Synchronization of secretory protein traffic in populations of cells

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To dissect secretory traffic, we developed the retention using selective hooks (RUSH) system. RUSH is a two-state assay based on the reversible interaction of a hook protein fused to core streptavidin and stably anchored in the donor compartment with a reporter protein of interest fused to streptavidin-binding peptide (SBP). Biotin addition causes a synchronous release of the reporter from the hook. Using the RUSH system, we analyzed different transport characteristics of various Golgi and plasma membrane reporters at physiological temperature in living cells. Using dual-color simultaneous live-cell imaging of two cargos, we observed intra- and post-Golgi segregation of cargo traffic, consistent with observation in other systems. We show preliminarily that the RUSH system is usable for automated screening. The system should help increase the understanding of the mechanisms of trafficking and enable screens for molecules that perturb pathological protein transport.

It is now clear that there are many routes for proper transport, modification and addressing of proteins in the secretory pathway of cells. This had been anticipated because of the diversity of target compartments (for example, plasma membrane, endosomes and lysosomes) but also because of the existence of plasma membrane subdomains[1–3]. Even for a simple transport route (such as the endoplasmic reticulum (ER) to plasma membrane) in nonpolarized cells, several independent pathways support the traffic of different subsets of protein cargo[4–7]. A comprehensive view of the mechanisms and dynamics of cargo sorting in these multiple secretory pathways requires assays that allow the dissection of the routes specific cargos follow. Ideally, these assays should be adaptable to a large diversity of cargos, allow quantitative and real-time trafficking observations and be amenable to large-scale experiments. Such assays would enable more detailed study of the large number of diverse trafficking regulators, such as families of small GTPases or SNAP receptors, the majority of which remain functionally unannotated.

Several approaches are currently used to image secretory traffic. For instance, short-term reporter expression, either after DNA microinjection or after photoconversion or photobleaching of a fraction of the target protein in single cells, has been used for this purpose. These methods are frequently coupled with temperature block and release to synchronize the reporter pool and allow traffic through the secretory pathway to be followed quantitatively. Temperature block (for example, 15 °C to block proteins in the ER or 20 °C to block proteins in the Golgi[8,9]) has proven a powerful tool for studying intracellular traffic, including in living cells, but it also imposes a temporary arrest of virtually all biosynthetic pathways in a mammalian cell. Another classical and powerful method for cargo synchronization relies on a thermosensitive viral glycoprotein (VSVGtsO45) that cannot exit from the ER at the restrictive temperature of 39.5 °C (refs. 10,11). Synchronous transport and processing at 32 °C can be monitored in living cells[12–14]. However, the restrictive and permissive temperatures for this system are not fully physiological and complicate the use of this system for systematic screening. In addition, the VSVGtsO45 system cannot be easily adapted to the analysis of a large diversity of cargos. An alternative method[15] relies on the fusion of the protein of interest with conditional aggregation domains, resulting in the aggregation of the fusion protein in the ER. This aggregation is reversed by the addition of a small ligand, allowing synchronous and controlled secretion of soluble or transmembrane proteins[15–18]. However, this approach is not applicable to proteins that cannot be tagged on their luminal domains and cannot be used for synchronization after the ER. A regulated trafficking system has also been developed that depends on the induction of reporter expression in *Drosophila melanogaster* cells[19]. It has been used for screening[20,21] but because of slow kinetics would be less adapted to real-time analysis of early trafficking steps (such as ER export).

Thus there is still an unmet need for a versatile trafficking assay that allows efficient synchronization of diverse cargo, under physiological conditions, permits quantitative live cell imaging and is amenable to automated screening. Here we describe such a system, named RUSH, which relies on the selective retention and release of cargo molecules from a donor compartment. We showed that the RUSH system can be used to study and quantify the trafficking of diverse proteins in live cells or in end-point assays, and that it shows potential for automated quantitative imaging and screening in the future.

