Supplementary Table 1. Readout probes used for barcode readout. A list of the readout sequences used for the "0" and "1" values of each bit, the dye to which each probe was conjugated, and the specific hybridization round in which the probe was hybridized.

Bit number	Bit value	Readout sequence	Dye	Hybridization round
1	0	ATCCTCCTTCAATACATCCC	Cy5	1
1	1	TATCTCATCAATCCCACACT	Cy5	7
2	0	ACACTACCACCATTTCCTAT	ATTO565	1
2	1	AAACACACACTAAACCACCC	ATTO565	6
3	0	ACTCCACTACTACTCACTCT	Alexa750	1
3	1	AACTCATCTCAATCCTCCCA	Alexa750	6
4	0	ACCCTCTAACTTCCATCACA	Cy5	2
4	1	AATACTCTCCCACCTCAACT	Cy5	8
5	0	ACCACAACCCATTCCTTTCA	ATTO565	2
5	1	TCTATCATCTCCAAACCACA	ATTO565	7
6	0	TTTCTACCACTAATCAACCC	Alexa750	2
6	1	TCCAACTCATCTCTAATCTC	Alexa750	7
7	0	ACCCTTTACAAACACACCCT	Cy5	3
7	1	TTCCTAACAAATCACATCCC	Cy5	9
8	0	TCCTATTCTCAACCTAACCT	ATTO565	3
8	1	ATAAATCATTCCCACTACCC	ATTO565	8
9	0	TATCCTTCAATCCCTCCACA	Alexa750	3
9	1	ACCCAACACTCATAACATCC	Alexa750	8
10	0	ACATTACACCTCATTCTCCC	Cy5	4
10	1	TACTACAAACCCATAATCCC	Cy5	10
11	0	TTTACTCCCTACACCTCCAA	ATTO565	4
11	1	ACTTTCCACATACTATCCCA	ATTO565	9
12	0	TTCTCCCTCTATCAACTCTA	Alexa750	4
12	1	TTCTTCCCTCAATCTTCATC	Alexa750	9
13	0	ACCCTTACTACTACATCATC	Cy5	5
13	1	AATCTCACCTTCCACTTCAC	Cy5	11
14	0	TCCTAACAACCAACTACTCC	ATTO565	5
14	1	ACCTTTCTCCATACCCAACT	ATTO565	10
15	0	TCTATCATTACCCTCCTCCT	Alexa750	5
15	1	TCCTCATCTTACTCCCTCTA	Alexa750	10
16	0	TATTCACCTTACAAACCCTC	Cy5	6
16	1	TCAAACTTTCCAACCACCTC	Cy5	12
17	0	TTACCTCTAACCCTCCATTC	ATTO565	11
17	1	ACACCATTTATCCACTCCTC	ATTO565	12
18	0	TCCCAACTAACCTAACATTC	Alexa750	11
18	1	ACATCCTAACTACAACCTTC	Alexa750	12
19	0	ATCCTCACTACATCATCCAC	Cy5	13

19	1	TCTCACACCACTTTCCTCAT	Cy5	14
20	0	TCCCTATCAATCTCCATAAC	ATTO565	13
20	1	TTATCCATCCCTCTTCCTAC	ATTO565	14
21	0	TCACCTCTAACTCATTACCT	Alexa750	13
21	1	TCCTACAACATCCTTCCTAA	Alexa750	14
22	0	ATCTCCCTTCTCTTCTCATA		Not measured
22	1	ATTACACCTCAACCCACACA		Not measured

Supplementary Table 2. Sequences of the Isolated Mutants. A list of the amino acid sequences for the isolated YFAST mutants characterized in Figure 3 and Supplementary Fig. 3. The mutated residues are marked in bold.

Mutant name	Amino acid sequence
Mutant 1	MEHVAFGSEDIENTLAKMDDGQLDGLAFGAIQLDGDGNILQYNAAEGDITGRDPKQVIGKN**L**FKD VA**C**GT**R**S**S**EFYGKFKEGVASGNLNTMFEWMIPTSRGPTKVKVHMKKALSGDSYWVFVKRV
Mutant 2	MEHVAFGSEDIENTLAKM**N**DGQLDGLAFGAIQLDGDGNILQYNAAEGDITGRDPKQVIGKN**L**FKD VA**C**GT**R**S**S**EFYGKFKEGVASGNLNTMFEW**T**IPT**K**RGPTKVKVHMKKALSGDSYWVFVKRV
Mutant 3	MEHVAFGSEDIENTLAKMDDGQLDGLAFGAIQLDGDGNILQYNAAEGDITGRDPKQVIGKN**L**FKD VA**E**GT**R**S**S**EFYGKFKEGVASGNLNTMFEW**T**IPT**K**RGPTKVKVHMKKALSGDSYWVFVKRV

Supplementary Notes:

Considerations when designing a high-throughput screen. There are several aspects that should be take into account when designing a high-throughput screen, including, for example, the number of bits in the barcodes, the fraction of possible barcodes used, and the number of cells that should be measured per variant to allow phenotypes to be measured accurately. Here we summarize some important points that should be considered when designing a screen to measure the phenotypic variability within a given library.

