

Measuring genetic interactions in human cells by RNAi and imaging

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Published online 11 September 2014; doi:10.1038/nprot.2014.160

Observation of how genetic interactions modulate phenotypes is a powerful method for dissecting their underlying molecular and functional networks. Whereas in model organisms genetic interaction studies are well established, systematic analysis of genetic interactions in human cells has remained challenging. Here we provide a detailed protocol for large-scale mapping of genetic interactions in human cells using a high-throughput phenotyping approach. Pairwise gene product depletion is induced by siRNA-mediated knockdown, and the resulting phenotypes are quantified by automated imaging and computational analysis to provide the basis for detecting genetic interactions between all pairs of genes tested. The whole workflow, depending on the size of the experiment, takes 3 or more weeks to complete.

INTRODUCTION

Genetic interactions have an important role in understanding the link between genotypes and phenotypes. They shape complex phenotypes, including diseases such as cancer, and they may have a role in explaining the ‘missing heritability’ in genome-wide association studies¹. The systematic mapping of genetic interaction networks has emerged as an important approach to understanding the interplay of genes on a systems level. Much of our current understanding comes from genetic interaction studies in yeast^{2–4} and *Escherichia coli*⁵, which have been based on combining libraries of deletion strains. The combinatorial effects of pairwise gene knockouts have been analyzed in terms of fitness-related phenotypes, as represented, for example, by colony size. Such an analysis has enabled the generation of a comprehensive map of the genetic interactions in yeast⁶. In other model systems, combinatorial loss-of-function analysis has been more challenging. We developed a genetic interaction analysis method in metazoan cells (*Drosophila melanogaster*), which is based on combinatorial, transient gene knockdown and subsequent multivariate phenotype analysis by imaging⁷. Recently, we have established a method to measure genetic interaction in human cells by extending the methods originally developed in *Drosophila*⁸.

Overview of the method

Large-scale pairwise gene knockdown in human cells is induced by combined transfection of two siRNAs, each targeting one gene. For each targeted gene, two distinct, nonoverlapping siRNAs are assayed, resulting in four siRNA combinations for each gene-pair tested. Phenotypes after knockdown are analyzed by staining with markers of DNA and cell morphology, followed by automated imaging in three channels. By using image analysis, over 100 descriptors are extracted, representing quantitative phenotypes. The resulting multivariate phenotype profiles are analyzed computationally to identify genetic interactions.

Here we describe the detailed protocol that we used for a genetic interaction screen of all pairwise combinations of 323 genes against 20 genes in the human colorectal cancer cell line HCT116 (ref. 8). For the larger gene set—the ‘long’ side of the interaction matrix, which we call target genes—we selected a set of chromatin

regulators, including chromatin-modifying enzymes and chromatin-binding proteins; chromatin biology is rich in molecular and genetic interactions⁶. For the smaller set—which we term query genes—we selected a subset of 20 genes from the targets, on the basis of the observation that a suitable selection of queries can substantially reduce experiment size compared with a full quadratic design, while providing most of the information content⁹. With two independent siRNA designs for each gene, our experiment comprised 80 384-well plates, and was performed in two biological replicates (160 plates). The workflow of the procedure is shown in **Figure 1**.

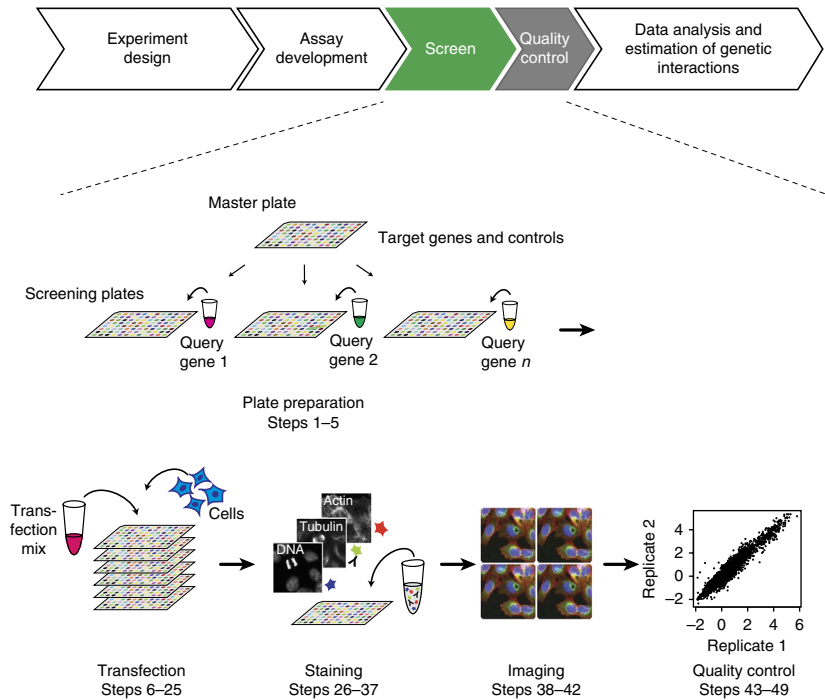
Advantages and limitations of the method

An advantage of this method is its multiparametric imaging read-out. Analyzing multiple shape, morphology and cell cycle-related phenotypes detects more interactions, and possibly more specific interactions, compared with analyzing only measures of overall cell viability (indicated, e.g., by the total cell number, or total ATP or DNA content). Previous analyses of genetic interactions in yeast have focused on cell fitness, which represents the integrated output of many cellular processes and is simple to assay. However, many interactions we found were specifically detected in certain phenotypes, reflecting that interactions between genes manifest themselves not only through cell growth and survival but may also show more immediate and more direct effects, e.g., on cell morphology or the arrangement of intracellular structures.

We recommend screening with individual siRNA reagents, using at least two independent siRNAs per target gene. In this way, potential nonspecific or off-target effects of siRNAs can be detected if the data resulting from the two siRNAs are discordant. We assay each pair of genes with all four combinations of the two designs, which allows for an assessment both of systematic siRNA-dependent biases and of experimental noise.

Other methods have been proposed for measuring genetic interactions in mouse or human cells, using endonuclease-prepared small interfering RNAs (esiRNAs) or a pooled shRNA format for pairwise gene knockdown^{10,11}. All currently used approaches have divergent advantages and disadvantages regarding scalability, the

Figure 1 | Workflow of a genetic interaction screen with combinatorial RNAi and automated imaging.



complexity of phenotypic information and are therefore suitable for complementary genetic interaction screening approaches¹². The protocol we developed can, in principle, be applied on a genome-wide scale. However, there are certain limitations in scalability, as the costs of imaging increase with experimental size. In addition, microscopy is a more time-consuming readout than, e.g., sequencing as used in a pooled shRNA screening approach, but in contrast it generates information on genetic interactions across many phenotypes. Depending on the staining and imaging parameters, time might also be a limiting factor in the scalability of this approach.

Experimental design

The procedure described here was developed for HCT116 cells and fit to the size and composition of the gene set we had chosen. For screens with different cell types, phenotypes, staining methods or gene set choices, some of the steps will require adaptation. In that case, we recommend thorough testing of each individual step to ensure that the overall procedure works reliably. The following section highlights points to be considered when planning a large-scale genetic interaction screen. In addition to staining for specific proteins or cellular structures of interest (actin, tubulin or others), we recommend always staining the nucleus (e.g., Hoechst or DAPI) to facilitate cell counting and segmentation.

