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dpcR: web server and R package for analysis of digital PCR experiments

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ABSTRACT

The digital Polymerase Chain Reaction (dPCR) enables an absolute quantification of nucleic acids. Different statistical analysis frameworks were proposed. However, most analysis is done in closed source software as provided by the vendors. This makes it harder to compare results, such as the confidence interval estimates. An unified open software framework for reproducible research is not available.

To perform dPCR analysis we implemented peer-review statistical methods and plots into the dpcR framework, based on the sophisticated statistical computing environment **R**. dpcR is versatile open source cross-platform software framework, which provides functions to process dPCR data independent of the hardware. Our software can be used for data analysis and presentation, as framework for novel technical developments and as reference for statistical methods in dPCR analysis. Features such as functions to estimate the underlying Poisson process, calculation of confidence intervals based on single samples as well as on replicates, a novel Generalized Linear Model-based procedure to compare dPCR experiments and a spatial randomness test for assessing plate effects have been integrated. We use a plug-in like architecture and abstraction layers to make the framework usable for droplets and (real-time) chamber based technologies.

dpcR is implemented with interfaces to the command-line, graphical user interfaces and interactive web application. Therefore, it can be used by novices in a graphical user interface or by experts via a command-line interface. The dpcR framework can be used to build a custom-made analyser according to the user requirements. dpcR is an open framework, which can be easily adapted to the growing knowledge in dPCR.

INTRODUCTION

The digital PCR (dPCR) is an important contender for precise nucleic acids quantifications. Applications include investigation of allele frequencies, single-cell analysis, gene expression analysis and absolute quantification of PCR products. The chemical basis (e.g., buffer, primer) of the dPCR and thermal cycling is similar to PCR. Approaches based on isothermal amplification were also developed (**???**). A first proposal for a dPCR-like approach and the use of the Poisson distribution to quantify the number of molecules on a "sample" was shown by Ruano *et al.* 1990 (PNAS) with the single molecule dilution (SMD) PCR (**?**). In 1999 Vogelstein *et al.* (PNAS) described the first true dPCR (**?**). Since approximately ten years the dPCR is rapidly gaining momentum in the mainstream user-base. There is an intensive research on dPCR platforms with the overall aim to make to technology broadly usable, cheap, robust and to enable high sample throughput (**???**).

A dPCR reaction consists of multiple amplifications occurring in numerous small "partitions" (e.g., nL volume droplets of water oil emulsions, chambers on micro structured chips). The result of dPCR is a binary vector describing states of partitions (positive in case of detected amplification, negative otherwise). The amplification in positive partitions indicates the presence of one or more template molecules. It is assumed that distribution of template molecules over partitions is described appropriately by the Poisson distribution. This probability distribution is parametrized using only single parameter, λ, which may be interpreted as the mean number of template molecules per partition. The relationship between λ, the number of positive partitions k and the total number of partitions n is as follows:

$$\lambda = -\log\left(1 - \frac{k}{n}\right) \quad (1)$$

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Knowing the λ and volume of the single partition, v, the computation of the concentration of a template in the sample (c) seems to be straightforward ($c = \frac{\lambda}{V}$), yet methods of defining the uncertainty of λ or concentration are still emerging (**?**).

The variety of existing procedures is disproportionate to the number of software packages dedicated to analysis of dPCR experiments. Most of the software comes in form of closed-source packages distributed by vendors of dPCR systems. The proprietary software without open source code prohibits validation the implementation of the assumed methodology and therefore has a limited usage in research. Moreover, vendors usually tie tightly software with their hardware, which hinders a comparison of results from different systems.

The lack of a dedicated software for analysis of dPCR results leads to the rapid development of custom scripts in **Mathematica** (Wolfram Research) (**?**), **MS EXCEL** (Microsoft) (**?**) or **R** (**??**). In addition, web-servers for analysis of dPCR data, as the **definetherain** (**?**), are emerging. However, these efforts have limited use, since they are tied to a specific problem and do not form an unifed framework.

The described situation is unfavorable for studies involving dPCR data. The absence of graphical user interfaces prohibits researchers not comfortable with the command line interfaces from using the custom-made frameworks. Others have to implement methods on their own, shifting their focus from an investigation to programming.

