nancy L. Allbritton**[b], and **Julie A. Sosa**[c]

[a]Department of Surgery, Duke University Medical Center, Durham, N.C.

[b]Department of Biomedical Engineering, University of North Carolina and N.C. State University, Raleigh, North Carolina, USA

[c]Departments of Surgery and Medicine, Duke Cancer Institute and Duke Clinical Research Institute, Durham, North Carolina, USA

Abstract

Purpose of review—In this review, we summarize recent developments in single-cell technologies that can be employed for the functional and molecular classification of endocrine cells in normal and neoplastic tissue.

Recent findings—The emergence of new platforms for the isolation, analysis, and dynamic assessment of individual cell identity and reactive behavior enables experimental deconstruction of intratumoral heterogeneity and other contexts, where variability in cell signaling and biochemical responsiveness inform biological function and clinical presentation. These tools are particularly appropriate for examining and classifying endocrine neoplasias, as the clinical sequelae of these tumors are often driven by disrupted hormonal responsiveness secondary to compromised cell signaling. Single-cell methods allow for multidimensional experimental designs incorporating both spatial and temporal parameters with the capacity to probe dynamic cell signaling behaviors and kinetic response patterns dependent upon sequential agonist challenge.

Summary—Intratumoral heterogeneity in the provenance, composition, and biological activity of different forms of endocrine neoplasia presents a significant challenge for prognostic assessment. Single-cell technologies provide an array of powerful new approaches uniquely well suited for dissecting complex endocrine tumors. Studies examining the relationship between clinical behavior and tumor compositional variations in cellular activity are now possible, providing new opportunities to deconstruct the underlying mechanisms of endocrine neoplasia.

Keywords

endocrine neoplasia; molecular classification; single-cell methods

Correspondence to James Koh, PhD, Department of Surgery, Duke University Medical Center, 3873, Durham, NC 27705, USA. Tel: +1 919 684 0892; james.koh@dm.duke.edu.

Conflicts of interest

N.L.A. discloses a financial interest in Cell Microsystems, a licensee of microraft technology. The other authors have no conflicts of interest.

INTRODUCTION

Understanding the origin and consequences of intratumoral heterogeneity is an issue of critical translational importance in the management of nearly all human cancers [1]; it potentially has acute impact on the management of endocrine neoplasia, where tumor-specific abnormal hormonal secretory behavior and cell signaling derangements are key functional drivers of physiological disruption. A growing body of evidence demonstrating intratumoral diversity in the clonal status, functional behavior, radiographic characteristics, and cellular composition of endocrine tumors such as parathyroid adenomas [2,3], thyroid lesions [4], neuroendocrine tumors [5], and pituitary adenomas [6] among others suggests that the biological properties of endocrine neoplasias must be considered products of a heterogeneous rather than monolithic tumor burden. Gaining an effective mechanistic understanding of the functional and genomic complexity of tumors such as these will require approaches capable of dissecting their constituent cellular components and relating variations in tumor composition to functional behavior. It has become increasingly apparent, however, that conventional bulk tumor methods can obscure compositional complexity, compressing genomic and phenotypic clonal variation into aggregate population averages that do not convey the specimen's underlying cellular and functional heterogeneity [7▪▪]. Accurately recognizing and accounting for spatially distributed regional heterogeneity or for numerically underrepresented tumor subclonal populations constitutes a major challenge for personalized cancer therapeutics. Evidence from multiple human cancers not limited to known 'mutator' subtypes [8,9] indicates that even prior to therapy, tumors can contain complex mixtures of genetically distinct subclones, a reservoir of diversity likely to include variants that can emerge under selective pressure to drive posttreatment relapse and disease progression [10▪,11–14]. Genomic identification of such variants from bulk tumor tissue is difficult even using advanced computational deconvolution approaches, as on the order of 1000× sequencing depth is required for high confidence detection of mutations arising from a subclone present at 1% of the overall tumor population [15].

The challenges of interpreting endocrine bulk tumor genomic data are illustrated clearly in a recent whole-exome study of 22 anaplastic thyroid carcinomas (ATCs), where the authors found a highly heterogeneous and complex spectrum of mutational patterns in the sequenced cohort, including a subset of tumors exhibiting MutL homolog lesions with consequent genomic instability [16▪]. Although a number of recurrent mutations clustering in the MAPK and Ras/ErbB pathways were documented, known oncogenic driver mutations could not be detected in more than 20% (5/22) of the ATCs examined. These findings indicate that ATCs harbor significant intra and intertumoral genomic diversity, highlighting the need to identify and attribute specific functional behaviors to the individual subpopulations resident in the intact tumors.