Plate design. We achieved good results by using a target-query design in which the target gene siRNAs and all control siRNAs were arrayed in two master template plates, one plate for each target siRNA set (Fig. 2a). The siRNAs on the master template plates were then aliquotted into assay plates by a liquid handling robot as many times as there were query siRNAs (and negative control siRNAs; see ‘Selection of control siRNAs’). Each query gene siRNA (or negative control siRNA) was then added to all wells (except some negative and all positive control wells; see ‘Selection of control siRNAs’) on the assay plates (Fig. 2b). In this way, each plate provides one column of data for the interaction matrix. Target gene siRNAs and controls (if possible) should be arrayed in (quasi) random order, and genes with similar functions should be spatially separated in order to avoid confounding effects of interest with spatial gradients or plate effects. In particular, one should avoid ordering the genes by name, because genes with similar names often belong to the same complex or pathway (e.g., ribosome or proteasome components), and thus they frequently show similar phenotypes (some commercial siRNA libraries are sorted by gene name). In third-party libraries, the flexibility of the layout is usually limited, with only certain rows or columns reserved for controls. When placing the controls, keep in mind potential plate or edge effects and avoid placing controls in the

outermost rows and columns. Furthermore, controls should be distributed across the plate, and replicates of controls are more informative if they are in nonadjacent wells. In practice, positive controls (which are screened without combination with the query siRNA) usually need to be placed in the same column to facilitate pipetting.

Selection of siRNAs. Commercially available siRNA libraries typically comprise several siRNAs targeting each gene. Although this increases the screen size, we recommend working with at least two independent siRNAs per gene, applied individually (rather than in pools) even at the primary phase of a genetic interaction screen⁸. In this manner, the four distinct combinations per gene-pair can provide experimental replication and opportunity to detect incongruous results and help avoid excessive effort on follow-up experimentation with false positives. In Laufer *et al.*⁸, the vendor’s library that we used provided three siRNAs for each gene; we selected two of the three siRNAs based on matching their knockdown performance, which we measured by quantitative PCR (qPCR).

Selection of control siRNAs. A good choice of control siRNAs greatly improves the chances of success for an RNAi experiment. Negative controls serve to reveal unspecific effects caused by the transfection procedure or by the presence of siRNAs in cells. For this purpose, siRNAs that do not target a known transcript are available; they should match in all parameters (especially concentration and sequence length) the siRNAs that target the gene set of interest. Positive controls serve to control transfection and incubation efficiency throughout the screen. We recommend not only using positive controls with strong viability effects but also using multiple different positive controls, including ones with intermediate effects and with effects on specific phenotypes of interest. Ideally, the positive controls should cover the full range of effect

Figure 2 | Design and pipetting of master and screening plates. (a) Two master template plates are prepared; each one comprises one individual set of target siRNAs sequences (blue). Negative (yellow) and positive control siRNAs (red) are included on both plates. For technical reasons, the controls that will be transfected alone (not in combination with the query siRNA) are placed in one column. Empty wells (gray) are placed in the first row and last column. (b) After aliquotting target siRNAs into assay plates, query siRNA is added to columns 1–22. Thus, each combination of the target gene set with one query gene results in four assay plates.

sizes and phenotypes of interest. In Laufer *et al.*⁸, we chose four positive control genes (checkpoint kinase 1 (*CHEK1*), coatomer protein complex, subunit β -2 (*COPB2*), polo-like kinase 1 (*PLK1*) and ubiquitin c (*UBC*)) that are known to affect cell viability to different degrees and through different mechanisms.

The control siRNAs should be spotted on every plate (i.e., they should be included on the master plate in which target gene siRNAs are spotted—see ‘Plate design’ section). In this way, one can control the quality of the screen over time. If necessary, the controls may also serve to adjust the readout data for drifts and biases (‘normalization’). In general, we recommend using several wells/plate for each control siRNA. Note that control siRNAs are prevalidated and therefore only one siRNA (or siRNA pool) is required per control gene. We recommend that each positive control siRNA be present in at least two wells per plate. Each positive control siRNA should be transfected as a single siRNA targeting an individual gene (single gene knockdown); i.e., when transferring query siRNA to the target gene siRNA wells on each screening plate, no query siRNA should be added to the positive control wells.

A set of nontargeting negative control siRNAs also needs to be included for accurate measurement of genetic interactions. Each negative control siRNA is transfected in combination with each query siRNA and each target siRNA to measure their individual knockdown effect. We recommend that the effect of each individual query siRNA is measured by co-transfection with a nontargeting negative control siRNA at least four times. In addition, at least four wells per plate should be reserved for transfection of only nontargeting control siRNA. To assess the single knockdown effects of each target gene siRNA, an additional screening plate can be prepared, which will be queried with the nontargeting control siRNA; this assumes that one screening plate for each target gene siRNA and two negative control siRNAs, four additional screening plates will be required per biological replicate.

Replicates. We recommend screening at least two biological replicates. Replicates allow for quality control both at the level of the whole screen and of individual observations (see ANTICIPATED RESULTS); these benefits outweigh the additional effort required. For each replicate, make sure to thaw a fresh batch of cells that ideally differ somewhat in time point and passage of freezing, and to use freshly prepared media. Given that the time required to screen a technical replicate is comparable to the time necessary to screen another biological replicate, we recommend screening additional biological replicates.

Screen planning. When setting up a large-scale screen, small details can influence the outcome of the experiment. Thorough planning is crucial for success. A detailed schedule helps identify and avoid bottlenecks regarding equipment or materials.

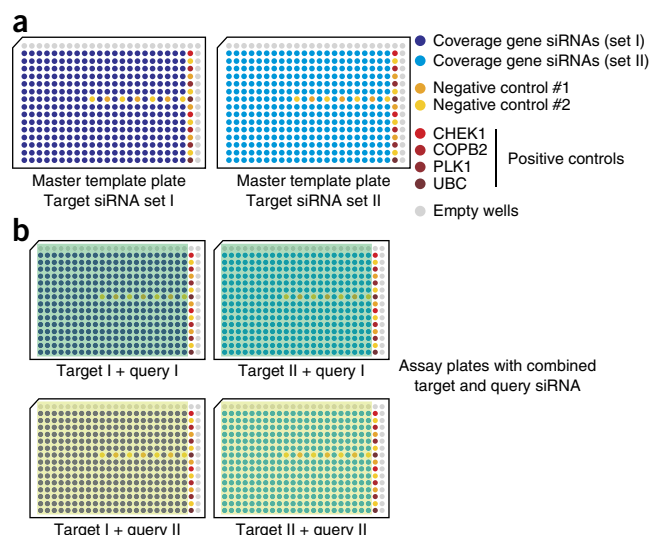


Figure 3 shows the schedule for the screen described in the PROCEDURE. Depending on the screen size and on the equipment used, the screen may need to be split into several batches. For example, in our genetic interaction screen, the transfection was conducted in three batches (27–27–26 plates). The amount of plates per batch here was limited by the transfection protocol and the incubation time of siRNAs and transfection reagent before cell seeding. Similarly, the amounts of required reagents and equipment need careful planning, especially regarding materials that are not usually present in the laboratory at the required amounts. List everything that will be needed throughout the screening procedure (for all replicates), and plan some excess for everything that cannot be replaced instantly. To reduce technical variation between biological replicates, prepare all storable reagents and solutions for both replicates together. If it is not possible to prepare the total amount in one flask, prepare in separate flasks, mix both in a large vessel after preparation and re-distribute into separate flasks.