In 2013 we started the development of the *dpcR* framework to perform analysis of dPCR experiments (**?**). We have chosen R software environment (**?**), because it is already extensively used in studies of qPCR data (**??**), both pre-processing ((**??**)) and analysis ((**??**)). Furthermore, R is freely available for the MacOS, Linux/Unix and Windows operating systems. It supports literate programming (e.g., *knitr*) and tools for reproducible research (e.g., *rctrack*) (**?**).

Since the R's command-line interface might not meet the needs of all potential users, we added a web server *dpcReport* using the *shiny* package. It allows access to the most of *dpcR* functionalities through the Graphical User Interface (GUI). *dpcReport* can be also installed as the stand-alone software.

MATERIALS AND METHODS

Implementation of *dpcR*

The *dpcR* package is an open source extension to **R**. It employs object-oriented programming paradigm and the `underscore_sep` naming convention (**?**).

Any processing of dpcR data is inevitably tied to the information loss, so we suggest to start analysis with raw data rather than summaries exported by vendor-provided software. If it is not possible, part of *dcpR* functionalities will be not available, but most of the study can be still successfully conducted using the same commands as in the case of unprocessed data.

Such universality results from the usage of uniform data format in all computations inside the workflow. Datasets, regardless of their origin (array- or droplet-based dPCRs) and type (total number of positive partitions, states of partitions, CQs of partitions), are stored in the standardized class. Subclasses further define the more specific features of objects as the spatial organization of chambers in case of the array-based dPCR.

The workflow of *dpcR* encloses complete data analysis, starting from data import or simulation and ending with report generation (Figure 1). The package employs peer-reviewed methods of computing the λ value and assess its uncertainty. We also implemented previously published methods of statistical analysis of dPCR reactions.

Data import

Import functions limit availability of the package by determining which datasets can be easily processed using the provided framework. To address the needs of potential users, *dpcR* not only covers data import from most popular systems, but also facilities data simulation and conversion of qPCR datasets.

Since the RDML format for dPCR is not yet established, we wrote function *read_dpcr* streamlining data import from several systems (see Table 1). To cover experimental or not yet included systems, we created a "raw data" format (see Supplementary Files for description). The user can manually arrange his data in this format and import it to the *dpcR* package. Such input files can be created in a spreadsheet program or a text editor.

Moreover, *dpcR* accepts other **R** objects, which is useful if the data required some further pre-processing not covered by the package (e.g., removal of missing values, smoothing (**?**)). Such objects may be integrated into the workflow using the *df2dpcr* or more flexible *create_dpcr* functions.

Data simulation We found two methods of simulating results of dPCR (**??**). The former is based on randomly distributing the exact number of template molecules over defined number of identical partitions. It results in the vector of partitions with known number of template molecules.

The procedure in Jacobs *et al.* 2014 models more complicated situation, fluorescence values of the passing droplets. The number of template molecules in particular partition as well as the volume of the partition are determined by separate probability distributions. The number of molecules per partition is further converted to the fluorescence values considering the random effects affecting amplification efficiency. The simulation also includes such phenomena as "rain", droplets that cannot be unambiguously assessed as positive or negative.

We complement these methods with our own, where the number of template molecules distributed over identical partitions is not exactly specified, but determined by the Poisson distribution with specific parameters. It is best

Table 1. Structured vendor export data formats handled by *dpcR* v. 0.5 and later.

Vendor	System	Format	Type
Bio-Rad	QX100 & QX200	CSV	Summary export
Fluidigm	BioMark	CSV	Summary export
Formulatrix	Constellation Digital PCR	CSV	Summary export

The number of structured export data formats handled by *dpcR* is growing. CSV, comma separated values.

Figure 1. *dpcR* workflow. The diagram shows main functions available at each step of a dPCR data analysis.