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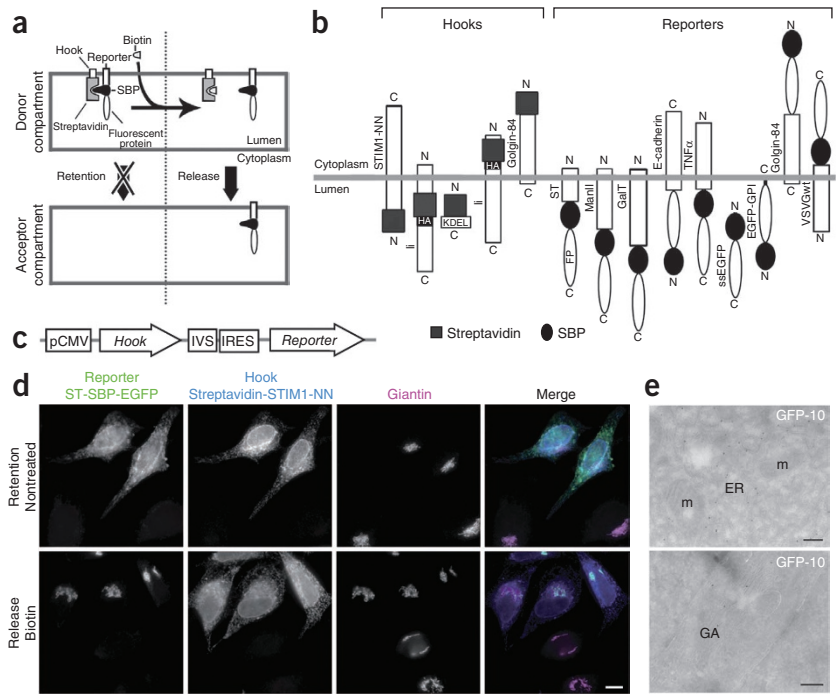

Figure 1 | The RUSH system. (**a**) A schematic of the principle illustrates that the reporter is retained in the donor compartment via its interaction with the hook. This interaction is mediated by the core streptavidin and the SBP. Release is induced by addition of biotin to allow trafficking of the reporter to its acceptor compartment. A fluorescent protein is fused to the reporter. (**b**) Schematics of hooks containing STIM1-NN, Ii or KDEL for ER retention or Golgin-84 for Golgi retention fused to streptavidin, and of reporters containing SBP fusions with Golgi proteins ST, ManII, GalT or Golgin-84, plasma membrane proteins VSVGwt, E-cadherin, TNFα or EGFP-GPI and secreted protein SBP-ssEGFP. HA, hemagglutinin tag; FP, fluorescent protein. (**c**) Schematics of genes coding for the hook and the reporter, expressed under the same *CMV* promoter (pCMV), separated by a synthetic intron (IVS, intervening sequence) and an internal ribosome entry site (IRES). (**d**) Micrographs of HeLa cells expressing streptavidin-STIM1-NN (blue) and ST-SBP-EGFP (green) with and without biotin treatment. The Golgi apparatus is indicated with staining for giantin (magenta). Scale bar, 10 µm. (**e**) Ultrathin cryosections of HeLa cells expressing Ii-streptavidin and ST-SBP-EGFP without biotin (top) or with biotin for 1 h (bottom) and immunogold-labeled with an antibody to GFP. One hour after biotin addition (bottom), ST-SBP-EGFP was localized to the Golgi apparatus (GA). m, mitochondria; GFP-10, 10-nm gold particles. Scale bars, 200 nm.

RESULTS

Principles of the RUSH trafficking assay

The RUSH system is based on the expression of two fusion proteins: the hook, stably expressed in a donor compartment, and the reporter, which reversibly interacts with the hook. Upon reversion of the interaction, the reporter is free to resume its journey to its final compartment. The core streptavidin is fused to the hook, and the SBP is fused to the reporter (**Fig. 1a**,**b**). The SBP binds streptavidin with high affinity and can be outcompeted by biotin[22,23], a natural, nontoxic vitamin, which diffuses freely in cell compartments. At steady state, the reporter protein is kept in the donor compartment owing to the streptavidin-SBP interaction. Synchronous release of the reporter occurs upon the addition of biotin to the medium. To monitor the trafficking of the reporter and allow real-time imaging in living cells, the reporter is also fused to a fluorescent protein (for example, enhanced (E)GFP or monomeric (m)Cherry).

As a hook in the ER, we used a mutant of stromal interaction molecule 1 (STIM1-NN; a type I protein) that localizes in the ER but that cannot bind microtubules[24] and an isoform of the human invariant chain of the major histocompatibility complex (Ii; a type II protein) that has an N-terminal arginine-based motif, which retains it in the ER[25]. We fused the hooks with the core streptavidin in their luminal or cytoplasmic domain depending on the reporter studied. We also generated a minimal ER hook by simply fusing streptavidin to a C-terminal ER retention signal (Lys-Asp-Glu-Leu; KDEL) (**Fig. 1b**). Finally, we selected Golgin-84 to be used as a cytoplasmic Golgi hook (**Fig. 1b**).