Plate preparation (Steps 1–5). When distributing the siRNAs into the assay plates, accuracy is crucial to avoid technical variation between plates. For this, automated pipetting is preferred. This implies that plate preparation has to be done in a nonsterile environment. To avoid contamination, spray down all equipment with 70% (vol/vol) ethanol, cover the plates at all times except when they are being filled and avoid rapid movement near open plates. Taking these precautions, we found only ~10 out of >27,000 wells contaminated in each replicate of our screen. For the target gene siRNA set, we recommend distributing the siRNAs (including controls) into 384-deep-well plates and transferring them into the assay plates using a liquid handling robot such as the Biomek FX (master plate generation and aliquotting). For the distribution of the siRNAs targeting the query genes, we used a NanoDrop II, which takes up large amounts of given solutions from a reservoir and precisely spots small volumes into multiwell plates (query gene siRNA distribution).

Transfection (Steps 6–25). The transfection procedure follows a reverse transfection protocol¹³. The siRNAs are plated first; this way, the most laborious pipetting steps can be done independently and before screening. Next, the transfection

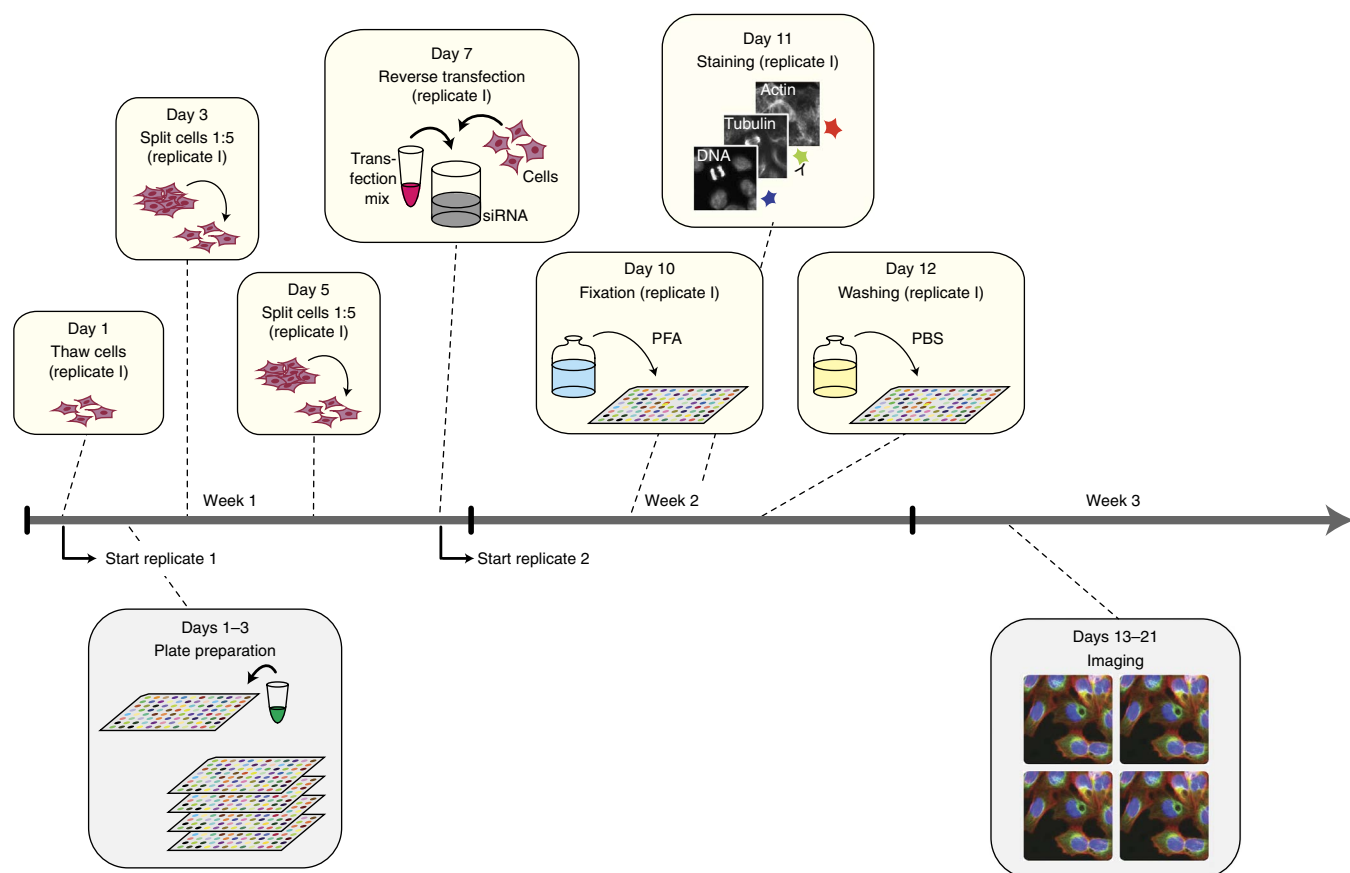


Figure 3 | Day-by-day schedule of the genetic interaction screen performed over 3 weeks.

reagent is added to the siRNAs using a multidrop dispenser and incubated with the siRNAs according to the manufacturer's instructions. During this incubation the cells are trypsinized, counted and diluted. After incubation, the cells are seeded using the multidrop dispenser. Proper cell treatment before screening is important for screening success. We recommend thawing fresh cells and passing them twice such that they grow dense but do not enter the plateau phase (this is dependent on the cell line used). In the last passage before the screen, perform several cell dilutions to ensure perfect conditions on the day of transfection. For example, HCT116 cells are passaged 1:5 every 2 d, and the last passage should include 1:5 and 1:6 dilutions. As these cells are small, one T75 flask usually produces enough cells for one transfection batch (26 or 27 384-well plates) described here. However, the exact transfection, cell treatment and expansion procedures depend very much on the cell type used, and they will require thorough testing and adaptation. In addition, the amount of cells seeded will vary with cell size and growth behavior. After 3 d of knockdown, cells transfected with nontargeting siRNA should have grown dense without reaching the plateau phase. In this way, sufficient dynamic range is available to detect phenotypic effects in both directions (decreased and increased viability). HCT116 cells are small but they grow fast, so seeding 1,750 cells per well of a 384-well plate ensures optimal cell density after 3 d.

Fixation (Steps 26–31). After 3 d of incubation with siRNAs, cells are fixed with paraformaldehyde (PFA). Cells are first washed once with PBS, and subsequently fixation and permeabilization

are performed in one step. We achieved good results with an incubation time of 45 min, resulting from the batch-wise processing of plates: using the Biomek FX, removing PBS and adding fixation solution takes ~10 min for ten plates. Including additional time to exchange solutions, handling one batch takes ~40 min. Therefore, to ensure equal timing for all batches, we set the incubation time to 45 min. However, this timing may be varied according to the desired protocol. When a different cell line is used, the protocol may have to be tested and modified. For example, HCT116 cells strongly attach to the plates, which allows a washing step before fixation. If the desired cell type detaches easily upon mechanical stimulation, fixation without a prior washing step might be an option. In addition, when performing a smaller screen, it is possible to continue directly with the staining procedure after washing out the fixation solution.

