

# High Content RNAi Screening Using Printed Libraries

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## Abstract

RNA interference is a research tool used to assign function to genes and has proven to be a powerful tool to study gene function by silencing transcription. RNA interference (RNAi) is a biological process in which RNA molecules inhibit gene expression, typically by causing the destruction of specific mRNA molecules [1-3]. Gene silencing with siRNA is one of a set of functional genomics technologies that enable researchers to identify the precise role a gene plays in a specific biologic process, which is one of the key steps in identifying therapeutic targets with well understood mechanisms of action.

While phenotypic screening is undergoing a resurgence, its use in conjunction with RNAi remains an expensive research strategy, mostly driven by costly assay reagents and the liquid handling and high content imaging instrumentation required to perform screening. Recently, these barriers have been lowered through the development of affordable, high quality automated microscope/imaging systems and the invention of miniaturized RNAi screening technologies that eliminate the requirement for automation infrastructure and reduce cost/data point by orders of magnitude. It is now possible to screen entire RNAi libraries at the bench, eliminating any need for liquid handling robotics.

Persomics technology is fundamentally different than the multi-well microplates that have become the norm in RNAi screening. Persomics plates replace the experimental well with a printed array of spots on an optical glass insert embedded in the base of an SBS standard plate format. Each of these spots is a dried spot of siRNA that also contains all the reagents necessary to silence genes in cells grown over them. This library screening solution requires a limited number of workflow steps for phenotypic screening using a cellular assay and an automated fluorescence imaging system, such as the Cytation. Unlike microplate wells, there is no physical barrier between the individual RNAi experiments, making workflows highly parallel and technically straightforward.

## ImageArray™ Plate



**Figure 1. ImageArray™ Microplate.** SBS compliant plate that has a glass slide fused into the central portion of the molded plastic reservoir plate.

## ImageArray vs. Multi-Well Screening

	ImageArray Screen	Multi-Well Microplate Screen
Infrastructure and Setup	Off the Shelf/ Turnkey	Significant
Automation	None Required	Required
Cost (including overhead)	Low	Considerable
Ease of Use	Easy	Dedicated Experts
Data Points/Well	3200	1
Hands-On Time	45 Minutes	Significant
Pipette Tips	1 - 5	1000s
Plates	1	10s-100s
Imaging Time	15 to 60 Minutes	Hours-Days-Weeks
Data Analysis Time	Similar	Similar