To address these issues, a number of powerful new technologies have been developed that provide a means for deconstruction of complex tumors at single-cell resolution (Fig. 1). These tools allow for examination of key etiological events and mutational drivers [17–20], identification of numerically rare but clinically significant tumor subclones that can drive therapeutic resistance, relapse, and disease progression [21,22], and quantitative tracking of clonal evolution and genetic drift [23–26]. The purpose of this review is to provide an

overview of these emerging methods and offer insights into how single-cell approaches can be used to improve the classification of endocrine tumors.

Single-cell enrichment and capture for determining genomic identity and transcriptional signatures in circulating endocrine tumor cells

Determining the provenance and expression patterns of circulating epithelial cells detected in the peripheral blood of patients with endocrine tumors could be an important tool for evaluating and managing metastatic risk. However, because these circulating cells typically are present at very low abundance, analysis through conventional molecular characterization methods can be challenging. Sorg and colleagues [27▪] addressed this issue by utilizing fluorescent antibody tagging to identify and capture cells expressing the epithelial cell marker EpCam in plasma from patients with differentiated thyroid cancer. Individual labelled cells were identified via fluorescence microscopy, captured in capillary micropipets, and subsequently probed via quantitative real time PCR for the expression of a panel of thyroid-specific transcripts. Despite the limitations of the small sample size in this exploratory study, positive expression of the TSH-receptor in 44/48 cells from three different patients is consistent with the thyroid origin of these cells and indicates that an approach based on single-cell enrichment and capture of circulating tumor-derived cells could potentially be adapted for assessment of metastatic risk in the management of thyroid cancer.

To enrich rare circulating tumor cells (CTCs) without prior knowledge of identifying markers, Ting and colleagues [28▪▪] utilized a combination of size-based hydrodynamic sorting and a microfluidics chamber negative inertial focusing device to selectively deplete the lymphocytic component from the whole blood of mice bearing human tumor patient-derived xenografts or genetically-induced endogenous murine tumors. Using this unbiased enrichment system, the authors were able to isolate and capture individual CTCs for subsequent single-cell RNA-seq analysis. The CTCs exhibited a transcriptional profile related to but distinct from the parental tumors, with enrichment for stromal-derived extracellular matrix proteins such as SPARC, a factor subsequently shown to be essential for tumor cell migration and invasiveness. Moreover, three distinct CTC expression clusters emerged from the overall analysis, suggesting the existence of multiple alternate pathways for metastatic cell evolution.

Such microfluidic platforms for CTC isolation possess a number of advantages, including the capacity for selective CTC capture based on different cellular attributes and a closed architecture preventing sample contamination; however, low throughput or poor cellular recovery limited the practical utility of early versions of these systems. Highly efficient cell capture devices capable of rapidly screening large blood volumes offer the ability to retrieve very low numbers of CTCs shed from small, localized tumors. Using a thermoplastic device compatible with high throughput manufacturing, Kamande and colleagues [29] collected an average of 11 CTCs/ml of blood from patients with locally resectable pancreatic tumors. The use of a modular system incorporating CTC collection, counting, and staining diminished cell loss enhanced purity and minimized assay time. Utilization of these new integrated tools to capture and characterize cells shed from endocrine neoplasias offers the

real possibility of identifying patients with early disease as well as for the detection of micrometastases.

An essential feature of single-cell approaches is the ability to isolate and distinguish tumor-derived cells from colocalized normal tissue components. A recent meta-analysis of papillary thyroid cancer transcriptional profiling data has demonstrated the critical importance of cellular-level resolution in the molecular classification of suspected nodal metastases [30▪▪]. Comparative global transcriptome data from primary thyroid tumors and associated nodal metastases proved to be of limited value because of the inability to attribute differential gene expression patterns to metastatic tumor cells as opposed to normal lymphoid tissue components present in the bulk nodal tissue. A candidate set of 1074 probes found to be more highly expressed in metastatic nodes than in the parental thyroid tumor were found by gene set enrichment analysis to be associated primarily with immune system function. Moreover, the fold changes observed for these candidate probes in the nodal metastasis relative to the parental tumor proved to be highly correlated ($P < 2 \times 10^{-16}$) with a control comparative analysis of normal thyroid tissue versus normal lymph node. This result demonstrates that the 1074 probes presumptively upregulated in nodal metastases were most likely enriched as a consequence of the confounding effect of normal lymphoid tissue as opposed to being associated with the acquisition of thyroid tumor metastatic potential. Similarly, the presence of normal thyroid tissue in bulk thyroid tumor tissue subjected to global gene expression analysis was found to give rise to an artifactual apparent downregulation of thyroid differentiation genes. The difficulty of controlling for variations in tumor versus normal cell content in bulk tissue specimens highlights the value of single-cell approaches for improving the resolution and specificity of molecular classification efforts.