Bottlenecking barcodes. We only include a small fraction of all possible *N*-bit binary barcodes in a library, and this bottlenecking strategy serves two purposes: (i) to limit the frequency with which the same barcode might be associated with two or more different genetic variants and (ii) to introduce an error robustness into our barcode-to-genotype identification process. In our construction of the barcoded genetic variants, barcodes are associated with individual genetic variants randomly, hence the probability that a given barcode could be assigned to multiple different genetic variant could be high. While this situation would be detected via next-generation sequencing when we build the barcode-to-genetic variant lookup table, these barcodes would have to be discarded from the library screen measurement since cells containing such barcodes could not be unambiguously assigned to a given genotype. If a large fraction of the used barcodes were associated with multiple genetic variants, the number of barcodes that would need to be discarded would be high. To overcome this problem, we restricted the number of barcodes used in the library to be less than 10% of the total number of possible *N*-bit binary barcodes. Specifically, after the barcoded genetic variants are assembled, we bottleneck the size of the barcoded genetic variants library such that the number of genetic variant-barcode pairs in the library is <10% of the total number of possible *N*-bit binary barcodes. Because only such a small fraction of barcodes are included, most barcodes will be present only once in the library, and the chance that a barcode is present more than once (hence allowing the possibility of being paired with more than one genetic variant) will be very small (<10%). The remaining small fraction of barcodes that are paired with more than one variant can be detected by sequencing and discarded in further analysis.

The second reason why we bottleneck is to introduce error robustness into our genotype identification process. Specifically, if only a relatively small fraction of all possible barcodes are used, barcode measurement errors will more likely produce a barcode that is not present in the library, i.e. an invalid barcode. Because we know the exact barcodes that are present in the library via next-generation sequencing, it is possible to identify the invalid barcodes that resulted from errors during barcode imaging and discard them. This ability greatly reduces the rate at which we misidentify the genotype of a given cell. For example, if we bottleneck the barcode number such that <10% of the total possible barcodes are present in the library, the chance that a barcode imaging error will lead to genotype misidentification will be reduced to <10%.

In our experiments, we chose a degree of bottlenecking such that only 1-10% of the possible 21-bit binary barcodes are present in our libraries. The bottlenecking was achieved experimentally by selecting a small, random subset of cells after transforming *E. coli* cells with the barcode-mutant plasmids under the condition that each cell contains a unique barcode-mutant pair (see Online Methods). For example,

to achieve a bottlenecking degree of 4%, we select the number of cells that is 4% of the number of possible 21-bit binary barcodes.

Determining the number bits in the barcodes. The number of bits in the barcode is determined by the number of gene variants that we need to screen. While optimizing YFAST, we created mutant libraries in two ways: (1) The first type of libraries contained a defined, relatively small number of mutants that we hope to screen exhaustively; (2) The second type of libraries contained a very large number of possible mutants where screening only a random subset of these mutants would already be very informative. When we created the first type of libraries, we chose a barcode diversity such that the number of barcodes in the library was 5 times more than the number of unique mutants to ensure that each mutant (or at least the vast majority of them) was present in the library at least once. Because of the bottlenecking strategy describe above, namely the number of barcodes in the library being < 10% of the total number of possible *N*-bit binary barcodes, we then needed the total number of possible barcodes to be 50 times more than the number of mutants to screen. Based on this number, we determined the desired number of bits. For example, if we plan to screen 20,000 specific mutants, we will need more than 1 million possible barcodes, and hence we would use a 21-bit barcoding scheme that can give ~2 million possible barcodes. When we created the second type of libraries in which only a subset of possible mutants will be screened, we selected a library size to be equal to the number of mutants that we intended to subsample from the larger library; in this case, each mutant in the library was only associated with a single barcode and the number of barcodes in the library was equal to the number of mutants to be screened. The number of possible *N*-bit binary barcodes and hence the number of bits required are then likewise determined based on the bottlenecking strategy.

Determining the desired number of measured cells per genetic variant. In the library screens, the number of cells that need to be measured for each genetic variant is largely determined by the accuracy of the phenotype measurement. As the number of cells measured for each genotype increases, the accuracy with which that phenotype is measured improves. The desired cell number per genetic variant is set by the noise properties of the screened phenotype and the measurement accuracy that is needed to discriminate phenotype variations.