Staining (Steps 32–37). The timing of the staining protocol described here was adapted to a high-throughput screen of a certain size. After blocking unspecific antibody binding, all three staining reagents (Hoechst, FITC-conjugated α -tubulin antibody, TRITC-conjugated phalloidin) are combined in the staining solution for one single staining step. Staining is performed overnight at 4 °C to reduce staining background. Furthermore, the subsequent washing procedure takes ~6 h, so incubation overnight relaxes the schedule. Again, this procedure can be adapted as some staining reagents might not require a blocking step, or staining might give better results when performed at room temperature (21–23 °C) for a short time period.

Imaging (Steps 38–42). Depending on the available plate handling system and the data storage solution, a screen may be imaged completely in one run or split into batches. Microscopy settings, including magnification, image sites, offset and illumination time should be determined before screening along with setting up the staining procedure. For example, antibody dilution (a cost factor) will influence illumination time and therefore total imaging time. When starting the imaging procedure either for the whole screen or for a batch, the determined settings should be verified using at least four plates that were not processed consecutively, e.g., the first and the last plate of the batch and some in between. Always check four or five wells that are distributed across the plates, as conditions can vary with position. When setting the illumination time, quantitatively check the image histogram, not only the preview picture. Make sure that gray values are well distributed and the foreground can be separated from the background, but allow for brighter images that can appear throughout the screen.

The amount of images taken per well defines the amount of data that need to be stored and analyzed. We imaged HCT116 cells at 10× magnification in three channels and with four image sites covering each well fully⁸. Thus, with 12 images per well and 8.4 MB per image, our screen produced 5.6 TB of image data, captured on three 2-TB hard drives.

Imaging HCT116 cells at 10× magnification allows for the resolution of intracellular structures while allowing a sufficiently large number of cells to be imaged. We have found the number of cells imaged to be a crucial factor for genetic interaction analysis. As HCT116 cells are relatively small, we imaged, on average, ~7,000 cells per experimental condition, and this number was crucial for detection sensitivity⁸. Data subsampling simulations indicated drastic loss of statistical power if smaller numbers of cells are analyzed⁸. When screening with larger cells, the sufficiency of number of cells imaged should be carefully evaluated.

MATERIALS

REAGENTS

- Cell line of choice (American Type Culture Collection or other supplier). The PROCEDURE as written has been optimized for HCT116 cells
- siRNAs (Ambion Silencer Select, Dharmacon or other suppliers)
- Ethanol (Sigma-Aldrich, cat. no. 32205)
- BSA type H1 (Gerb, cat. no. 1063,0100)
- DharmaFECT 1 transfection reagent (Thermo Scientific, cat. no. T-2001)
- DMSO (Genaxxon, cat. no. M6323)
- FBS (Biocrom, cat. no. S0115)
- Hoechst 33342 (Life Technologies, cat. no. H1399)
- KCl (Sigma-Aldrich, cat. no. P9541)
- KH_2PO_4 (Sigma-Aldrich, cat. no. P9666)
- McCoy's 5a modified medium (Gibco by Life Technologies, cat. no. 26600-023) or another medium suitable for the cells in use
- NaCl (Sigma-Aldrich, cat. no. 31434)
- Na_2HPO_4 (Sigma-Aldrich, cat. no. S0876)
- Paraformaldehyde (PFA; Sigma-Aldrich, cat. no. P6148) **! CAUTION** PFA is toxic; use skin and eye protection. Handle the powder under a fume hood. Dispose of it as a hazardous chemical.
- PBS for cell culture (Gibco by Life Technologies, cat. no. 10010-015)
- Phalloidin, TRITC-conjugated (Sigma-Aldrich, cat. no. P1951)
- **! CAUTION** Phalloidin is highly toxic. Use skin and eye protection.
- RPMI 1640 (Gibco by Life Technologies, cat. no. 31870-025)
- Sodium azide (Sigma-Aldrich, cat. no. 71290) **! CAUTION** Sodium azide is toxic; use skin and eye protection.
- Triton X-100 (Sigma-Aldrich, cat. no. T8787)
- Trypsin-EDTA, 0.25% (wt/vol) (Gibco by Life Technologies, cat. no. 25200-056)
- Tubulin antibody (mouse monoclonal, clone DM1 α), FITC-conjugated (Sigma-Aldrich, cat. no. F2168)
- CASYton solution (Roche Applied Science, cat. no. 05651808 001)

EQUIPMENT

- Multichannel pipette, Finnpiptet (Thermo Scientific)
- Multichannel pipette tips, Finntips (Thermo scientific)
- Stericup-GP filter units, 0.22 μm , 500 ml (Millipore, cat. no. SCGPU05RE)
- Cell culture flasks T75 with filter cap (BD Falcon, cat. no. 353136)
- Cell culture flasks T75 and T175 (Greiner, cat. nos. 660160 and 658170)
- Serological pipettes, 5, 10, 25 and 50 ml (BD Falcon)
- Pipetboy (Integra)
- CASY model TT cell counter and analysis system (Innovatis)
- CASY cups for cell counter (Roche Applied Science, cat. no. 05651794001)
- 96-deep-well storage plates (Thermo Scientific, cat. no. AB-0661)
- 384-deep-well plates (Greiner, cat. no. 781270)
- 384-well cell culture plates, black, clear bottom (BD Falcon, cat. no. 353962)
- Plastic lids for multiwell plates (Greiner, cat. no. 656161)
- Centrifuge for tubes and plates (Eppendorf)

- Liquidator 96 pipetting system (Mettler Toledo)
- Biomek FX workstation with 384-tip head (Beckman Coulter). Programs used in the PROCEDURE are detailed in **Box 1**
- Biomek FX AP384 P30XL 50- μl tips (Beckman Coulter, cat. no. A22288)
- Innovadyne NanoDrop II high-precision dispenser
- PlateLoc Velocity 11 heat sealer (Agilent Technologies)
- Multidrop dispenser, type 836 (Thermo Scientific, cat. no. 5840300)
- Cassettes for multidrop dispenser
- Screening racks for multiwell plates
- InCell Analyzer 2000 with wide-field camera (GE Healthcare)
- KINExd scara plate handling robot (PAA)
- Data mass storage >6 TB
- Multiprocessor computer server (e.g., a cluster computer with 128 CPUs and a minimum of 4 GB of RAM per CPU)

REAGENT SETUP

FBS For heat inactivation, incubate FBS at 56 °C for 30 min. Prepare 50-ml aliquots and store them at –20 °C. Do not exceed storage times recommended by the manufacturer.

Growth medium Supplement McCoy's 5a medium (or other, as necessary) with 10% (vol/vol) FBS. Filter the medium through a 0.22- μm filter and store it at 4 °C. Do not exceed storage times recommended by the manufacturer.

PBS, 20× Mix to a final concentration of 2.74 M NaCl, 54 mM KCl, 200 mM Na_2HPO_4 and 40 mM KH_2PO_4 . Prepare the solution with molecular biology-grade water. Dilute it to 1× PBS for use in preparation of fixation, blocking and staining solutions and for all washing steps during fixation and staining. Store it at room temperature for 1 year.