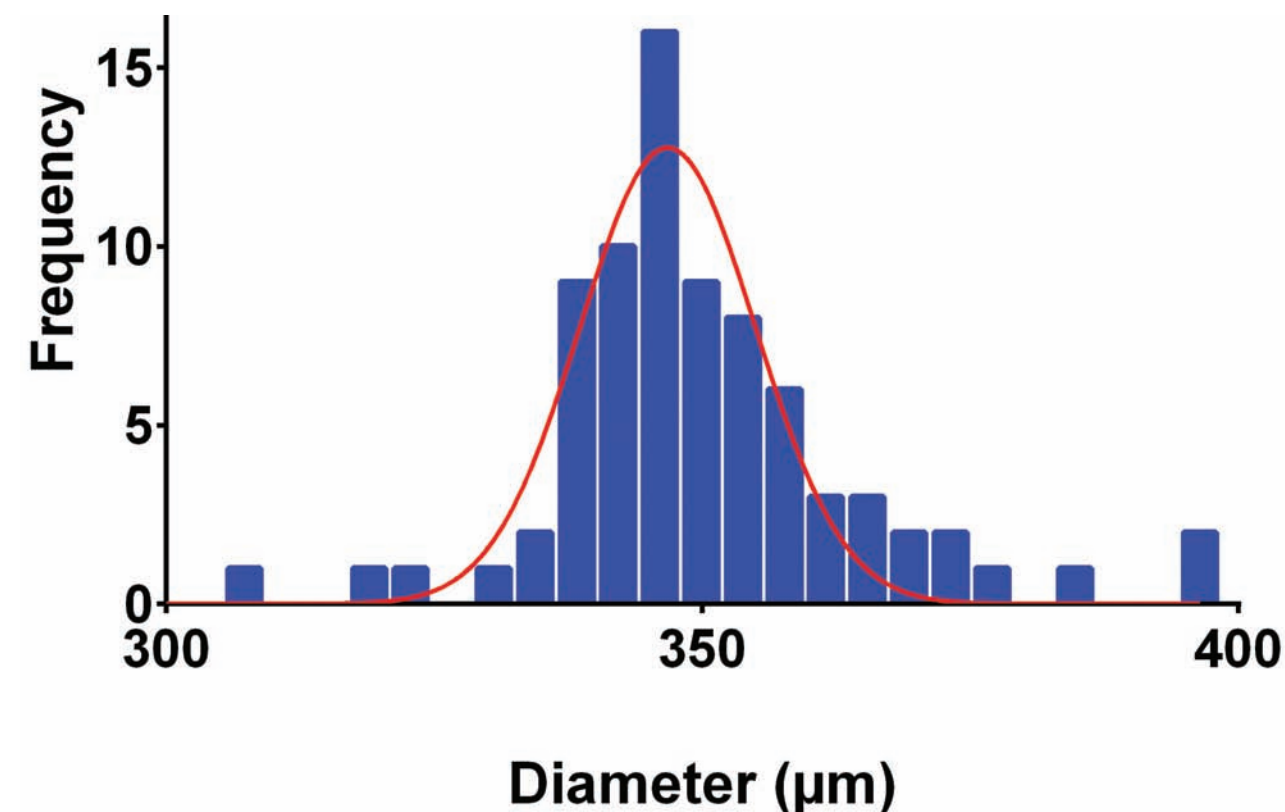
**Figure 2. Comparison of ImageArray™ and Multi-Well Screening.** Printed RNAi arrays save considerable amounts of time and reagents as compared to traditional microplate screening techniques. In addition considerable less instrument infrastructure is required.

## ImageArray™ Spot Constituents



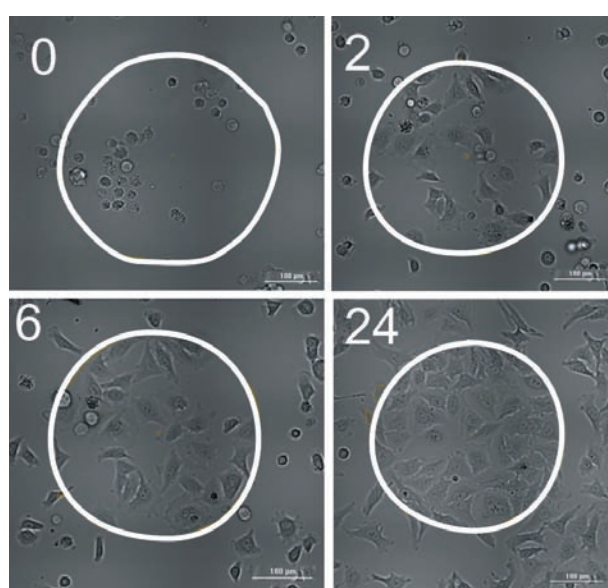
**Figure 3. Constituents of a Printed RNAi Microspot.** Microspots positions are identified with a fluorescent label in addition to specific siRNAs, transfection and an encapsulation reagents. Each spot is a specific RNAi reaction with surrounding regions serving as untreated controls.