We then adapted reporters belonging to different classes of proteins (type I, type II transmembrane, glycosylphosphatidylinositol (GPI)-anchored proteins and soluble secretory proteins) to the RUSH system (**Fig. 1b**) by fusing them to a SBP tag and to a fluorescent protein. We cloned the genes encoding the hook and the reporter in a bicistronic expression plasmid to simplify the experimental setup and ensure expression of both proteins. We placed the hook before an internal ribosome entry site (**Fig. 1c**) so that enough hook was expressed to retain every reporter molecule.

To validate the use of the RUSH system, we analyzed the retention and release of the targeting domain of a Golgi enzyme, β-galactoside α-2,6-sialyltransferase 1 (ST) in HeLa cells. At steady state, ST-SBP-EGFP and streptavidin-STIM1-NN were found in the ER (**Fig. 1d**). One hour after biotin addition, we detected ST-SBP-EGFP in the Golgi apparatus, its target compartment, while the hook was still localized in the ER (**Fig. 1d**). We obtained similar results using Ii-streptavidin as a hook (**Supplementary Fig. 1**).

Immunoelectron microscopy (**Fig. 1e**) showed that, in the absence of biotin, the reporter was localized in a network of tubular elements characteristic of the ER. Note that the ER's overall structure did not seem affected by cargo accumulation. One hour after biotin addition, we observed labeling in the Golgi, indicating that the reporter was efficiently transported from the ER to the Golgi. We tested whether stable or transient expression of the RUSH proteins induced ER stress. We observed that expression of either protein alone or expression of both hook and reporter did not lead to an increase in the amount of the chaperone BiP (**Supplementary Fig. 2a**). Moreover transfection with a RUSH plasmid did not impair the trafficking of VSVGtsO45-GFP (**Supplementary Fig. 2**).

We examined the kinetics of release of the reporter from the hook. We analyzed the interaction between the hook and the reporters ST-SBP-EGFP or α-mannosidase II (ManII)-SBP-EGFP (**Fig. 2a**,**b** and **Supplementary Fig. 3**) by immunoprecipitation using an antibody to GFP at different time points after the addition of biotin. The interaction was rapidly lost after biotin