For our screen of YFAST variants, we observed a large cell-to-cell variance in the fluorescence intensity measurements between cells expressing the same genotype. This variance was observed even within a monoculture of the original YFAST. This observation indicated that the measurement accuracy of this type of phenotype from a single cell was low and, thus, required us to screen many more cells than mutants to increase this accuracy. In addition, we found that different mutants appear in different abundance within our libraries, and this natural variation arose because of the random processes of constructing the plasmid-mutant libraries and transforming *E. coli*. To ensure that the majority of mutants are measured with a desired number of cells, this abundance variation further increases the oversampling requirement. For the YFAST measurements, we aimed to measure ~100 cells on average per mutant.

Finally, in our genotype (barcode) measurements, a substantial fraction of the cells are discarded by readout intensity thresholding and by the rejection of barcodes that do not match the valid barcodes

present in the library, as described in the main text. In our measurements, ~66% of the measured cells were discarded because of the above procedures. As a result, we needed to measure, on average, 300 cells per YFAST variant to achieve of the goal of ~100 cells per mutant. We therefore measured 20 million cells to screen 60,000 YFAST variants.

Since the noise properties of the screened phenotype and the measurement accuracy that is needed to discriminate phenotype variations both depend on the phenotype to be screened, the number of cells that needs to be measured per genotype depends critically on the phenotype to be screened. Thus, we recommend that pilot measurements be conducted to determine the noise observed for the desired phenotype. With these measurements, it should then be possible to estimate the number of cells required to discriminate different phenotypes to a given accuracy. Also, it is worth noting that given the reproducibility between phenotypes measured for the same genotype in separate screens, it may also be possible to increase the number of cells measured on average for a given library by simply replicating the screen multiple times with the same library and pooling the results so as to improve the accuracy of phenotype variability if it is determined not to be sufficient from a single measurement.

Estimate of the maximum plausible library size of the genetic variants. There are multiple factors that determine the maximum library size of genetic variants that can be screened. The first potential limitation to the size of the library is the number of unique barcodes that can be measured. We have demonstrated the ability to image 21-bit barcodes, and we did not observe a degradation in the image quality between the last imaged bit and the first imaged bit. Thus, we envision that adding more bits to the barcode should be possible. For example, 25-bit barcodes are likely readily measurable. Moreover, given such a modest extension in the length of the barcode, we envision that there will be no challenges to constructing plasmids that contain 25-bit barcodes, or creating the barcode-mutant lookup table using existing next-generation sequencing approaches (Illumina HiSeq or NovaSeq). 25-bits would produce ~30 million possible barcodes. Based on our bottlenecking strategy, we typically select <10% of the possible barcodes to include in the library, which means <3 million barcodes to include in the library. By utilizing high-competency *E. coli* strains, as we have done here, it should be possible to create 10-fold more transformants than library members, a sufficient coverage level, by pooling a few transformation reactions. If we aim to see each mutant (or the vast majority of them) at least once, we would like to have 5 times more barcodes in the library than the number of genetic variants, which, in this case, means the library could contain up to ~600,000 genetic variants. Assuming that we would like to measure 10-100 cells per variant on average (depending on the phenotype measurement accuracy requirement), and based on our current settings of the barcode readout intensity threshold, in which 1/3 of cells pass the threshold and generate correct barcodes, we would need to measure ~18 - 180 million cells. In the measurements we demonstrated here, we characterized ~1-2 million *E. coli* cells in a 40-hour long screen. However, in these measurements we used a relatively low density of *E. coli* on our coverslips so as to minimize the chance of cells contacting each other. This density could be increased as high as 10-fold without producing substantial cell-cell contact, and improvement in cell-segmentation algorithms should also allow contacting cells to be properly segmented. Thus, we anticipate that it should be possible to measure ~18 – 180 million cells with a reasonable imaging time (2-18 days).

Moreover, there are multiple ways that our protocols could be modified so as to further increase throughput. For example, improved hybridization approaches might reduce the number of dim or dark cells, allowing more of the measured cells to be utilized in the screen. Alternatively, it may also be possible to use lower magnification objectives to measure much larger fields of view and hence allow substantial improvements in the measurement throughput. We can use the low magnification for genotype (barcode) imaging while keeping the use of high magnification for the high-resolution phenotype measurements because the phenotype measurements are typically fast and the total imaging time of the screen is dominated by barcode imaging which requires many rounds of hybridization. In parallel, we anticipate that it should also be possible to increase the number of barcodes by either increasing the number of bits in the binary barcode scheme or by using higher order barcoding schemes, such as ternary or quaternary schemes. Thus, we anticipate that further advances in methodology could extend the throughput of our image-based screening method substantially.