## Array Microspot Size Distribution



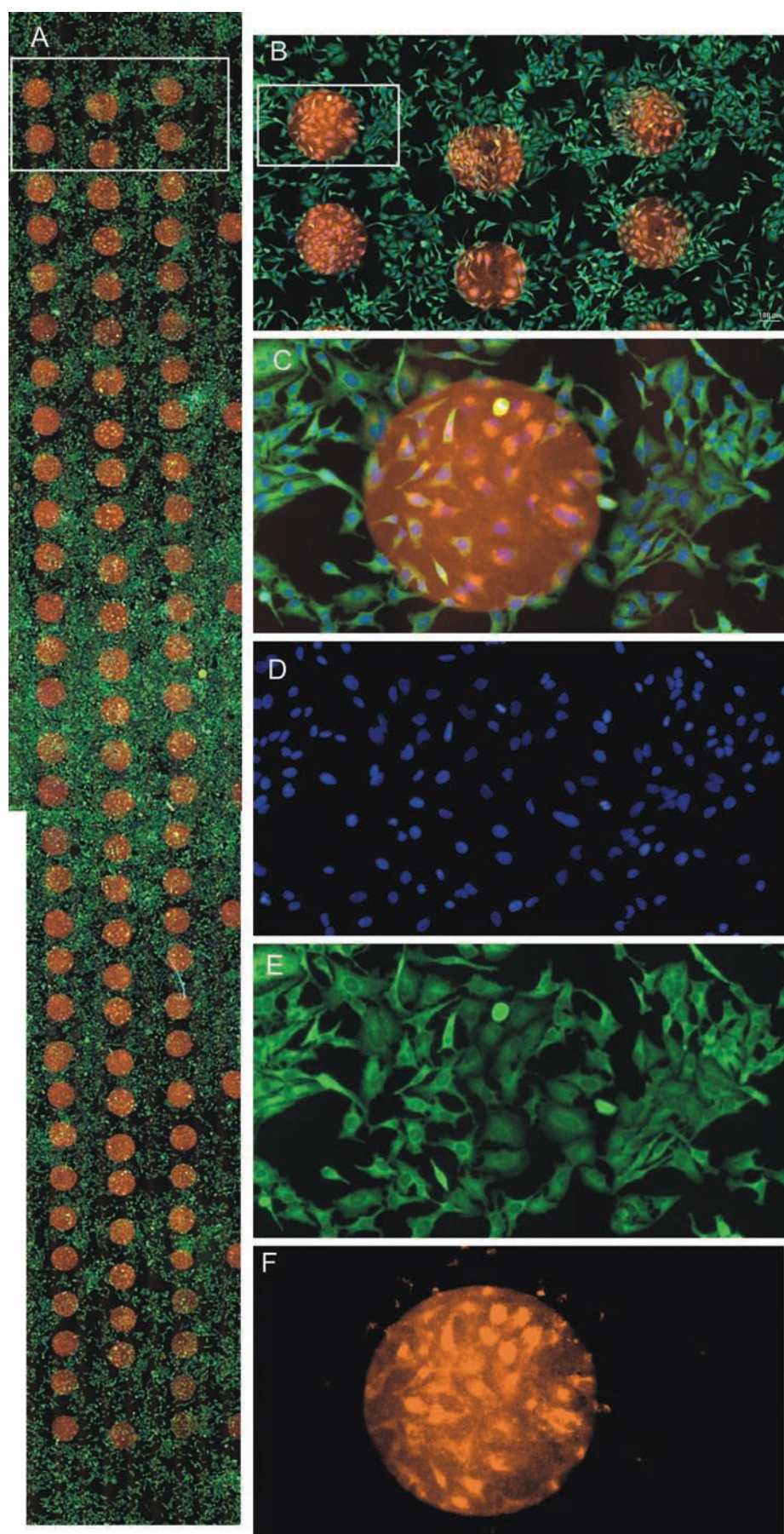
**Figure 4. Determined Microspot Size.** The diameter of 100 microspots was determined using object oriented image analysis of the RFP channel. Data was then expressed as a frequency histogram.