Fixation/permeabilization solution Dissolve 5% (wt/vol) PFA in PBS (stir it overnight, and heat it to 65 °C if necessary). Add 0.2% (vol/vol) Triton X-100 and fill it up with PBS to the end volume; mix the solution and distribute it into flasks. Store the solution at 4 °C protected from light for 6 months. **! CAUTION** PFA is toxic. Wear skin and eye protection and use a fume hood when preparing this solution.

Blocking solution Mix 3% (wt/vol) BSA and 0.05% (vol/vol) Triton X-100 in PBS. Prepare sufficient solution for the complete experiment (including staining solution); mix the solution and distribute it into flasks. Store the solution at 4 °C protected from light for 6 months.

Staining solution, 2× Dilute Hoechst 33342 (10 mg/ml stock solution) 1:2,500, phalloidin-TRITC (0.1 mg/ml stock solution in DMSO) 1:750 and α -tubulin-FITC antibody 1:375 in blocking solution. Staining solution should be prepared freshly each day and stored at 4 °C protected from light until use. **! CAUTION** Phalloidin is highly toxic. Wear skin and eye protection. Use a fume hood when dissolving phalloidin in DMSO.

Box 1 | Biomek FX Workstation programs used in the PROCEDURE

Program 1 (aliquotting)

- Load the tips.
- Mix the source three times by aspirating (–4 mm from liquid, 100 µl/s) and dispensing (–5 mm from liquid, 100 µl/s) 30 µl.
- Aspirate 17 µl from the source (2 mm from bottom, 90 µl/s) and dispense 2.5 µl to plates 1–5 (0.8 mm from bottom, 90 µl/s). Touch the wells with tips to avoid drop formation on tip ends. Empty the tips in the source.
- Repeat the previous step with plates 6–10.
- Unload the tips.

Program 2 (washing, fixation/permeabilization and blocking)

- Load the tips.
- Aspirate 50 µl from plates 1–10 (0.2 mm from liquid, follow liquid, 5 µl/s), and dispense to waste.
- Wash the tips three times by aspirating and dispensing 50 µl of water at the washing station.
- Aspirate 50 µl from the source (plastic container), and dispense it to plates 1–10 (2 mm from bottom, 5 µl/s).
- Wash the tips three times.
- Unload the tips.
- At the first washing step before fixation, the volume per well is only 50 µl. Remove only 40 µl to leave 10 µl in the wells.

Program 3 (staining)

- Load the tips.
- Aspirate 50 µl from plates 1–10 (0.2 mm from liquid, follow liquid, 5 µl/s), and dispense to waste.
- Wash the tips three times by aspirating 50 µl of water at the washing station.
- Aspirate 10 µl from 384-deep-well plate (0.2 mm from liquid, follow liquid, 5 µl/s) and dispense it to plates 1–10 (0.1 mm from liquid, follow liquid, 4 µl/s).
- Wash the tips three times.
- Unload the tips.

EQUIPMENT SETUP

Multidrop dispenser Use a standard cassette. Before use, flush the cassette with 70% (vol/vol) ethanol, leave the ethanol in the tubing for 5 min and flush it thoroughly with RPMI 1640 medium. For transfection (Step 16), set the dispensing speed to ‘high’ and the volume to 15 µl. Define the columns used. Prime the tubing with transfection mix. Avoid bubble or drop formation on the tips, as this will result in uneven distribution of transfection mix. After dispensing, flush the tubing with RPMI 1640 medium and release the tubing until the next use. For cell

seeding (Step 23), set the dispensing speed to ‘low’ and the volume to 30 µl. Prime the tubing with the prepared cell suspension. Avoid the formation of bubbles or the formation of drops on the outer side of the tips.

CASY cell counter To count HCT116 cells, add 25 µl of cell suspension to 10 ml of CASYton solution. Set up the counter to the 1:400 cell dilution option, define three counting steps and set the threshold for viable cells between 9.60 and 30.00 µm.

PlateLoc heat sealer Set the temperature to 180 °C and sealing time to 1.8 s. Use the 290 metal pad.

PROCEDURE

Plate preparation for two biological replicates ● TIMING 3 d

▲ **CRITICAL** Although it is possible to split the plate preparation procedure and store the plates, processing the plates on subsequent days is recommended to avoid multiple freeze-thaw cycles of siRNAs.

▲ **CRITICAL** To avoid contamination, spray down all equipment with 70% (vol/vol) ethanol, cover the plates at all times, except when they are being filled, and avoid rapid movement near open plates.

- 1| Dilute all siRNAs to 200 nM.
- 2| Pipette target gene siRNAs into four 96-deep-well plates, 250 µl of each siRNA per well. Plan patterns according to the desired outcome on 384-well screening plates.
- 3| Transfer the target gene siRNAs and controls to a 384-well master plate using the Liquidator pipetting system, 110 µl per well. siRNAs are transferred in a way that, e.g., the siRNAs in the four individual wells A01 on the four 96-well master plates are pipetted into the adjacent wells A01, A02, B01 and B02 on the 384-well master plate (and on the screening plates after aliquotting). Prepare two 384-well master plates for each set of individual siRNAs targeting the gene set (one plate for each of the two target gene siRNAs; the number of 384-well master plates will depend on the size of the screen). Seal the master plates and store them at 4 °C.
- 4| Transfer the target gene siRNAs from the master plate(s) into the black, clear-bottom 384-well screening plates, 2.5 µl per well, using the Biomek FX Workstation with program 1 (Box 1). Seal the plates and store them at 4 °C overnight.

5| Add the query gene siRNAs to the screening plates using the NanoDrop II dispenser, 2.5 µl per well. Seal the plates and store them at -20 °C. If the first replicate experiment is started on the next day, store the plates for replicate I at 4 °C.

■ **PAUSE POINT** If required, the prepared assay plates can be stored at -20 °C for up to 2 months.

Cell thawing ● **TIMING 15 min**

▲ **CRITICAL** Steps 6–36 describe the processing of one biological replicate. Transfection is carried out in batches; here we used three batches of 27, 27 and 26 screening plates each.

6| Prewarm the growth medium to 37 °C.

7| Quickly thaw the cells in a 37 °C water bath.

8| Add the cells to 10 ml of growth medium and mix carefully. The number of cells required will depend on the cell line (see Experimental design).

9| Spin down the cells for 5 min at 300g at room temperature.

10| Carefully aspirate the medium. Resuspend the cells in 10 ml of McCoy's 5a medium and put the cell suspension in a T75 cell culture flask with vented cap. Incubate the cells at 37 °C, 5% CO₂.

Cell propagation ● **TIMING 6 d from cell thawing**

11| 2 d after thawing, remove the growth medium from the cells and briefly wash the cells with 10 ml of PBS.

12| Replace the PBS with 2 ml of 0.25% (wt/vol) trypsin-EDTA and incubate the cells for 5 min at room temperature.

13| Add 8 ml of growth medium to the flask to stop the enzymatic reaction.

14| Put 8 ml of fresh growth medium into a new T75 flask; add 2 ml of cell suspension for a 1:5 cell split.

15| Repeat Steps 11–14 after 2 d, 2 d before the day of transfection. Per transfection batch, prepare one T75 flask with a 1:5 dilution and one with a 1:6 dilution, both in a total volume of 15 ml of growth medium.