Single-cell methods for functional profiling of endocrine tumor cells

Physiological disruption secondary to metabolically uncoupled or otherwise aberrant secretory behavior is a defining characteristic of many forms of endocrine neoplasia [31–36]. Gaining a clearer understanding of which cells or cell types within a given tumor are driving hormonal perturbation and identifying the specific molecular mechanisms linking neoplastic transformation to compromised endocrine signaling activity are therefore essential for designing rationally based, targeted therapies. Single-cell methods for interrogating cellular composition, signaling behavior, agonist responsiveness, and subcellular trafficking dynamics provide a powerful suite of tools for ex-vivo provocative testing of live endocrine tumor cells. When combined with spatially localized visualization and capture methods, single-cell readouts of cellular content or dynamic cellular behaviors can provide precise, individualized, functional assessment of endocrine tumor composition, enabling direct attribution of biological behaviors to specific cells or cell types within the aggregate tumor population.

Evaluating the real-time kinetics of dynamic signal transduction events in endocrine cells responding to physiological stimuli requires single-cell spatial and temporal resolution. A variety of innovative devices are being developed that can address this experimental need. For example, new process lines recently have been described for stimulating individual cells

with bioactive surfaces that can mimic cell–cell contact, particulate stimulation, or physiological ligand engagement. One such design employed a dielectrophoresis-based microfluidic system to enable the controlled initiation of a cellular stimulus, incorporating fluorescence indicator visualization of induced intracellular calcium transients as readouts of signal transduction at the single-cell level [37]. The continuous flow design of the microfluidics chamber allowed for the controlled delivery of agonist-loaded microparticles to simulate cell–cell contact and could be adapted for real-time assessment of hormone secretion or the release of other bioactive compounds in response to provocative physiological stimulation. In an endocrine context, one could envision challenging tumor cells with a physiological agonist such as extracellular calcium in the case of parathyroid adenomas to examine how neoplasia disrupts signaling behavior and perturbs the relative responsiveness of individual cells within the parental tumor.

Optically transparent lab-on-chip platforms and microfluidic chamber methods are rapidly becoming a mainstay of single-cell analysis, and these platforms can be fabricated into a wide array of designs for single-cell confinement, continuous fluid flow exchange and sampling, transient capture, and microarray plating surfaces [38]. Microfluidic systems have been employed as single-cell anticancer drug screening platforms [22], dispersion units for improving tumor dissociation to single-cell suspensions [39], and as specific metabolism and bioenergetics reactor vessels for the capture and assessment of live, single-cell reactivity in microscopically visualized fluorescence, chemostat, or brightfield assays [40]. Microfluidics technology has also been utilized to scale genomic methods to single-cell resolution. Using RNA-seq in combination with a novel clustering algorithm, complete transcriptome profiles and clonal lineages have been successfully generated from single cells captured in microfluidic chambers [7▪▪,41▪▪,42,43▪▪]. These methods represent a significant advance in throughput over dilution-based methods of manual capture and amplification prior to single-cell sequencing [18]. Other recent applications of single-cell microfluidics systems include digital PCR [44], microRNA quantitation [45], single-cell whole genome haplotyping [46], and analysis of secretory and chemotactic behaviors [47]. The primary limitations to microfluidics systems in their current iterations are high unit cost, high complexity, and often poor reliability, although these considerations are rapidly improving as the designs and instrumentation mature. For example, Reid and colleagues [48] successfully measured hormone secretion at 6-s intervals from live single pancreatic islets over 24 h, demonstrating unprecedented device reliability. On the other hand, these devices can easily be scaled, promising high-throughput analyses of cells, enabling customized assays of a variety of cell types or biological materials, and offering a range of potential adaptations specifically engineered for cell-limited samples, such as fine needle aspirates or minimal volume biopsy specimens.

In contrast to purely molecular methods, single-cell imaging allows for dynamic live-cell assessment of cellular behavior in response to a variety of environmental challenges or manipulations. Image-based spatiotemporal tracking of secretory vesicle membrane fusion events via time-lapse total internal reflection fluorescence microscopy has recently been used to demonstrate regionally localized ‘hotspots’ of exocytosis in specific areas of pancreatic islet cells [49▪]. The authors derived an automated algorithm for identifying and quantitating plasma membrane fusion events, incorporating a sequential scheme of

background subtraction, event localization, particle tracking, particle trajectory, and fusion detection as filtering criteria. By capturing the precise origin and movement of the secretory vesicles, a previously unappreciated cluster of circumscribed exocytosis domains could be visualized, indicating the existence of a dedicated and localized cytoskeletal-dependent transport mechanism for secretory vesicle delivery. This degree of resolution, which would not have been possible with conventional bulk cell imaging, indicates a new level of spatially ordered functional behavior as an essential component of endocrine cell secretory activity.