## Growth of Cells on Microspots



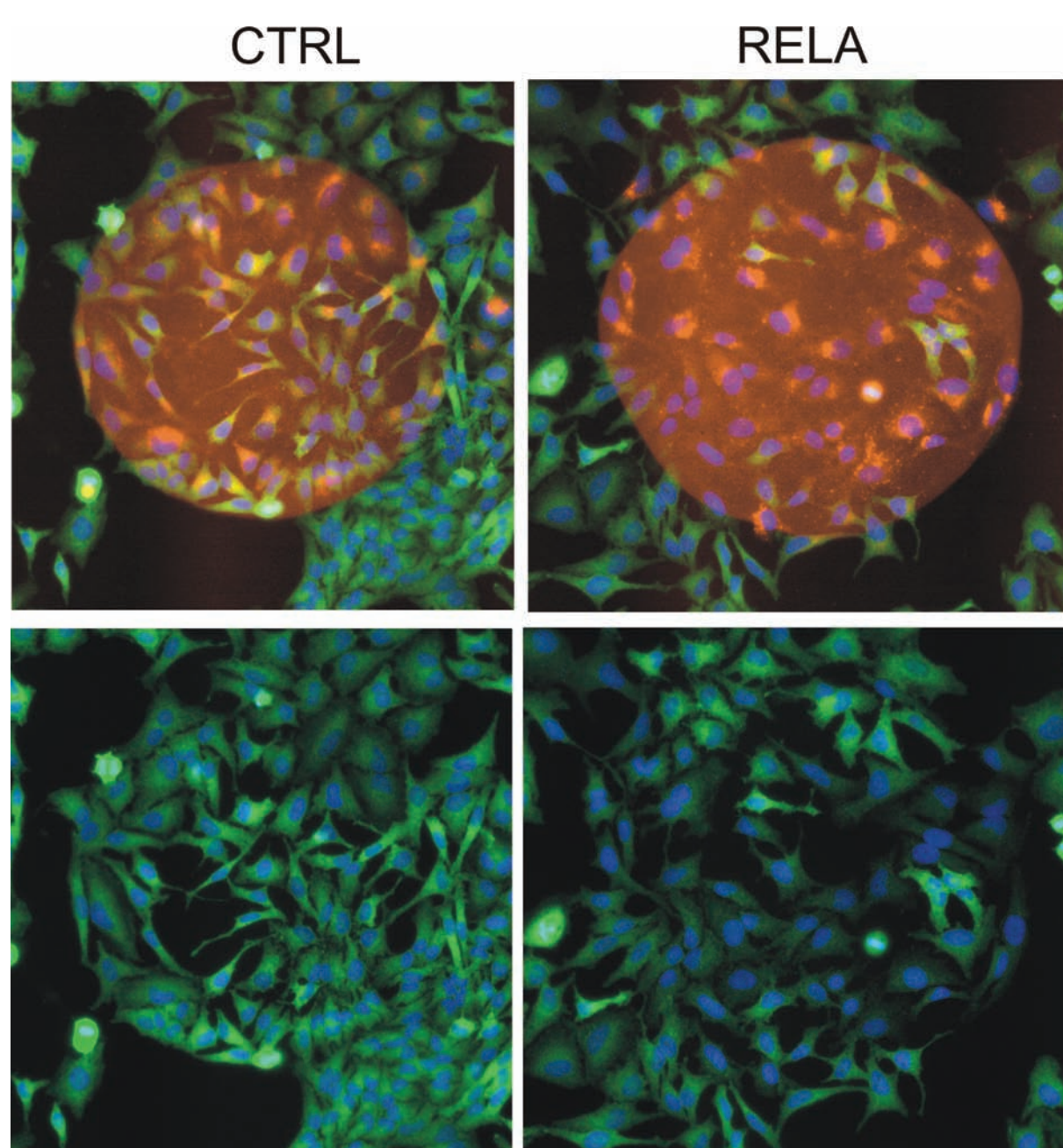
**Figure 5. Growth of Cells on Microspot locations.** HeLa cells were seeded in a Persomics plate and an individual spot was monitored over 24 hours using bright field imaging. The microspot (white outline) was identified by red fluorescence and subsequent threshold analysis of overlaid image. Time after cell seeding is indicated in each tile along with a magnification scale, which represents 100 µm.