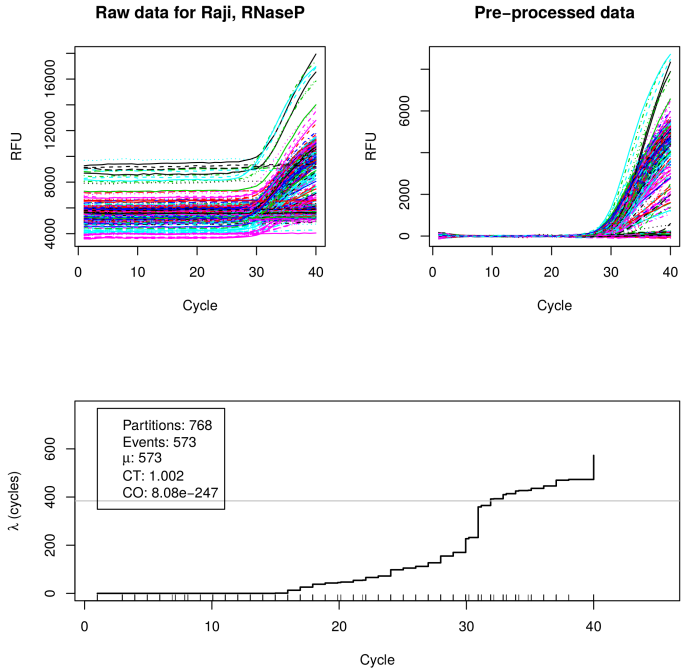

Figure 2. Uncover characteristics of dPCR data. Selected dPCR platforms are qPCR platforms at the same time. The function *qpcr2pp* uses the qPCR amplification curve data and interprets them as dPCR (Poisson process). A) Raw data of The function were B) preprocessed (baselined, smoothed) with functions from the *chipPCR* package and C) finally analysed (Cq calculation → binarize) with the *qpcr2pp* (qPCR to Poisson process) function from the *dpcR* package.

described as the variant of procedure introduced elsewhere (**?**), but without the assumption of having the constant number of template molecules over all samples. All three methods are available under *sim_dpcr* command.

Integration of qPCR data High-Throughput qPCR is a well established and robust technology, which allows precise quantification of DNA material in high throughput fashion. However, the quantification by qPCR is challenging at very low and very high concentrations. In addition, pre-processing and data analysis is a affected by numerous adverse effects (**??**).

The recently proposed solution (**?**) overcomes these complications by using the dPCR methodology to analyze qPCR data. Briefly, quantification points (Cq) are computed using the real-time measurements of several amplification curves. Next, the Cq values are binarized and treated as the status of partitions effectively converting multiple qPCR experiments into a dPCR. This functionality is supported by the *qpcr2pp* function (Figure 2).

Statistical analysis

Calculation of the uncertainty To determine the uncertainty of the estimated λ we employ two previously published peer-reviewed methods The first (**?**) uses the normal approximation to compute the confidence intervals for binomially distributed $\frac{k}{n}$. Next, the confidence intervals are altered using Equation **1**.

The other method (**?**) is based on the uncertainty of the measurement and includes also the uncertainty caused by the variation of volume. The exact formula for the uncertainty of the λ value is:

$$u_\lambda = \sqrt{\frac{k}{n^2(1-\frac{k}{n})}} \tag{2}$$

and for the uncertainty of the concentration of the template molecules:

$$u_c = C\sqrt{\left(\frac{1}{\log 1-\frac{k}{n}}\sqrt{\frac{k}{n(n-k)}}\right)^2+\left(\frac{u_V}{V}\right)^2} \tag{3}$$

where

Both methods are implemented in the *summary* function.

Moments It is assumed that the Poisson distribution describes the distribution of template molecules over partitions. The *dpcR* package enables computation of four first moments (mean, variance, skewness, kurtosis) of the distribution from which the data is sampled. It is also possible to compare these empirical moments with theoretical moments of the Poisson distribution calculated using the estimated λ (Table 2).

Comparison of dPCR experiments The *dpcR* package covers peer-reviewed methods of comparing results of dPCR experiments. Here, by the comparison we understand a procedure, where all data points from all runs are considered and affect the final outcome.

Two methods, GLM and MRT, conduct such analysis on the run level by comparing individual runs against each other (**?**). Both are accessible using the *test_counts* function.