Single-cell imaging with fluorescent intracellular calcium indicators such as Fluo-4-AM is especially useful for examining the dynamics of signal transduction in contexts such as cell–cell communication assays, where sufficiently precise spatial and temporal resolution of calcium transients cannot be achieved in bulk plate assays [37]. Calcium imaging with detection of signal amplitude, duration, and frequency along with cell adjacency and positional data can be captured in single-cell format to generate agonist response profiles as a means to identify the specific repertoire of expressed receptors in individual living cells [50]. The ability to perform ex-vivo provocative testing is particularly suited to the study of endocrine cell signaling. Adaptation of these image-based assays to single-cell format on a cell capture surface or in a microfluidics vessel chamber allows unique field address assignment for each functionally interrogated cell with locationally defined postassay in-situ staining or subsequent single-cell recovery for downstream genomic analysis. As the key feature of this type of analysis is observation of dynamic behavior, the resulting data incorporate both positional and temporal dimensions in addition to the readout for the assay output variable.

Efficient and objective quantitative analysis of the high content image data generated through single-cell imaging experiments requires systematic dimensional reduction and pattern recognition computational approaches to extract maximal informational content from the sequentially ordered image stacks. By employing computational approaches such as dynamic feature normalization to elicit fold change commonalities, in-silico synchronization to unmask temporal patterns that may otherwise be obscured because of technical artifact such as mixing time intervals and sister cell analysis for paired cell validation, spatiotemporal patterns can be brought into higher relief [51]. The development of robust and flexible computational analysis tools capable of evaluating multidimensional dynamic interactions, including cell–cell signaling events remains a largely unmet need, although analogous algorithms to quantitate cell dependencies have been described in transcriptome profiling systems [7▪▪].

Interrogation of the dynamic functional response of individual cells in register with post-assay biomarker profiling represents a powerful extension of live cell imaging in single-cell systems. The approach was utilized recently to examine pancreatic islet cell signaling responses to repetitive glucose challenge [52]. Two different kinetic and sensitivity threshold patterns of glucose response were detected in wild type pancreatic islet cells, and subsequent marker gene staining revealed that the respective response types could be distinguished on the basis of pancreatic alpha and beta cell markers. By combining biomarker, positional, and dynamic response data, several important and previously

unknown features of pancreatic islet cell biology emerged. The alpha and beta cells were found to diverge in their signaling responses only after the second and third consecutive glucose challenge, a provocative testing scenario that cannot be achieved in single point or fixed-cell systems. The distinction between alpha and beta cell reactivity would not be apparent in bulk cell assays, as cell type assignment would not be captured in register with functional activity. The ex-vivo interrogation system provided a means for direct comparison of the relative abundance and activity of different marker profile subsets in normal and diabetic pancreatic tissue. Finally, the regional localization of cells with different activity profiles in the pancreatic islets revealed a nonrandom distribution suggestive of a previously unappreciated level of tissue organization. The method of functionally defining individual cellular phenotypes in conjunction with marker profiling and spatial localization represents a highly versatile approach for evaluating both normal and diseased tissues, especially in endocrine settings, where specific agonist/response relationships are well defined.

CONCLUSION

The characterization of endocrine neoplasias using single-cell technologies is a rapidly developing field with numerous opportunities for direct translational applicability. The conceptual approach of ex-vivo functional classification will provide an important new tool for interrogating the dynamic activity of a given tumor specimen rather than relying solely on static histomorphological assessment. The integration of dynamic functional readouts into scalable single-cell assay devices could eventually be developed as a platform for real-time diagnostic assessment of biopsy material or suspected endocrine tumor tissue. Significant technical challenges remain, including the development of automated and adaptable high-dimensional data analysis algorithms for efficient and streamlined interpretation of dynamic signal transduction activity. Rigorous evaluation of the prognostic and predictive power of ex-vivo live-cell functional assessment of endocrine tissue will await future clinical data registry studies where discrete patterns of cellular activity can be tested for association with clinical presentation and outcome.

Acknowledgements

The authors acknowledge financial support from the NIH through grants 5R01DK088188–05 (to J.K.), 1R21CA192004–01 (to J.K. and J.A.S.), and R01CA177993 (to N.L.A.).

Financial support and sponsorship

None.