## Microspot Analysis

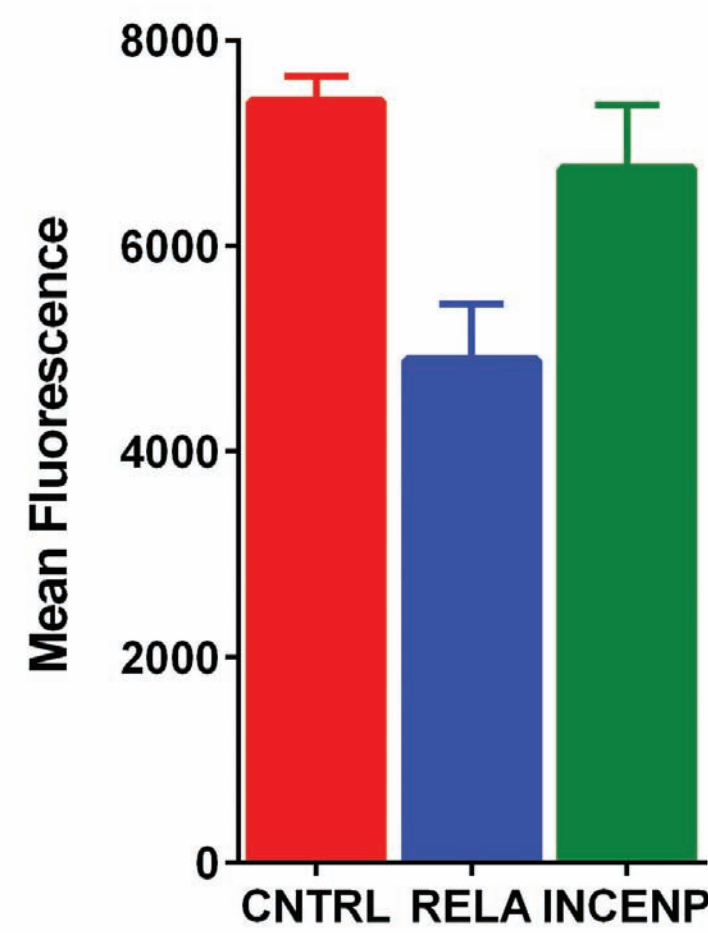


**Figure 6. ImageArray™ Screen.** (A) Image montage of a portion of an ImageArray. (B) Enlarged image demonstrating 6 microspots of the boxed portion depicted in figure 3A. (C) Magnified three color image of a single microspot (boxed region of 3B). (D-F) Image shown if figure 3D shown as separate blue, green and red images.

## RELA Knockdown



**Figure 7. Three color images of Control and RELA RNAi Microspots.** Overlaid images of discrete image Array microspots show qualitative differences in green fluorescent antibody staining of HeLa cells grown over RNAi spots. Top images depict region of the microspot, while lower images have red fluorescence removed for better visualization.



**Figure 8. Quantitative Analysis of RELA knockdown.** The mean green fluorescence of 50 microspots from each a control, RELA and INCENP RNAi microspots where determined and compared.

## Materials and Methods

### Cell Culture

HeLa cells were cultured in Advanced DMEM supplemented with 10% fetal bovine serum and penicillin-streptomycin at 37 °C in 5% CO<sub>2</sub>. Cultures were routinely trypsinized (0.05% Trypsin-EDTA) at 80% confluency. For RNAi experiments, 1 x10<sup>6</sup> cells in 10 mL of Optimem supplemented with 5% FBS and penicillin-streptomycin were plated into the ImageArray™ dish and incubated at 37 °C in 5% CO<sub>2</sub> for 48 hours.