Reverse transfection (batch-wise) ● **TIMING 3 d, including incubation time**

16| Take the siRNA screening plates for batch 1 (from Step 5) out of the cold room and spin them down briefly. Remove the seals under the cell culture hood and cover the plates with plastic lids. Allow the plates to reach room temperature during this step.

17| Prepare transfection mix in a tube, calculating 0.05 µl of DharmaFECT 1 in 4.95 µl of serum-free RPMI medium per well, and incubate it for 10 min at room temperature.

▲ **CRITICAL STEP** To account for pipetting errors, always calculate 400 wells per 384-well plate when preparing transfection mix, and also prepare 10–15 ml of additional transfection mix to account for the multidrop dispenser's dead volume and priming.

18| Add an additional 10 µl per well of serum-free RPMI medium to the tube from Step 17, and mix by carefully inverting the tube. Transfer 15 µl per well of this transfection mix to the siRNAs in the screening plates using the multidrop dispenser. Incubate for 30 min at room temperature. During the incubation of transfection mix and siRNAs, prepare the cells (from Step 16) for plating, as described in Steps 19–22.

? **TROUBLESHOOTING**

19| Aspirate the growth medium from the plates from Step 16 and wash the cells briefly by adding 10 ml of PBS to the flask to remove serum. Replace the PBS with 2 ml of 0.25% (wt/vol) trypsin-EDTA and incubate for 5 min at room temperature.

20| Stop trypsinization by adding 8 ml of growth medium. Carefully pipette the cell suspension up and down eight to ten times to separate the cells. Check cell separation by visual inspection.

▲ **CRITICAL STEP** Avoid bubble formation in the cell suspension.

PROTOCOL

21| Count the cells on the CASY cell counter. Perform three CASY cleaning cycles before counting. Count three independent replicates and calculate the average.

22| Per well, prepare a cell suspension with 1,750 cells in 30 μ l of growth medium.

▲ CRITICAL STEP To account for pipetting errors, always calculate 400 wells per 384-well plate when preparing cell suspensions, and also prepare 10–15 ml of additional transfection mix to account for the multidrop dispenser's dead volume and priming.

23| After 30 min of incubating the transfection mix and siRNAs (Step 18), seed the cells onto the plates with the multidrop dispenser at low speed.

▲ CRITICAL STEP When handling the plates during transfection, keep them covered with plastic lids at all times, except when dispensing the transfection mix and cell suspension. Avoid moving anything over the open plates.

? TROUBLESHOOTING

24| Seal the plates and place them in screening racks and put them in the incubator. Note the time of the start of incubation. Incubate the cells at 37 °C, 5% CO₂ for 3 d.

25| Repeat Steps 16–24 with the remaining batches of screening plates.

Fixation/permeabilization ● TIMING 6 h (2 h per batch)

26| Take the first batch of screening plates out of the incubator.

27| Remove the plate seals and observe each plate for contamination of wells. Wash the plates using the Biomek FX workstation with 50 μ l of PBS per well with program 2 (**Box 1**), ten plates at a time.

? TROUBLESHOOTING

28| Remove the PBS and add 50 μ l per well of fixation/permeabilization solution using program 2, ten plates at a time. Incubate each plate at 37 °C for ~45 min while processing the rest of the batch.

! CAUTION PFA is toxic. Wear skin and eye protection. Dispose of PFA-containing waste as hazardous chemical.

29| 45 min after adding fixation/permeabilization solution to the first plate, start washing with 50 μ l of PBS per well using program 2, ten plates at a time.

30| Wash again with PBS, ten plates at a time. Seal the plates and store them at 4 °C.

31| Repeat Steps 26–30 with the remaining batches of screening plates.

■ PAUSE POINT The plates can be stored at 4 °C until staining for up to 2 weeks.

Staining ● TIMING 5 h (plus overnight incubation)

32| Prepare the staining solution for all batches of screening plates. Prepare master plates for staining by pipetting 170 μ l of staining solution into each well of a 384-deep-well plate. One master plate contains staining solution for 16 plates (from Step 30).

33| Remove the PBS from the plates (from Step 30) and add 50 μ l per well of blocking solution using program 2, eight plates at a time. Incubate the plates for 30 min at room temperature.

34| Remove the blocking solution and add 10 μ l per well of 2× staining solution using program 3 (**Box 1**).

35| Seal the plates and store them at 4 °C overnight.

36| Repeat Steps 32–35 with the remaining batches.

Processing the second biological replicate ● TIMING 12 d

37| Repeat Steps 6–36 for the second biological replicate (from Step 5).

Imaging ● TIMING 8–9 d

38| Calculate the data storage space needed for the images, and calculate how many plates can be imaged until one hard drive is filled. Split the plates into batches accordingly.

39| Allow the plates (from Step 35) to reach room temperature.

40| Clean the outer side of the plate bottoms with 70% (vol/vol) ethanol and lint-free paper towels.

41| For each batch, take some plates (e.g., first, last and some in between) and fine-tune the microscopy settings. Place the plates in a plate stacker and set the plate handling robot according to the manufacturer's advice.

? TROUBLESHOOTING

42| Start automated imaging, according to the manufacturer's instructions. Control the process regularly to detect any focussing issues.

? TROUBLESHOOTING

Image processing and data analysis ● TIMING 14 d

▲ **CRITICAL** A complete data analysis workflow in the form of an executable or 'reproducible' document is provided in the R/Bioconductor¹⁴ package HD2013SGI (ref. 8; <http://www.bioconductor.org/packages/release/data/experiment/html/HD2013SGI.html>). The workflow includes all required steps including image segmentation, feature extraction, statistical analysis and the calling of genetic interactions.

43| Perform cell segmentation and feature extraction using the R/Bioconductor software EImage¹⁵ and imageHTS (<http://www.bioconductor.org/packages/release/bioc/html/imageHTS.html>). Summarize single-cell features by taking the mean (and optionally, other statistics) over all cells in each well to obtain a per-experiment feature vector. For a fully detailed description, refer to Section 4 ('Image segmentation and feature segmentation') of the HD2013SGI vignette.

▲ **CRITICAL STEP** Depending on the staining and cell type, this step will need adaptation, for which adequate effort and time should be budgeted.

? TROUBLESHOOTING

44| Check the extracted features for plate effects (**Fig. 4a**). Feature values should be distributed evenly across the plates. Any visible pattern, such as a spatial gradient or extreme values concentrated at the edges, probably reflects technical issues during screening (e.g., less cell growth in the plate edges) and uneven distribution of transfection mix or cell suspension during transfection. If necessary, spatial gradients can be removed computationally, e.g., with the R/Bioconductor software cellHTS2 (ref. 16; <http://www.bioconductor.org/packages/release/bioc/html/cellHTS2.html>).

? TROUBLESHOOTING

45| Perform further feature quality control. Depending on each feature's distribution of values, feature transformation can be appropriate, for instance, by taking the logarithm of the feature values (see the HD2013SGI vignette, Section 6.2, 'Transform features and screen normalization'). Features should have the properties of a continuous measure (i.e., a feature's range should not be limited to a small set of discrete values). Features need to be controlled for reproducibility across biological replicates (**Fig. 4b**). We recommend using only features with a correlation coefficient (r) of more than 0.6 between replicates (**Fig. 4c**; HD2013SGI vignette, Section 6.3, 'Quality control of features').