Table 2. Empirical and theoretical moments of the Poisson distribution. $\mu = \frac{1}{n}\sum_{i=1}^{n} x_i$. $m_j = \frac{1}{n}\sum_{i=1}^{n}(x_i-\mu)^j$. $s = \sqrt{m_2}$

Moment	Empirical	Theoretical
Mean	μ	λ
Variance	s^2	λ
Skewness	$\frac{m_3}{s^3}$	$\frac{1}{\sqrt{\lambda}}$
Kurtosis	$\frac{m_4}{s^4}$	$\frac{1}{\lambda}$

The GLM method is named after Generalized Linear Model, which is used to estimate the λ values for all runs. Next, results are compared against each other using the max-t test. Thanks to the method of computation, the obtained p-values of comparison does not require further comparisons.

The MRT method compares runs using the uniformly most powerful ratio test to compare the value of λ between two runs. The uniformly most powerful test has the highest probability of rejecting the false null hypothesis among all tests with the same confidence level.

Additionally, we also implemented a method for comparing multiple experiments (**?**) as the *test_pooled* function. It uses Generalized Linear Model to compute directly the estimate of the concentration of the template in the sample from the pooled data of replicates.

Spatial distribution Array based dPCR experiments provide information about spatial distribution of partitions. Procedures belonging to spatial statistics verify if the status (positive, negative) of partition depends on its location. To address such questions, we implemented a Complete Spatial Randomness test for dPCR arrays. Moreover, we provide functionalities to export array dPCR data to **R** packages specialized in spatial statistics as described elsewhere (**?**).

Export of analysis results

REDUNDANT Since *dpcR* is based on the **R** environment all facilities for a report generation are usable as described before (**?**).

A commonly available data exchange format is a perquisite for reproducible research. So far no cross-platform and system-independent format has been introduced for dPCR experiments. Therefore, we decided to use `RData` as our default format. `RData` works across all **R** environments and saves the variable names along the content of multiple variables, which can be restored in any workspace.

Public datasets

dpcR includes datasets or refers to additional R packages for testing purposes. The data originate from different dPCR and qPCR systems and were either published previously (**?????**) or *de novo* generated.

Figure 1 provides an overview of important functions available at each step of a dPCR analysis. The first step is to import sample data into the **R** session. *dpcR* accepts various data structures. This includes matrices of raw data and predefined structures provided by the different vendors (see Table 1).

Documentation

The concise documentation of all functions and classes included in the *dpcR* package helps users in adapting our work to their needs. We use included datasets to create meaningful examples showing the input and output of implented procedures. The documentation is available as standard **R** package reference manual and as a vignette, additional guide helping in understanding the basics of the package.

According to the dMIQE guidelines (**?**) we used following notation:

- λ: average molecule numbers per partition,
- k: number of molecules per partition,
- m: total number of the molecules.

The following convention was kept in the documentation, the source code and the outputs of all functions.

dpcReport: graphical user interface

For the convinience of researchers less familiar with **R**, we implemented the core functionalities of *dpcR* package in form of the graphical user interface (GUI).

We aimed for a form factor (e.g., smart phone, tablet, desktop PCR) and operating system independent implementation of a graphical user interface. *dpcReport* is based on *shiny* technology and offers an intuitive user interface, which can be accessed by browsers (e.g., Google Chrome, Mozilla Firefox). Semi-interactive charts are based on *ggplot2* (see Supplemental Files). All tables are also interactive, facilitating filtering and selecting specific records.

An interesting feature of the *shiny* technology is the automatic integration in environments, which support `HTML5` and `ECMAScript`. The *dpcReport* integrates not only into modern web browsers, but also **R** Integrated development environments (IDEs) such as **RKWard** (Figure 3) (**?**) or **RStudio**.

The first panel of GUI, "Input file", is responsible for loading the input data. Since it utilizes *dpcR* functions, it is as versatile as data import functionalities of our package. Moreover, it is possible to edit the input dataset using the mini-spreadsheet included in the panel. All important features, starting with experiment names and ending with results, can be freely modified allowing manual correction of the input.

The descriptive summary of the data in form of interactive tables and plots is included in the panel "Data summary". Estimated λ values and their uncertainties are computed using the *summary* function. Box-and-whisker diagrams cumulatively describe relationships between experiments and their λ value or concentration of the template, while scatter charts allow individual representation of runs.