REFERENCES AND RECOMMENDED READING

Papers of particular interest, published within the annual period of review, have been highlighted as:

▪ of special interest

▪▪ of outstanding interest

1. McGranahan N, Swanton C. Biological and therapeutic impact of intratumor heterogeneity in cancer evolution. Cancer Cell. 2015; 27:15–26. [PubMed: 25584892]
2. Shi Y, Hogue J, Dixit D, et al. Functional and genetic studies of isolated cells from parathyroid tumors reveal the complex pathogenesis of parathyroid neoplasia. Proc Natl Acad Sci U S A. 2014; 111:3092–3097. [PubMed: 24510902]
3. Ritter CS, Haughey BH, Miller B, Brown AJ. Differential gene expression by oxyphil and chief cells of human parathyroid glands. J Clin Endocrinol Metab. 2012; 97:E1499–E1505. [PubMed: 22585091]
4. Kim SJ, Chang S. Predictive value of intratumoral heterogeneity of F-18 FDG uptake for characterization of thyroid nodules according to Bethesda categories of fine needle aspiration biopsy results. Endocrine. 2015
5. Lapa C, Werner RA, Herrmann K. Visualization of tumor heterogeneity in neuroendocrine tumors by positron emission tomography. Endocrine. 2015
6. Mertens F, Gremeaux L, Chen J, et al. Pituitary tumors contain a side population with tumor stem cell-associated characteristics. Endocr Relat Cancer. 2015; 22:481–504. [PubMed: 25921430]
7 ▪▪. Shalek AK, Satija R, Shuga J, et al. Single-cell RNA-seq reveals dynamic paracrine control of cellular variation. Nature. 2014; 510:363–369. [PubMed: 24919153] The seminal study demonstrates the capacity of single-cell transcriptomics to delineate the extent of gene expression variation between cohorts of phenotypically matched, identically stimulated dendritic cells. By comparing gene expression profiles in single cells stimulated collectively versus cells stimulated in isolated microfluidics chambers, the authors showed that the range of variability was constrained in the absence of cell–cell contact, revealing the importance of direct cellular communication in coordinating complex and heterogeneous transcriptional programs within a population of seemingly identical cells.
8. Swanton C, McGranahan N, Starrett GJ, Harris RS. APOBEC enzymes: mutagenic fuel for cancer evolution and heterogeneity. Cancer Discov. 2015; 5:704–712. [PubMed: 26091828]
9. Roberts SA, Gordenin DA. Hypermutation in human cancer genomes: footprints and mechanisms. Nat Rev Cancer. 2014; 14:786–800. [PubMed: 25568919]
10 ▪. Meyer M, Reimand J, Lan X, et al. Single cell-derived clonal analysis of human glioblastoma links functional and genomic heterogeneity. Proc Natl Acad Sci U S A. 2015; 112:851–856. [PubMed: 25561528] The study describes the generation and subsequent functional and genomic profiling of single-cell clonal isolates derived from FACS-sorted human glioblastoma tumor specimens. Significant phenotypic and genomic heterogeneity was discovered within each tumor, with temozolomide-resistant clones found even in naïve patient samples, demonstrating that resistance to this therapeutic agent is present in a subset of tumor cells prior to treatment. Ex-vivo functional profiling of tumor-derived subclones could potentially be used to inform treatment strategies tailored to the clonal composition of a given tumor.
11. Bedard PL, Hansen AR, Ratain MJ, Siu LL. Tumour heterogeneity in the clinic. Nature. 2013; 501:355–364. [PubMed: 24048068]
12. Johnson BE, Mazor T, Hong C, et al. Mutational analysis reveals the origin and therapy-driven evolution of recurrent glioma. Science. 2014; 343:189–193. [PubMed: 24336570]
13. Shah SP, Roth A, Goya R, et al. The clonal and mutational evolution spectrum of primary triple-negative breast cancers. Nature. 2012; 486:395–399. [PubMed: 22495314]
14. Navin N, Kendall J, Troge J, et al. Tumour evolution inferred by single-cell sequencing. Nature. 2011; 472:90–94. [PubMed: 21399628]
15. Ding L, Wendl MC, McMichael JF, Raphael BJ. Expanding the computational toolbox for mining cancer genomes. Nat Rev Genet. 2014; 15:556–570. [PubMed: 25001846]
16 ▪. Kunstman JW, Juhlin CC, Goh G, et al. Characterization of the mutational landscape of anaplastic thyroid cancer via whole-exome sequencing. Hum Mol Genet. 2015; 24:2318–2329. [PubMed: 25576899] The study presents the first whole-exome analysis of ATC, a rare but frequently intractable endocrine malignancy. Exome sequencing was performed in matched tumor and normal bulk tissue samples from a cohort of 22 ATC cases. Ontology analysis of the somatic mutation profile data revealed clustering into three broad groups, including tumors with BRAF V600E mutations or Ras-family lesions suggestive of a common origin with well

differentiated thyroid carcinomas. The high degree of heterogeneity in ATC genetic backgrounds implies an active process of intratumoral evolution in these endocrine malignancies.