? TROUBLESHOOTING

46| Perform quality control for each pair of individual siRNAs targeting the same gene. The features observed after knockdown of each of the two siRNAs should be highly similar to each other. We recommend only accepting genes for which the phenotypic profiles of both siRNAs are correlated, with $r > 0.7$ (**Fig. 4d**). Depending on the level of variation in the screening data, the achievable correlation can vary, and the threshold may need to be adapted. Noncorrelation of the phenotype profiles of an siRNA pair might reflect nonspecific effects, or lack of effect, of one or both siRNAs; conversely, high correlation between two siRNAs across many different features and query genes is only possible when both siRNAs have the same strong effect, and it can indicate that the effect is target-specific.

? TROUBLESHOOTING

47| Estimate the main effects of single siRNAs (single knockdown effects). By following ref. 17, we fit the model

$$d_{ijk} = m_i + n_j + g_{ijk} \quad (1)$$

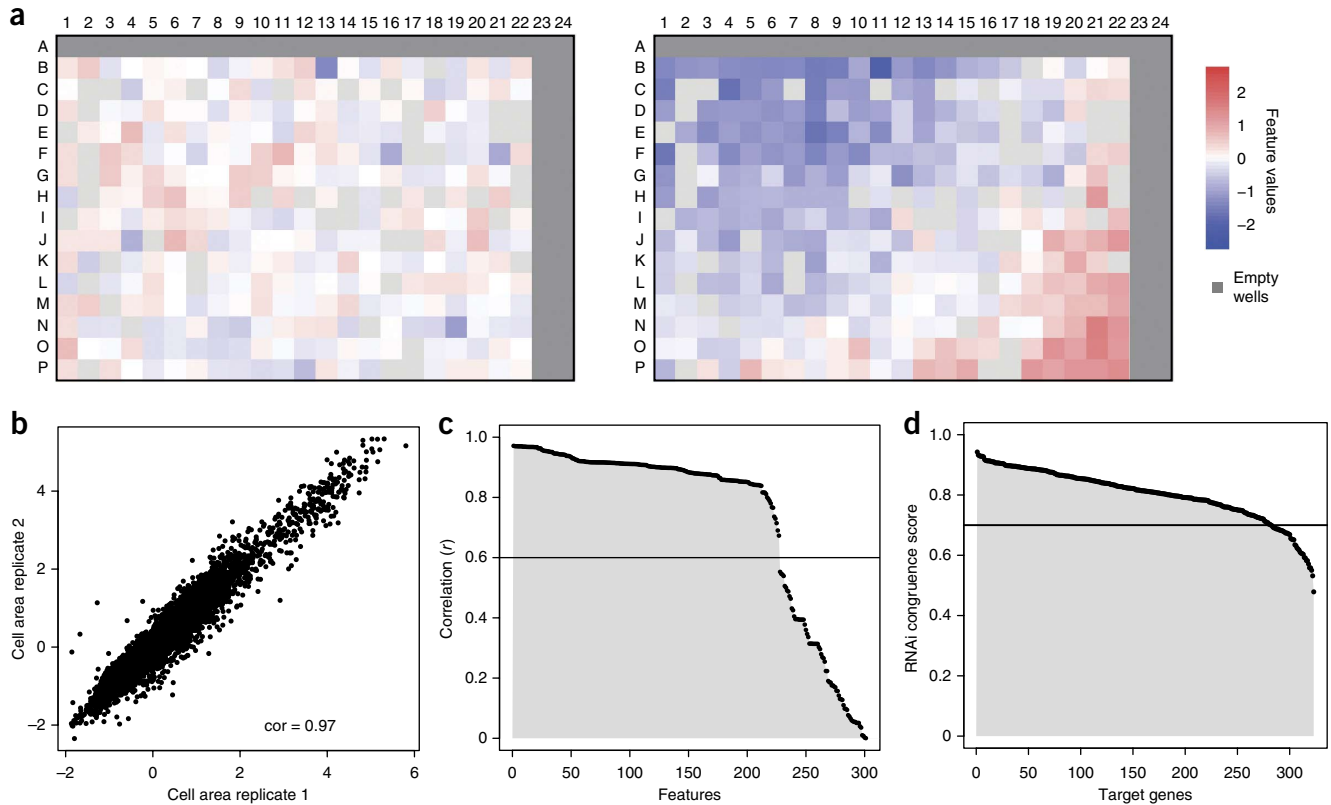


Figure 4 | Quality control for screen readout, extracted features and siRNAs. **(a)** Exemplary plate plots of raw data to illustrate potential plate effects. Each well is colored according to the per-experiment value of the given feature. The left plot shows an example with no visible spatial trends. In the right plot, a strong gradient is visible, with low values concentrating in the upper left corner of the plate and high values concentrating in the lower right corner. Such spatial effects can reflect technical issues during screening; in mild cases, they can be computationally adjusted for, and in other cases they necessitate data removal or repetition of the measurement. **(b)** Example of high correlation of the feature ‘cell area’ between two biological replicates. **(c)** Correlation coefficients (r) between biological replicates of all extracted features. We recommend setting a threshold of $r > 0.6$ and excluding features with lower correlation from subsequent analysis. Figure from ref. 8, Nature Publishing Group. **(d)** Correlation coefficients (r) of phenotypic profiles (RNAi congruence score) between siRNA pairs targeting the same gene. We recommend excluding from the analysis genes with siRNA pair correlation of $r < 0.7$. Figure from ref. 8, Nature Publishing Group.

The single knockdown effects m_i and n_j are estimated from the screening data (for each individual target gene siRNA i and query gene siRNA j) by minimizing a suitable norm of the tensor g_{ijk} . When the screen contains a large proportion of target and query gene siRNAs that show negligible effects, the target and query siRNA main effects can be estimated by the row and column medians of the matrix obtained by averaging d_{ijk} over the replicates k . When target or query gene siRNAs contain many genes with measureable knockdown effects, the estimation of the main effects needs to be restricted to the subset of data corresponding to nontargeting control partners. If batch or plate effects are notable, the estimates can be made separately per batch or per plate. The earlier-mentioned choices imply trade-offs between estimation bias and variance, which need to be assessed in a data set-dependent manner. In any case, the nontargeting negative control siRNAs can be used for quality control.

48 | Subtract the single siRNA knockdown effects m_i and n_j (estimated in Step 47) from the measured data d_{ijk}

$$g_{ijk} = d_{ijk} - m_i - n_j \quad (2)$$

to obtain the genetic interaction measurements g_{ijk} . In this way, genetic interaction measurements are computed for each siRNA combination and each replicate.

49 | Use the estimated genetic interactions g_{ij1} and g_{ij2} of the two (or more) biological replicates per siRNA combination to test for statistical significance, if the genetic interaction g_{ij} is different from zero with a moderated t -test¹⁸. A fully detailed example analysis is provided in the R/Bioconductor package HD2013SGI (ref. 8; Section 6.6, ‘Pairwise interaction scores’ and Section 6.7, ‘Statistical testing of interaction terms’).