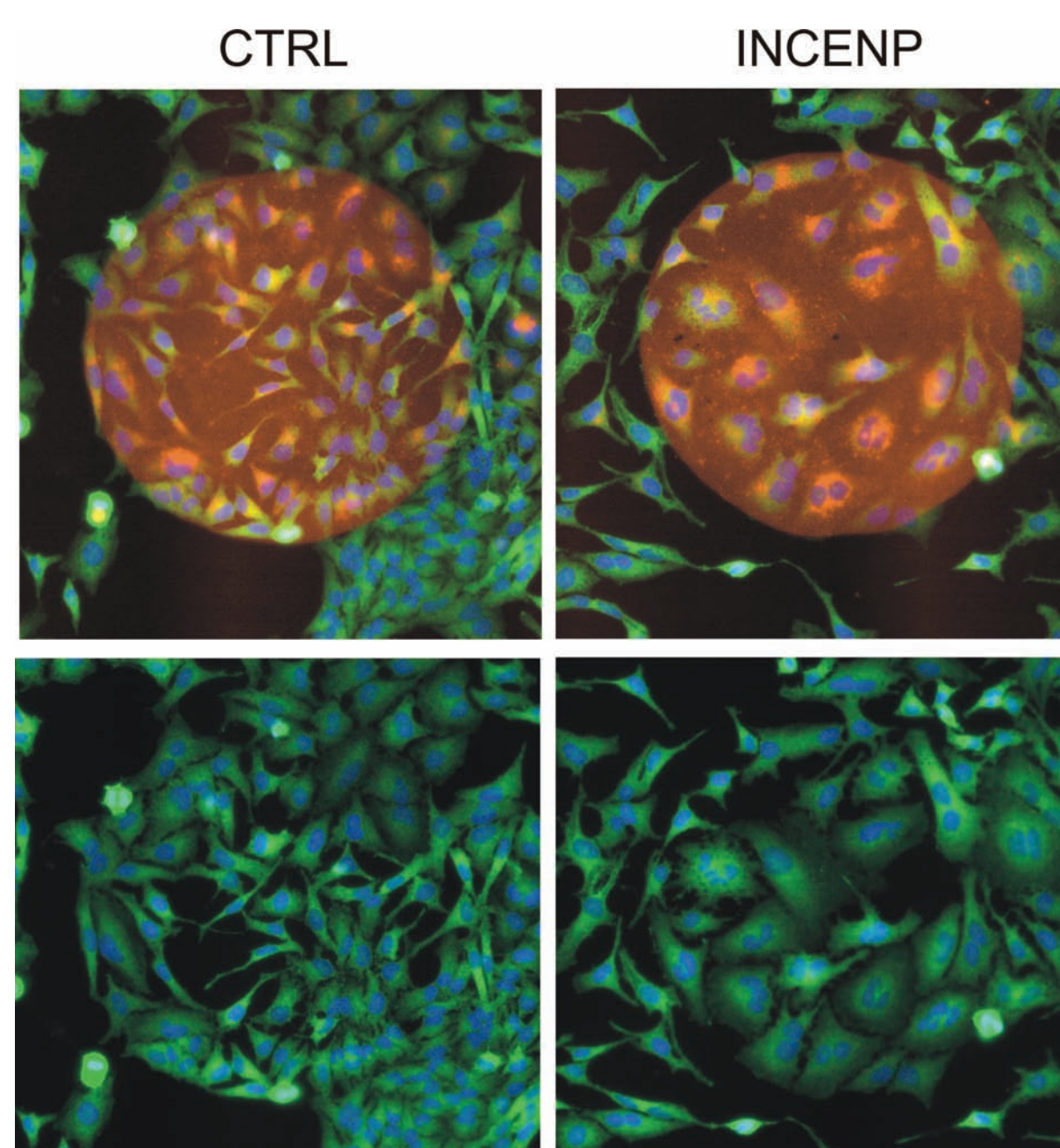
### Imaging

RNAi transfected cultures were imaged using a Cytation 5 Microplate Imager (BioTek Instruments, Winooski, VT) Configured with DAPI, GFP and RFP light cubes. The imager uses a combination of LED light sources in conjunction with band pass filters and dichroic mirrors to provide appropriate wavelength light. The DAPI light cubes uses a 337/50 excitation filter and a 447/60 emission filter, GFP light cube uses a 469/35 excitation filter and a 525/39 emission filter, while the RFP light cube uses a 531/40 excitation and 593/40 emission filters.