17. Bendall SC, Nolan GP. From single cells to deep phenotypes in cancer. Nat Biotechnol. 2012; 30:639–647. [PubMed: 22781693]
18. Sun HJ, Chen J, Ni B, et al. Recent advances and current issues in single-cell sequencing of tumors. Cancer Lett. 2015; 365:1–10. [PubMed: 26003306]
19. Eirew P, Steif A, Khattra J, et al. Dynamics of genomic clones in breast cancer patient xenografts at single-cell resolution. Nature. 2015; 518:422–426. [PubMed: 25470049]
20. Janiszewska M, Polyak K. Clonal evolution in cancer: a tale of twisted twines. Cell stem cell. 2015; 16:11–12. [PubMed: 25575078]
21. Wang E, Zaman N, McGee S, et al. Predictive genomics: a cancer hallmark network framework for predicting tumor clinical phenotypes using genome sequencing data. Semin Cancer Biol. 2015; 30:4–12. [PubMed: 24747696]
22. Wang Y, Tang X, Feng X, et al. A microfluidic digital single-cell assay for the evaluation of anticancer drugs. Anal Bioanal Chem. 2015; 407:1139–1148. [PubMed: 25433683]
23. Katsios C, Ziogas DE, Liakakos T, et al. Translating cancer genomes sequencing revolution into surgical oncology practice. J Surg Res. 2012; 173:365–369. [PubMed: 21195428]
24. Wills QF, Mead AJ. Application of single-cell genomics in cancer: promise and challenges. Hum Mol Genet. 2015; 24:R74–R84. [PubMed: 26113645]
25. Paguirigan AL, Smith J, Meshinchi S, et al. Single-cell genotyping demonstrates complex clonal diversity in acute myeloid leukemia. Sci Transl Med. 2015; 7:281re2.
26. Barber LJ, Davies MN, Gerlinger M. Dissecting cancer evolution at the macro-heterogeneity and micro-heterogeneity scale. Curr Opin Genet Dev. 2015; 30:1–6. [PubMed: 25555261]
27 ▪. Sorg S, Pachmann K, Brede-Hekimian K, et al. Determining tissue origin of circulating epithelial cells (CEC) in patients with differentiated thyroid cancer by real-time PCR using thyroid mRNA probes. Cancer Lett. 2015; 356(2 Pt B):491–495. [PubMed: 25304372] Single-cell capture and real-time PCR analysis were utilized to determine whether circulating epithelial cells detected in the blood of patients with differentiated thyroid cancer arise from the primary tumor. Single cells expressing the epithelial cell surface marker EpCAM were identified via immunofluorescence and captured individually for quantitative real time PCR assessment of thyroid gene expression. The study demonstrates the feasibility of using single cell profiling to identify CTCs and establish their provenance as a marker of endocrine tumor cell dissemination.
28 ▪▪. Ting DT, Wittner BS, Ligorio M, et al. Single-cell RNA sequencing identifies extracellular matrix gene expression by pancreatic circulating tumor cells. Cell Rep. 2014; 8:1905–1918. [PubMed: 25242334] The landmark study is a prototype for single-cell transcriptome analysis as a tool to illuminate functional diversity in a heterogeneous tumor cell population. The authors utilized a novel microfluidics enrichment approach and single-cell RNAseq analysis to examine the genomic profiles of CTCs. Unsupervised hierarchical clustering and principal component analysis revealed three distinct CTC populations, with further diversity within each group suggesting microenvironment-dependent specificity and the existence of multiple alternative pathways in achieving metastatic potential.
29. Kamande JW, Hupert ML, Witek MA, et al. Modular microsystem for the isolation, enumeration, and phenotyping of circulating tumor cells in patients with pancreatic cancer. Anal Chem. 2013; 85:9092–9100. [PubMed: 23947293]
30 ▪▪. Tarabichi M, Saiselet M, Trésallet C, et al. Revisiting the transcriptional analysis of primary tumours and associated nodal metastases with enhanced biological and statistical controls: application to thyroid cancer. Br J Cancer. 2015; 112:1665–1674. [PubMed: 25965298] The comprehensive meta-analysis of papillary thyroid cancer transcriptional profiling datasets compellingly demonstrates the confounding effect of poorly controlled variations in input tumor or nodal metastasis tissue in comparative genomic profiling studies. The authors present convincing evidence that efforts to distinguish node-positive from node-negative thyroid tumors produce signatures reflective of patient-specific rather than tumor characteristic-specific differences. Moreover, the transcriptomes of thyroid tumor nodal metastases proved to be heavily contaminated by lymphoid tissue contributions, completely defeating the original experimental intent of identifying gene expression changes associated with the acquisition of metastatic

potential. The results highlight the inherent limitations of bulk tumor studies where emergent molecular features or characteristics cannot be definitively attributed to specific tumor cells.