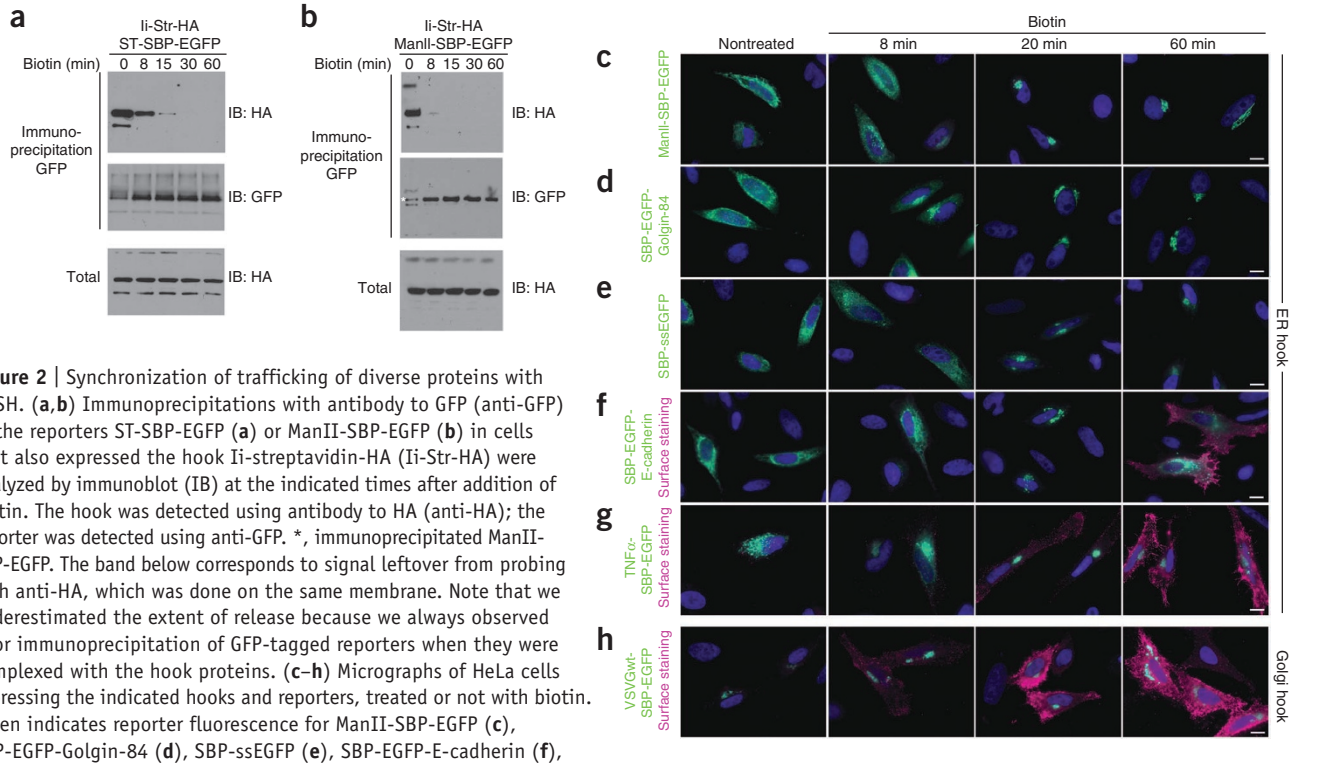

Figure 2 | Synchronization of trafficking of diverse proteins with RUSH. (**a**,**b**) Immunoprecipitations with antibody to GFP (anti-GFP) of the reporters ST-SBP-EGFP (**a**) or ManII-SBP-EGFP (**b**) in cells that also expressed the hook Ii-streptavidin-HA (Ii-Str-HA) were analyzed by immunoblot (IB) at the indicated times after addition of biotin. The hook was detected using antibody to HA (anti-HA); the reporter was detected using anti-GFP. *, immunoprecipitated ManII-SBP-EGFP. The band below corresponds to signal leftover from probing with anti-HA, which was done on the same membrane. Note that we underestimated the extent of release because we always observed poor immunoprecipitation of GFP-tagged reporters when they were complexed with the hook proteins. (**c**–**h**) Micrographs of HeLa cells expressing the indicated hooks and reporters, treated or not with biotin. Green indicates reporter fluorescence for ManII-SBP-EGFP (**c**), SBP-EGFP-Golgin-84 (**d**), SBP-ssEGFP (**e**), SBP-EGFP-E-cadherin (**f**), TNFα-SBP-EGFP (**g**), VSVGwt-SBP-EGFP (**h**). Either Ii (**c**,**e**,**g**) or STIM1-NN (**f**) were used as a luminal ER hook. Ii was used as cytoplasmic ER hook (**d**) and Golgin-84 as cytoplasmic Golgi hook (**h**). Magenta shows cell surface staining with anti-GFP (**f**,**g**) or with an antibody to VG (**h**); blue, DNA staining. Scale bars, 10 μm.

addition for both reporters, although it was more complete for ManII-SBP-EGFP. This fast release of the reporter from the hook can also be inferred from the rapid diffusion of reporters in the ER observed soon after biotin addition to cells, as described below, and enabled the analysis of early steps of export from the donor compartment.