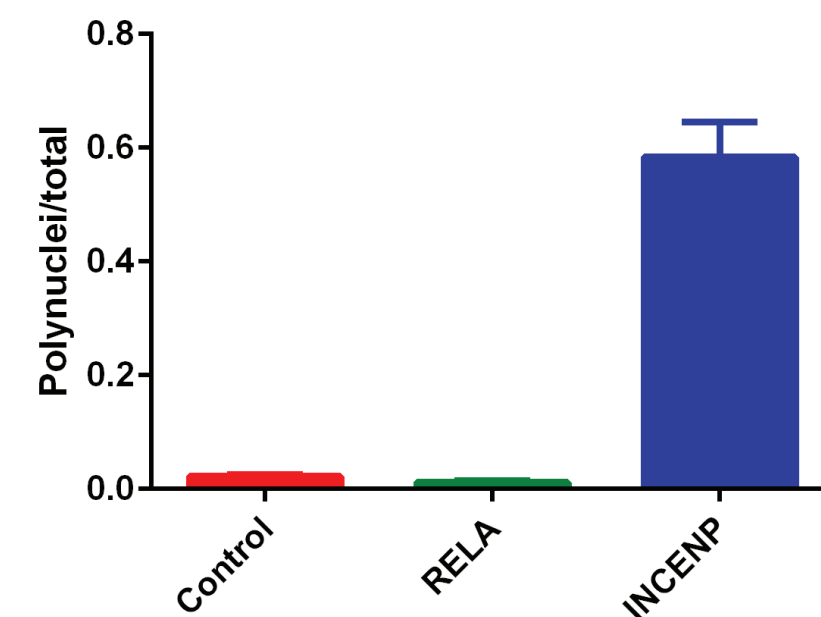
### Image Analysis

Multiple three-color overlaid digital images were electronically stitched using Gen5™ software. Object cell counting of the RFP channel was used to identify specific locations of microspots. Subpopulation analysis was used to determine the mean fluorescence intensity of the GFP channel as a means to assess RELA knockdown. Poly-nuclei determination was made by manual assessment of the number of nuclei in cells within the microspot.