31. Syro LV, Rotondo F, Ramirez A, et al. Progress in the diagnosis and classification of pituitary adenomas. Front Endocrinol (Lausanne). 2015; 6:97. [PubMed: 26124750]
32. Wojakowska A, Chekan M, Widlak P, Pietrowska M. Application of metabolomics in thyroid cancer research. Int J Endocrinol. 2015; 2015:258763. [PubMed: 25972898]
33. Raverot G, Vasiljevic A, Jouanneau E, Trouillas J. A prognostic clinicopathologic classification of pituitary endocrine tumors. Endocrinol Metab Clin North Am. 2015; 44:11–18. [PubMed: 25732637]
34. Ferrer-Garcia JC, Iranzo González-Cruz V, Navas-DeSolís S, et al. Management of malignant insulinoma. Clin Transl Oncol. 2013; 15:725–731. [PubMed: 23460559]
35. Duan K, Gomez Hernandez K, Mete O. Clinicopathological correlates of hyperparathyroidism. J Clin Pathol. 2015; 68:771–787. [PubMed: 26163537]
36. Cetani F, Pardi E, Borsari S, Marcocci C. Molecular pathogenesis of primary hyperparathyroidism. J Endocrinol Invest. 2011; 34(7 Suppl):35–39. [PubMed: 21985978]
37. Kirschbaum M, Jaeger MS, Duschl C. Measurement of surface-mediated Ca2+ transients on the single-cell level in a microfluidic lab-on-a-chip environment. Methods Mol Biol. 2015; 1272:247–256. [PubMed: 25563189]
38. Dittrich P, IbáñezF AJ. Analysis of metabolites in single cells-what is the best micro-platform? Electrophoresis. 2015
39. Qiu X, De Jesus J, Pennell M, et al. Microfluidic device for mechanical dissociation of cancer cell aggregates into single cells. Lab Chip. 2015; 15:339–350. [PubMed: 25377468]
40. Vasdekis AE, Stephanopoulos G. Review of methods to probe single cell metabolism and bioenergetics. Metab Eng. 2015; 27:115–135. [PubMed: 25448400]
41 ▪▪. Jaitin DA, Kenigsberg E, Keren-Shaul H, et al. Massively parallel single-cell RNA-seq for marker-free decomposition of tissues into cell types. Science. 2014; 343:776–779. [PubMed: 24531970] The study introduced the concept of massively parallel single-cell RNA sequencing as a means to define cellular heterogeneity within a mixed input sample population. The RNAseq profiles of 1000 individual cells were generated and analyzed by unsupervised clustering analysis, revealing distinct cellular groupings that emerged without any prior assumptions regarding marker gene expression.
42. Shalek AK, Satija R, Adiconis X, et al. Single-cell transcriptomics reveals bimodality in expression and splicing in immune cells. Nature. 2013; 498:236–240. [PubMed: 23685454]
43 ▪▪. Treutlein B, Brownfield DG, Wu AR, et al. Reconstructing lineage hierarchies of the distal lung epithelium using single-cell RNA-seq. Nature. 2014; 509:371–375. [PubMed: 24739965] A novel microfluidic single-cell isolation and RNA-sequencing approach was employed in this study to examine and compare the expression patterns in individual cells at four different stages of lung alveolar development. This approach could be applied in principle to any tissue for the identification and characterization of cellular subtypes, progenitors, and differentiation lineage relationships.
44. White AK, VanInsberghe M, Petriv OI, et al. High-throughput microfluidic single-cell RT-qPCR. Proc Natl Acad Sci U S A. 2011; 108:13999–14004. [PubMed: 21808033]
45. Jang JS, Simon VA, Feddersen RM, et al. Quantitative miRNA expression analysis using fluidigm microfluidics dynamic arrays. BMC genomics. 2011; 12:144. [PubMed: 21388556]
46. Fan HC, Wang J, Potanina A, Quake SR. Whole-genome molecular haplotyping of single cells. Nat Biotechnol. 2011; 29:51–57. [PubMed: 21170043]
47. Sackmann EK, Fulton AL, Beebe DJ. The present and future role of microfluidics in biomedical research. Nature. 2014; 507:181–189. [PubMed: 24622198]
48. Reid KR, Kennedy RT. Continuous operation of microfabricated electrophoresis devices for 24 hours and application to chemical monitoring of living cells. Anal Chem. 2009; 81:6837–6842. [PubMed: 19621896]
49 ▪. Yuan T, Lu J, Zhang J, et al. Spatiotemporal detection and analysis of exocytosis reveal fusion ‘hotspots’ organized by the cytoskeleton in endocrine cells. Biophys J. 2015; 108:251–260. [PubMed: 25606674] The study describes a novel image analysis platform for the automated

identification and analysis of plasma membrane fusion events associated with cellular secretion in pancreatic endocrine cells. Using total internal reflection fluorescence microscopy, spatiotemporal examination of insulin secretion in pancreatic cells reveals nonrandom clustering of exocytotic hotspots. The results indicate the presence of a previously unappreciated degree of subcellular regional specificity and cytoskeletal organization underlying endocrine secretory activity.