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
18, 23	Bubble or drop formation on multidrop tips	Minor damage to tips, dirty tips, bubbles in transfection mix or cell suspension	Soak a paper towel with 70% (vol/vol) ethanol, let it dry under the hood and wipe the tips until the drops are gone
27	Contamination of single wells or full plates	Contaminated medium or siRNA mix Contaminated cells Contamination during plate preparation	Contamination of a small number of isolated wells is normal; these wells should be flagged and excluded from subsequent analysis. Excessive contamination reflects problems throughout the procedure and might require repetition of a complete experiment Always prepare fresh cell culture medium for each screen replicate. Check the cells during screen preparation and use them only if they are free from contamination. Take good care during plate preparation and spray down all equipment with 70% (vol/vol) ethanol. Do not move anything over open plates and keep plates closed whenever possible
41	Strong background staining in one or more channels	Nonspecific staining or leftover staining reagents	Control blocking time to reduce nonspecific antibody binding. Increase the number of washing steps to ensure that staining reagents are washed off properly
42	Offset changes during imaging, resulting in loss of focus	Distortion of plastic plates when warming up cold plates	Always let plates reach room temperature before setting up the microscope
43	Poor transfection efficiency or low knockdown efficiency (indicated by unexpected behavior (growth) of cells transfected with positive (viability) controls)	Defective lot of transfection reagent	Always test every new lot of transfection reagent and do not change lots during the experiment
44 (analysis)	Strong spatial effects	Unequal distribution of reagents by dispensing device (Multidrop) Conditions during incubation are not equal in all wells	Thoroughly test liquid dispensers before experiment to ensure even distribution of all reagents Ensure equal incubation conditions for all plates and wells by storing plates in plate racks and keeping a minimum distance between plates for proper ventilation. Test the incubator for a possible temperature gradient
45	Very low overall reproducibility between biological replicates	Differences in the experimental procedure between replicates	For high experimental reproducibility, it is crucial to strictly follow the same protocol for each replicate. If possible, prepare solutions for both replicates together and do not change lots or concentrations during the experiment. Note every calculation, timing and so on for every step of the experiment to permit retracing of differences if necessary. Do not change personnel between replicates. If problems occur during one replicate that require changes (e.g., lower cell numbers per well), it can make sense to maintain these changes in subsequent replicates
46	Reproducibility between specific pairs of siRNAs targeting the same gene is low	Off-target effects for one or both siRNAs	Drop this target gene from the screen Investigate the individual efficacy of siRNA and specificity by reverse-transcription (RT)-qPCR or RNA-seq in the particular cells and use only the specific and efficient siRNA Design and validate new siRNAs for the target gene or transcript isoform of interest and use them in subsequent screening campaigns

● TIMING

Steps 1–5, plate preparation for two biological replicates: 3 d (1 d for master-plate preparation, 1 d for aliquotting and 1 d for query gene siRNA distribution)

Steps 6–10, cell thawing: 15 min

Steps 11–15, cell propagation: 6 d (15 min hands-on time, 2 and 4 d after thawing)

Steps 16–25, reverse transfection: 3 d including incubation time

Steps 26–31, fixation/permeabilization: 6 h

Steps 32–36, staining: 5 h (plus overnight incubation)

Step 37, processing of second biological replicate: 12 d

Steps 38–42, imaging: 8–9 d, but it can vary when screening conditions are changed (staining, screen size).

Imaging time also depends on the illumination time per channel and on the number of images taken per well (given by the number of channels and the magnification used; with higher the magnification, more sites need to be imaged per well to have enough cells)

Steps 43–49, image processing and data analysis: 14 d (The timing for data analysis can vary depending on prior experience in data analysis, on batch effects and spatial effects on each plate that have to be considered, and on available computing power.)

ANTICIPATED RESULTS

The microscopy output is a set of TIFF images of the stained nuclei and cellular structures of choice (**Fig. 5**).

The image analysis starts with the segmentation of nuclei, which is propagated to the cell bodies using a second staining¹⁹. In our screen, nuclei were stained with DAPI and cell bodies with phalloidin and α -tubulin. We recommend this approach, as nuclei tend to be well separated, and their segmentation is typically simpler than directly segmenting cell bodies.

For each of the identified cells, quantitative cellular features are extracted. The software package imageHTS provides a large choice of features that can be computed. Per-cell features are summarized to per-experiment features by averaging over all cells. In addition to averaging, which can lose properties of subpopulations, we recommend considering other summary statistics such as measures of dispersion and quantiles; in addition, higher moments or fits of multimodal distributions (mixture models) might be useful for some applications. A fully detailed description is also provided in Section 4.3, ‘Feature extraction’ of the HD2013SGI (ref. 8) vignette (<http://www.bioconductor.org/packages/release/data/experiment/html/HD2013SGI.html>). When a large number of features are acquired, some of them will be statistically redundant with others, and a feature selection process should be applied to select a subset of nonredundant features to reduce the complexity of the subsequent analysis. An example is provided in Section 6.5, ‘Selection of nonredundant features’, of the HD2013SGI vignette. We prefer direct feature selection methods to methods that select linear combinations, such as principal component or linear discriminant analysis, owing to the more straightforward interpretability of the former. To further enhance interpretability, we manually preselect features of particular interest (e.g., cell number, nuclear area) rather than rely on a fully data-driven algorithm.

There is more than one definition for genetic interaction. Multiple variants exist²⁰ and they could be used instead of model 1 (equation 1, Step 47). We have adopted the view that a definition is useful if the resulting genetic interaction matrix is sparse. This means that genetic interactions are relatively rare and that the noninteracting model (e.g., equation 1 without the g_{ijk} term) explains most of the data. If in different applications this sparsity criterion is not met, then, depending on their criteria, users will need to explore several options: (i) mapping features to a scale that is better described by additive effects as in equation 1, e.g., by a logarithmic transformation, (ii) applying alternative noninteracting models such as investigated by Mani *et al.*²⁰ or (iii) dropping the sparsity criterion.

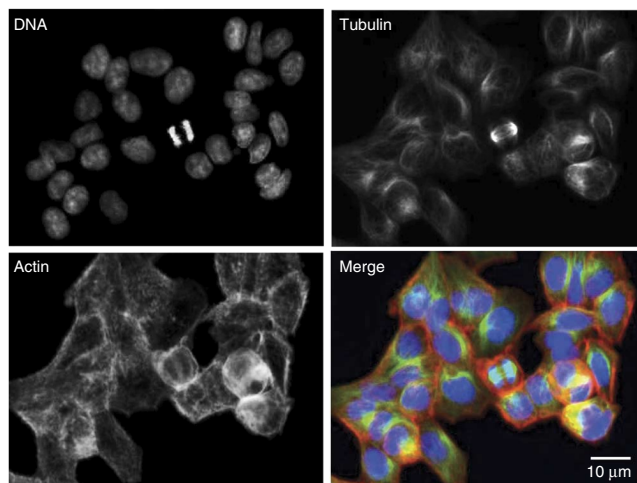


Figure 5 | Images and feature extraction. Sections of TIFF images acquired with an InCell Analyzer 2000 at 10× magnification in three channels, and a merged image. While covering a full well with four image sites, this magnification allows for detailed imaging of intracellular structures.

ACKNOWLEDGMENTS M.B. is supported by a European Research Council Advanced grant ('Syngene'). W.H. acknowledges support by the European Union project Systems Microscopy. C.L. and B.F. were supported by the CellNetworks Cluster of Excellence of the German Research Foundation (DFG).

AUTHOR CONTRIBUTIONS C.L., B.F., W.H. and M.B. designed the protocol and wrote the manuscript.

COMPETING FINANCIAL INTERESTS The authors declare no competing financial interests.

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