The third panel "Comparison of runs" facilitates comparison of multiple runs contained by the input data set. It employs *dpcR* function *test_counts* to provide user with information about significantly different values of λ. The proposed groups of runs are presented in tabular format and in charts.

The "Advanced analysis" panel consists of more specialized functionalities. Here an user may test the array data for randomness of spatial distribution of positive droplets. The interactive visualization of the array allows analysis of whole plate or manually selected region. The second part of the "Advanced analysis" panel is devoted to the investigation of distribution of the positive droplets in individual runs via computation of its confidence intervals and its moments. The highly customizable functionalities allows choice between few implemented methods of confidence interval estimation for binomial variable.

The last panel enables flexible report generation. The report can be customized by including various sections, which are equivalents of the GUI panels. The complete user input,

including manually adjusted parameters of test performed in GUI, are preserved and returned in the report to increase the reproducibility of the research study. An important option of *dpcReport* is an export of the **R** source code used for the report generation. This code can be used for recreating the analysis in the **R** environment or prototyping more complicated workflows.

RESULTS

In the following section we show applications of the *dpcR* package.

Vendor independent data analysis

QX100 series is no longer available. Instead, the newer version QX200 protocol consisting of the QX200 droplet generator (Bio-Rad, cat. no. 186-4002) and the QX200 droplet reader (Bio-Rad, cat. no. 186-4003) can be purchased, for which the protocol can be applied without changes. Alternative dPCR devices available from, e.g., RainDance Technologies, Life Technologies or JN Medsys (**?**).

Automatic report generation

In (**?**) we gave an example where we re-analyze droplet dPCR data from a Bio-Rad QX100 system with an early implementation of the *dpcR* package. As recommended in the dMIQE guidelines (**?**) we included key elements in the report.

Availability

The *dpcR* framework is available as open source software package (GPL-3 or later) as part of the Bioconductor project (**?**). The stable version is hosted at http://cran.r-project.org/web/packages/dpcR and the source code is available from https://github.com/michbur/dpcR.

DISCUSSION

Currently, there exist different dPCR analysis software solutions provided by the vendors. But most of the software packages are black boxes, which prevent deep insight into the data processing step. Other and we think that scientific software should be open (**??**). In addition, most of the software solutions are aimed to be used in very specific scenarios and a mutual exclusive to alternative platforms (e.g., droplet vs. chamber-based). We have chosen **R** because it is the *lingua franca* in biostatistics and broadly used in other disciplines (**?**). We developed the *dpcR* package, which is a software framework for analysis of dPCR. *dpcR* provides the scientific community a broadly applicable tool for teaching purposes, data analysis and theoretical research based on simulations. Our software framework can be used to accelerate the development of new approaches to dPCR.

Functions included may be used to simulate dPCRs, perform statistical data analysis, plotting of the results and simple report generation.

CONCLUSION

In conclusion, *dpcR* provides means to understand how dPCR works, to design, simulate and analyze experiments, and to verify their results (e.g., confidence interval estimation), which should ultimately improve reproducibility. We have built what we believe to be the first unified, cross-platform, dMIQE compliant, open source software framework for analysing dPCR experiments. Our *dpcR* framework is targeted at a broad user base including end users in clinics, academics, developers, and educators. We implemented existing statistical methods for dPCR and suggest the introduction of a standardized dPCR nomenclature. Our framework is suitable for teaching and includes references for an elaborated set of methods for dPCR statistics. Our software can be used for (I) data analysis and visualization in research, (II) as software framework for novel technical developments, (III) as platform for teaching this new technology and (IV) as reference for statistical methods with a standardized nomenclature for dPCR experiments. The framework enables the simulations and predictions of Poisson distribution for dPCR scenarios, the analysis of previously run dPCRs. Due to the plug-in structure of the software it is possible to build custom-made analysers.

Instead of implementing algorithms for clustering and "rain" (positive droplets) definition of droplet dPCR data, we focused on more specific and not yet well-accessible functionalities. We chose not to duplicate existing works and instead provide users of dPCR technology with a flexible workflow that incorporate most fundamental needs: estimation of the λ value, comparison of template concentration among several runs and quality control. For clustering of ddPCR data, we refer to **R** packages dedicated to flow-cytometer research. Implementations range from manual to automatic clustering (**????**).

