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Technical aspects and recommendations for single-cell qPCR

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ABSTRACT

Single cells are basic physiological and biological units that can function individually as well as in groups in tissues and organs. It is central to identify, characterize and profile single cells at molecular level to be able to distinguish different kinds, to understand their functions and determine how they interact with each other. During the last decade several technologies for single-cell profiling have been developed and used in various applications, revealing many novel findings. Quantitative PCR (qPCR) is one of the most developed methods for single-cell profiling that can be used to interrogate several analytes, including DNA, RNA and protein. Single-cell qPCR has the potential to become routine methodology but the technique is still challenging, as it involves several experimental steps and few molecules are handled. Here, we discuss technical aspects and provide recommendation for single-cell qPCR analysis. The workflow includes experimental design, sample preparation, single-cell collection, direct lysis, reverse transcription, preamplification, qPCR and data analysis. Detailed reporting and sharing of experimental details and data will promote further development and make validation studies possible. Efforts aiming to standardize single-cell qPCR open up means to move single-cell analysis from specialized research settings to standard research laboratories.

1. Introduction

1.1. The promises of single-cell analysis

Organisms, organs and tissues are complex mixtures of cells and extracellular molecules that together carry out all types of biological functions. Cells can perform functions individually as well as interacting with the surrounding microenvironment. They show highly variable molecular profiles that respond to internal and external stimuli making every cell unique. The cell's profiles are also dynamic, adapting to the ever-changing microenvironment. Thanks to recent technological advances to handle and analyze single cells we now have the means to characterize all cell types, their different states and start to decipher their functions. Omni-omics, which is the measurement of multiple analytes in the same cell is feasible. By characterizing all the cell types that make up a tissue or organ we will learn about cell-to-cell interactions and how the cells act in concert to perform the complex biology of organisms.

1.2. Pros and cons of qPCR

Quantitative PCR (qPCR) is one of the most versatile and commonly applied methods in molecular biology and is available to researchers in most biological and medical laboratories. Quantitative PCR is also used in diagnostics to quantify biomarkers and to detect pathogens. Many molecular biologists know how to design and perform qPCR experiments, handle and evaluate data and set up new applications, such as single-cell analysis. Numerous detection technologies and instrumentations are available that can be applied at single-cell level (Kubista et al., 2006). Single-cell qPCR has been used in a wide range of applications, including insulin producing beta cells (Bengtsson et al., 2005), the influence of single-nucleotide polymorphisms on gene-expression phenotypes (Wills et al., 2013), astrocyte activation (Rusnakova et al., 2013; Ståhlberg et al., 2011), neuron activity (Liss et al., 2001), breast cancer stem cells (Akrap et al., 2016), colon cancer stem cells (Dalerba et al., 2011), cancer associated fibroblast activation (Busch

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et al., 2017), cell fate decision in stem cells (Guo et al., 2010; Narsinh et al., 2011) and cell cycle regulation (Dolatabadi et al., 2017). Compared to most other single-cell techniques the effort to move from bulk to single-cell analysis is rather straightforward when using qPCR. Regular assays and standard instrumentation may be applied. Single-cell collection, preamplification and some modified data analysis are the additional steps needed. An advantage of qPCR is that DNA, all types of RNA and many proteins can be analyzed even in combination, all generating Cycle of quantification (Cq) readouts (Darmanis et al., 2016; Genshaft et al., 2016; Ståhlberg et al., 2012; Tang et al., 2006). Another major advantage compared to other single-cell technologies is that qPCR data analysis is well established and can be performed with standard qPCR analysis software with no need for advanced bioinformatics and writing of scripts (Ståhlberg et al., 2013a). Quantitative PCR can also be used in combination with next generation sequencing (NGS) taking advantage of the strengths of both technologies (Kroneis et al., 2017). A limitation of qPCR is that only a limited number of target molecules, usually 1 to 96, can be assessed in a workflow. When the number of targets is larger other techniques such as NGS are preferred. Quantitative PCR and NGS are related technologies, since most NGS protocols include PCR (Gawad et al., 2016; Wen and Tang, 2016). Hence, qPCR and NGS workflows share many experimental features and limitations. Expression data measured with NGS and qPCR correlate and the methods should be exchangeable (Wu et al., 2014). In qPCR, each assay is optimized and the workflow is simpler, contributing to higher sensitivity and reproducibility compared to NGS (Kroneis et al., 2017). Another advantage of qPCR over NGS is standardized analysis workflow. Quantitative PCR data can be analyzed by most non-specialists, while NGS data analysis is still to be standardized and results may depend on the tools used, the assumptions made and the particular analysis workflow. User friendly analysis packages for non-experts are still few and have limited functionalities. For most applications, qPCR is also more cost-effective than NGS when considering all steps from sample handling to analyzed data, even if the reagent cost per gene and cell may be higher for qPCR. Quantitative PCR may also be substituted by digital PCR in single-cell analysis (Albayrak et al., 2016; Ottesen et al., 2006), but the cost per digital PCR is high and the throughput is low with current platforms, limiting its use.