## INCENP Knockdown

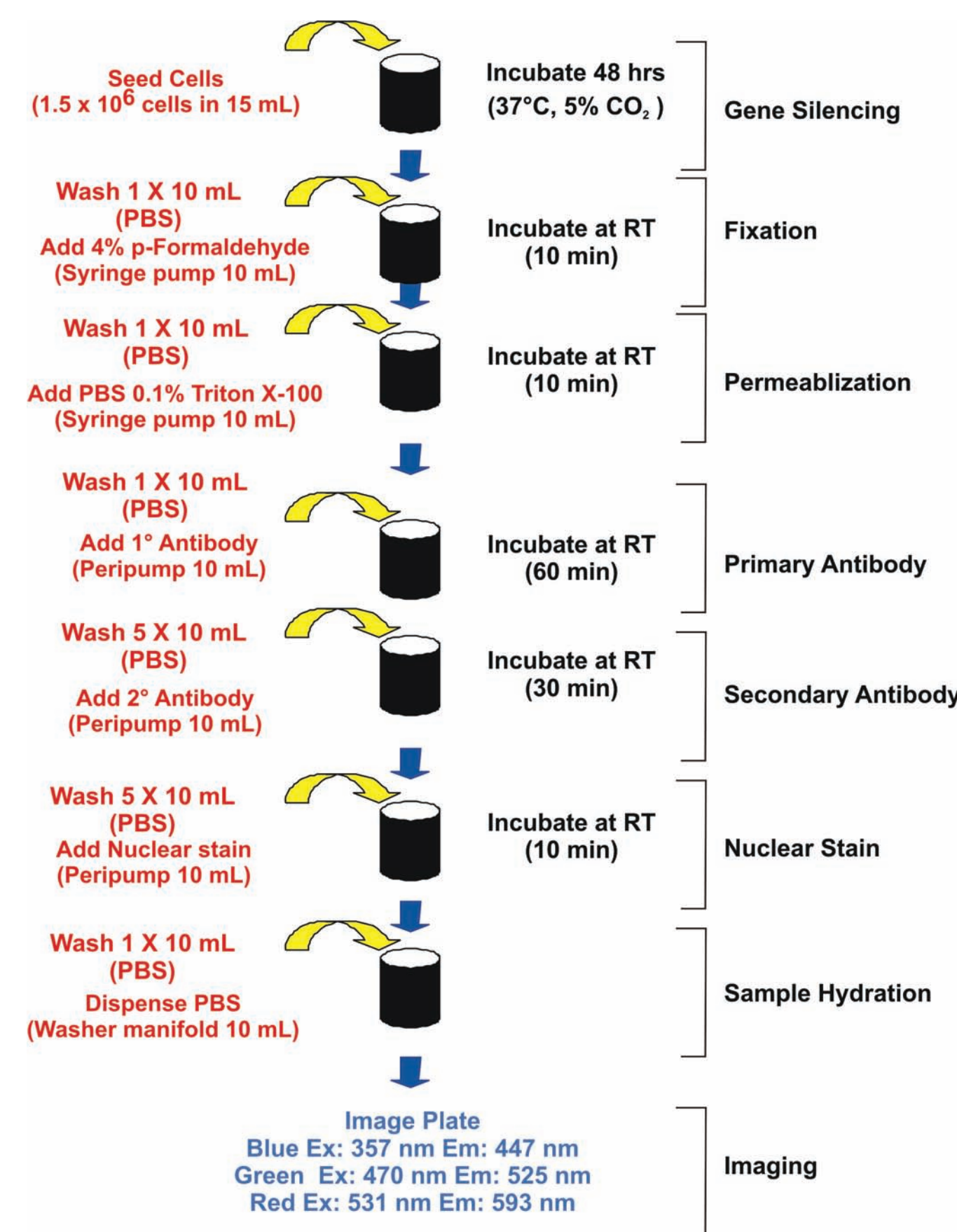


**Figure 9. Three color images of Control and INCENP RNAi Microspots.** Overlaid images of discrete image Array microspots show qualitative differences in the number of nuclei in each cell of HeLa cells grown over RNAi spots. Top images depict region of the microspot, while lower images have red fluorescence removed for better visualization.



**Figure 10. Quantitative Analysis of INCENP knockdown.** The mean green fluorescence of 50 microspots from each a control, RELA and INCENP RNAi microspots where determined and compared

## Automated Assay Process



**Figure 11. Schematic of the ImageArray™ Plate Processing Steps.** The MultiFlo™ FX was used to dispense the required reagents, as well as provide and aspirate PBS wash solution. Incubation timing was controlled by LHC™ software (BioTek Instruments). Following fixation and staining regions of the ImageArray™ plate were imaged with a 20X objective using a montage of images to assess multiple microspots in their entirety.

## Instrumentation



**Figure 12. MultiFlo™ FX Multi-Mode Dispenser washer.** The MultiFlo FX is a modular, upgradable reagent dispenser that can have as many as two peri-pump (8 tube dispensers), two syringe pump dispensers and a strip washer. The syringe and washer manifolds can be configured for plate densities from 6- to 384-well.



**Figure 13. Cytation™ 5 Cell Imaging Multi-Mode Reader.** Cytation 5 is a modular, upgradable multi-mode reader that combines automated digital microscopy and conventional microplate detection. Cytation 5 includes both filter- and monochromator-based detection; the microscopy module provides up to 60x magnification in fluorescence, brightfield, color brightfield and phase contrast. Incubation to 65 ° and plate shaking are standard features. The imaging module uses a turret to hold up to 6 objectives. Excitatory light and emission wavelengths for fluorescence microscopy are provided using LED light cubes in combination with specific band pass filters are dichroic mirrors. The imaging module holds up to 4 LED cubes. In conjunction with the multi-mode reader, Gen5™ software, which controls reader function, also provides image analysis and data reduction

## Conclusions

- Printed RNAi Arrays can be used to consolidate Genome wide Expression Screens
  - Significant Savings
    - Time
    - Reagents
- Quantitative and Phenotypic Changes can be Observed
  - INCENP knockdowns interfere with mitosis and can be observed by polynuclear HeLa cells
  - RELA knockdowns can be observed by reduction in specific fluorescent antibody staining to the NK-kappa-B p65 subunit protein
- MultiFlo FX Reagent Dispenser
  - Automates the Liquid handling tasks necessary for ImageArray™ Fixing and Staining
- Cytation 5 Imager has a number of features that enable live cell imaging
  - Auto-focus and Auto-exposure
  - Multiple color Imaging capabilities
- Quantitative Image Analysis Using Gen5 Software
  - Montage Stitching
  - Mean signal Determination
  - Population analysis