Moreover, discussion with our peers and the literature suggest that a consensus of an appropriate method for dPCR is not available (**?**). Our open framework includes to invitation to the scientific community to join and support the development of *dpcR*.

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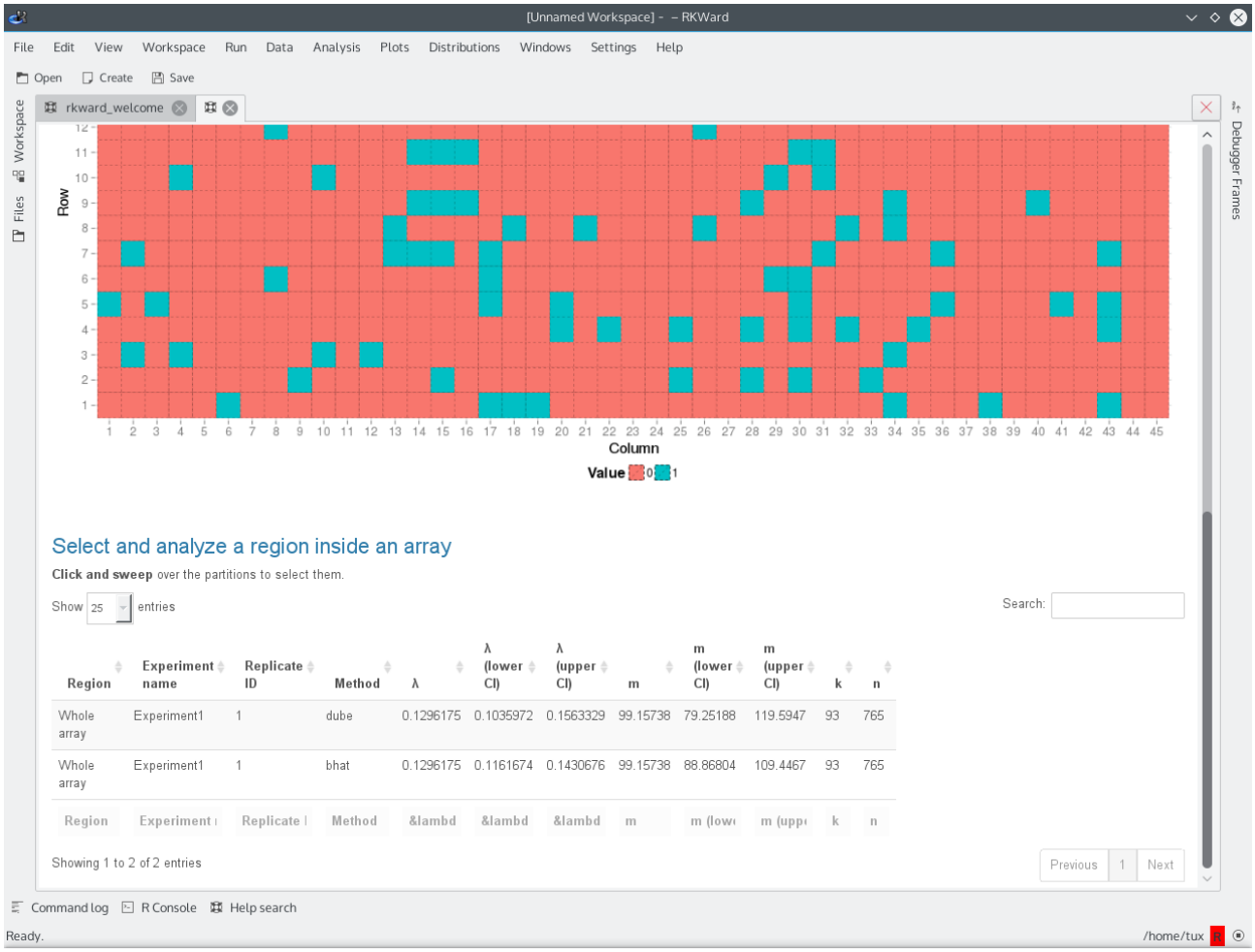

Figure 3. *dpcReport()* function running in the graphical user interface and integrated development environment **RKWard**.

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