2. Single-cell analysis using qPCR

2.1. The single-cell qPCR workflow

The single-cell field is still characterized by methodology development and biological proof-of-concept studies. Emerging data show that single-cell analysis can provide vital information about the cell that is not available when studying classical bulk samples. However, for single-cell profiling to become a mainstream methodology it needs to be transparent and to some degree standardized. To date, few reports and biological findings in the single-cell field have been verified in independent studies. A first step towards standardizing single-cell analysis is to report the entire experimental workflow in detail and make data publically available. This will make validation of results and confirmation of findings easier.

Quantitative PCR is a rather mature technology and MIQE (**M**inimum **I**nformation for publication of **Q**uantitative real-time PCR **E**xperiments) guidelines are available for its use on classical bulk samples (Bustin et al., 2009). Similar guidelines are also provided for digital PCR (Huggett et al., 2013). The goal of MIQE is to provide the basis for good experimental practice and encourage detailing protocols that allow for unbiased interpretation of qPCR data. Most aspects of the MIQE guidelines are relevant for single-cell analysis, but some experimental steps need adjustments and there are also some unique steps in single-cell analysis that must be considered (Table 1). Table 2 shows the additional and adjusted items that are specific to single-cell analysis.

Fig. 1 shows the workflow of single-cell qPCR analysis targeting nucleic acids. Experimental details of single-cell protein analysis have been detailed elsewhere (Darmanis et al., 2016; Genshaft et al., 2016; Ståhlberg et al., 2012). Single-cell DNA analyses using qPCR have been reported (Neves et al., 2014; Potter et al., 2013; Rygiel et al., 2015; Ståhlberg et al., 2012; Yang et al., 2014), but most single-cell studies so far has targeted RNA. The workflows for RNA and DNA analyses are similar, except that the RNA workflow requires a reverse transcription (RT) step, while the DNA workflow requires opening of the chromatin structure. Sample handling with cell dissociation and single-cell collection followed by direct lysis are specific to single-cell workflows. Single cells can be collected with several techniques, the most common being microaspiration, laser microdissection, and flow cytometry. Major focus is currently on throughput, where the introduction of droplet reaction containers has revolutionized the single-cell field (Ziegenhain et al., 2017). Attention is also on spatial information, linking cells molecular profiles to their localization in tissues and organs (Lee et al., 2014; Ståhl et al., 2016), and to specific compartments within the single cell (Sindelka et al., 2008). Preamplification is another common, but not an exclusive, single-cell step that is not discussed in the MIQE guidelines. Finally, data analysis is also in many ways unique for single-cell analysis. Carefully optimized protocols and adequate controls are important in all experimental work. In single-cell analysis the experimental setups are more challenging as the numbers of target molecules are very few. Successful single-cell workflows are characterized by efficient lysis and high reaction efficiencies as well as minimal material losses.

2.2. Experimental design

When designing single-cell experiments one of the first questions that arises is how many cells should be analyzed. In classical studies one sample per subject is usually collected, RNA is extracted and analyzed (Fig. 2). If a single gene target is analyzed its normalized expression in the studied groups is compared using univariate statistics to assess the significance of the measured difference. If multiple targets are analyzed multivariate methods are usually more powerful to classify the subjects. Technical replicates may be performed, where replicates upstream in the workflow, such as sampling replicates, reduce confounding variation more than downstream, such as qPCR replicates (Tichopad et al., 2009).

Table 1
MIQE checklist and additional single-cell items.

Item to check - MIQE	Single-cell level
Experimental design	Applicable
Sample preparation	Partly applicable
Nucleic acid extraction	Not applicable
Reverse transcription	Applicable
qPCR target information	Applicable
qPCR oligonucleotides	Applicable
qPCR protocol	Applicable
qPCR validation	Applicable
Data analysis	Partly applicable
Additional single-cell item to check	
Single-cell collection and direct lysis	
Preamplification target information	
Preamplification oligonucleotides	
Preamplification protocol	
Preamplification validation	

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