50. Glaser T, Castillo AR, Oliveira Á , Ulrich H. Intracellular calcium measurements for functional characterization of neuronal phenotypes. Methods Mol Biol. 2015

51. Davis DM, Purvis JE. Computational analysis of signaling patterns in single cells. Semin Cell Dev Biol. 2015; 37:35–43. [PubMed: 25263011]

52 ▪▪. Kenty JH, Melton DA. Testing pancreatic islet function at the single cell level by calcium influx with associated marker expression. PLoS One. 2015; 10:e0122044. [PubMed: 25853429] comprehensive study of pancreatic islet cell responsiveness to glucose challenge examined live-cell intracellular calcium flux in individual cells within intact islets as a readout of biochemical signaling, combining these measures with subsequent postassay biomarker profiling. The single-cell integration of biomarker, positional, and spatiotemporal dynamic response data in individual cells establishes a powerful new approach for ex-vivo functional analysis of endocrine cell behavior, identity, and lineage. The concept of aligning individual live-cell functional calcium flux response assessment with marker analysis or genomic profiling could have broad potential applicability to studying the underlying mechanisms of secretory dysregulation in multiple endocrine neoplastic diseases.

KEY POINTS

- Single-cell methods will be key tools for deconstructing the effect of intratumoral heterogeneity and differentially responsive cellular subpopulations on the behavior of normal and neoplastic endocrine tissue.
- The combination of image-based platforms and single-cell assay methods preserves positional, cell-adjacency, and spatial information that can be used to generate a more accurate understanding of signaling behavior, clonal outgrowth, and contextual cues in native tumors.
- Dynamic, ex-vivo functional assessment of endocrine cell responsiveness to physiological agonists or candidate therapeutic agents can reveal distinct kinetic patterns of signal transduction that can be tested for association with clinical presentation and disease course.
- The ability to align real-time live-cell interrogation of cellular responsiveness with postassay cell-type identification and characterization provides a means to attribute specific functional characteristics to individual cells and probe how variations in endocrine tumor content can influence biological behavior.
- The development of modular devices for analyzing real-time dynamic biochemical behavior at single-cell resolution with automated, address-specific cell selection and recovery for subsequent downstream molecular profiling has broad potential applicability for endocrine tumor cell functional characterization, especially in settings where cellular input numbers are limiting.

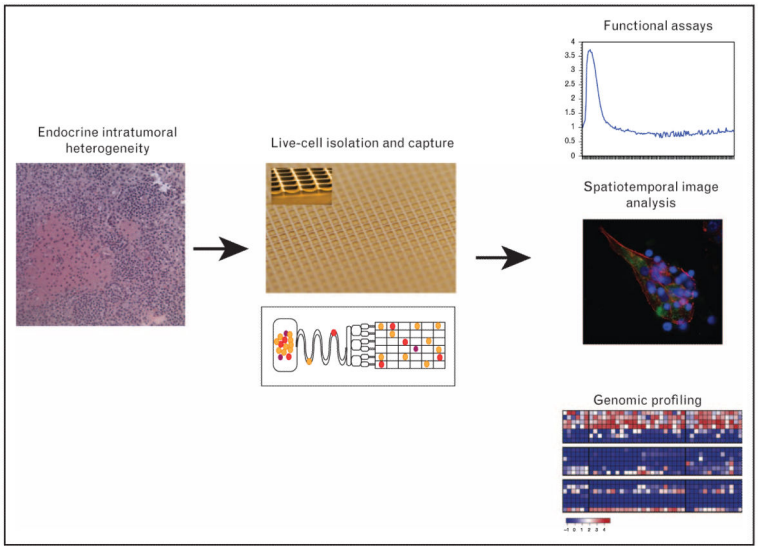

FIGURE 1.

Generalized workflow for single-cell analysis of tumor cells. Heterogeneous tumor material is recovered and distributed into a single-cell isolation array platform, imaging chamber, or microfluidics device (shown schematically). The live cells are then interrogated using in-situ functional assays or spatiotemporal image analysis, followed by downstream genomic profiling.