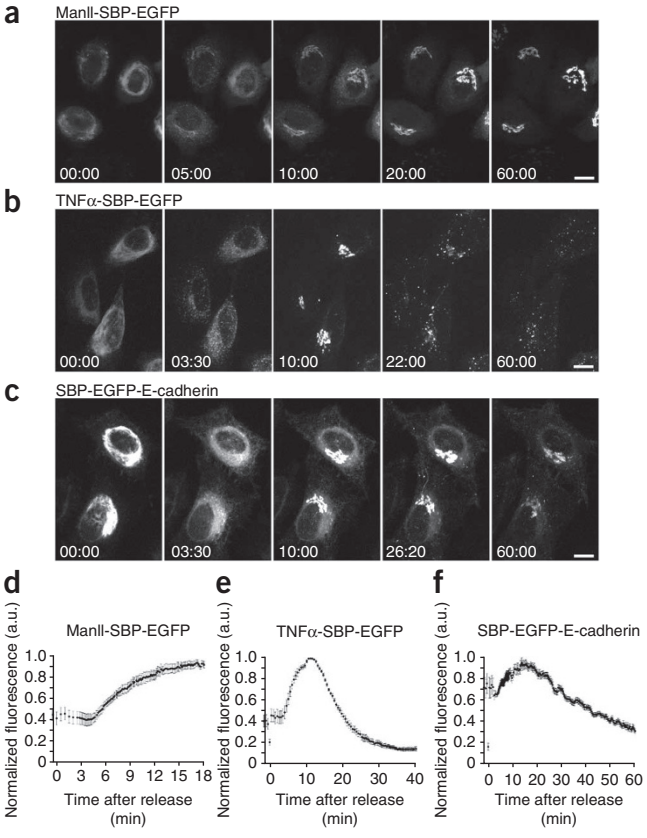

Figure 3 | Diverse cargos exhibited differences in kinetics and morphology of transport carriers. (**a**–**c**) Micrographs of HeLa cells expressing Ii-streptavidin as a hook and ManII-SBP-EGFP (**a**), TNFα-SBP-EGFP (**b**) or SBP-EGFP-E-cadherin (**c**) as a reporter. Biotin was added at 00:00 (minutes:seconds). A maximum intensity projection of optical sections is shown. Scale bars, 5 μm. (**d**–**f**) The plots show integrated fluorescence intensity in the Golgi region at each time point, corrected for background and normalized to the maximum value. Curves depict the measurement of at least seven cells of a representative experiment. Error bars, s.d.

The RUSH system applies to diverse cargos

We then tested the performance of the RUSH system on many secretory cargos (**Fig. 1b**). In nontreated cells, all reporters localized in the ER (**Fig. 2c**–**g** and **Supplementary Fig. 4**). Soon after the addition of biotin, the reporters started to leave the ER. We detected some in ER exit site–like structures, whereas others had already reached the Golgi apparatus (**Fig. 2c**–**g** and **Supplementary Fig. 4**). Twenty minutes after biotin addition, all reporters were clearly present in the Golgi complex (**Fig. 2c**–**g** and **Supplementary Fig. 4**), whereas the signal in the ER was strongly decreased, showing that the majority of the reporters moved from the ER to the Golgi along the secretory pathway.

At 60 min after biotin addition, a large fraction of the reporter proteins reached their target compartment. The Golgi enzymes ManII, ST and β-1,4-galactosyltransferase 1 (GalT) were concentrated in the Golgi apparatus at this time point (**Fig. 2c** and **Supplementary Fig. 4**). We also still detected the secreted soluble EGFP, SBP-ssEGFP, in the Golgi complex but its intensity

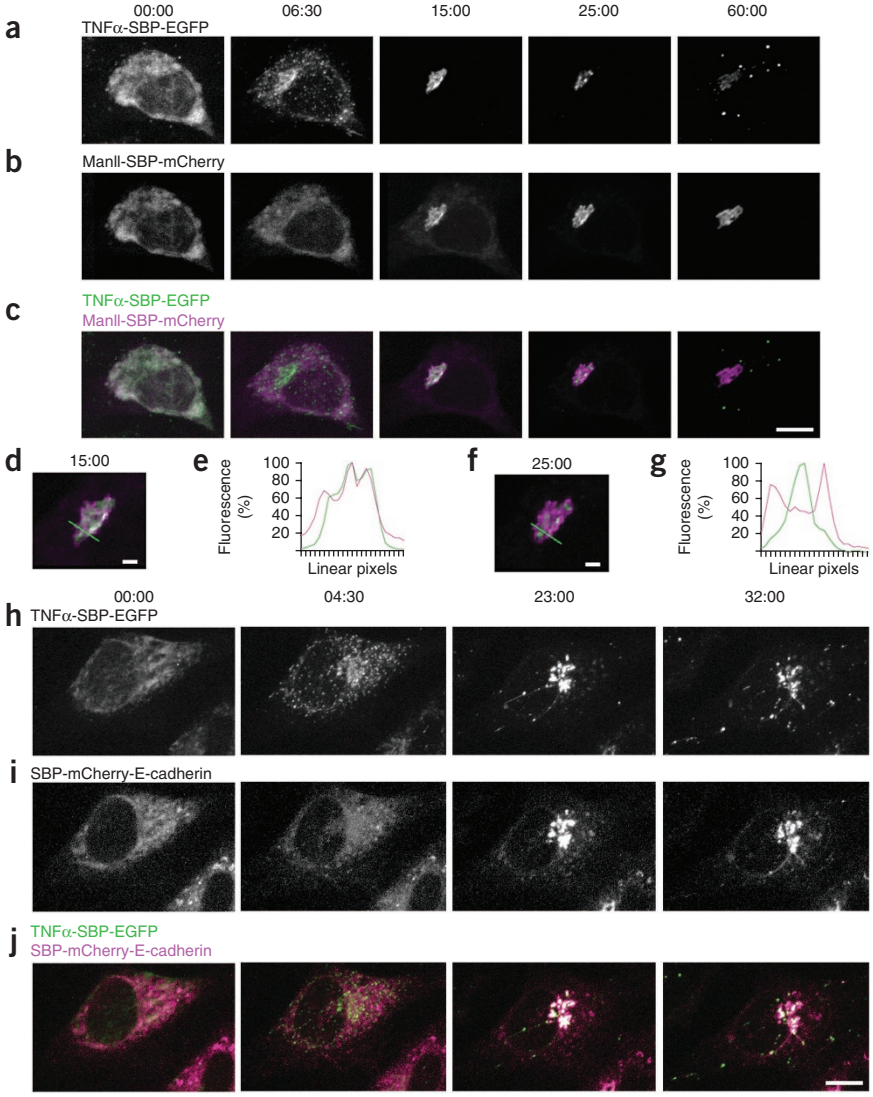

Figure 4 | Dual-color simultaneous imaging of RUSH-synchronized traffic of two cargos in living cells. (**a**–**g**) HeLa cells were transfected to express Ii-streptavidin as a hook, and TNFα-SBP-EGFP and ManII-SBP-mCherry as reporters. Micrographs show cells at the indicated time points after addition of biotin at time 00:00 (**a**–**c**). Scale bar, 5 µm. Analysis of TNFα-SBP-EGFP (green) and ManII-SBP-mCherry (magenta) fluorescence intensity (**e**,**g**) as a function of the line shown in **d** and **f** at 15 min and 25 min, respectively. Scale bars, 2 µm. (**h**–**j**) HeLa cells were transfected to express Ii-streptavidin as a hook, and TNFα-SBP-EGFP and SBP-mCherry-E-cadherin as reporters. The micrographs show cells at the indicated time points after addition of biotin at time 00:00. Scale bar, 5 µm.

was reduced compared to the 20 min time point suggesting that, as expected, part of SBP-ssEGFP was secreted (**Fig. 2e**). We confirmed its secretion by a western blot analysis of cellular and extracellular samples (**Supplementary Fig. 5**). The four plasma membrane cargos SBP-EGFP-E-cadherin, tumor necrosis factor-α (TNFα)-SBP-EGFP, wild-type VSVG (VSVGwt)-SBP-EGFP and SBP-EGFP-GPI were clearly expressed at the plasma membrane after 60 min (**Fig. 2f**,**g** and **Supplementary Fig. 4**).

Because the Golgi complex is a very dynamic organelle, it is more difficult to define efficient Golgi hooks than ER hooks. However, we could synchronize the transport of VSVGwt-SBP-EGFP from the Golgi complex to the plasma membrane using Golgin-84 as a Golgi hook. At steady state, VSVGwt-SBP-EGFP was retained in the Golgi complex by streptavidin-Golgin-84. After addition of biotin to the medium, the reporter was localized to the plasma membrane within 20 min (**Fig. 2h**).

Analysis of traffic kinetics and carrier morphology

Using the RUSH system we analyzed the synchronized trafficking of various cargos in living cells, using a spinning disk confocal microscope at 37 °C without prior temperature block. We imaged HeLa cells transiently expressing Ii-streptavidin as a hook and ManII-SBP-EGFP, TNFα-SBP-EGFP or SBP-EGFP-E-cadherin as reporters (**Fig. 3a**–**f**). Before treatment, we detected all reporters in the ER. Soon after biotin addition (2–5 min), ManII-SBP-EGFP diffused in the ER, and we observed it in the Golgi complex within less than 10 min, where it stayed until the end of the acquisition at 60 min (**Fig. 3a**,**b** and **Supplementary Video 1**).

The synchronized trafficking of TNFα-SBP-EGFP revealed a similarly fast ER export; we detected scattered dots corresponding to ER exit sites as early as 2–3 min after biotin addition (**Fig. 3b**, **Supplementary Fig. 6** and **Supplementary Video 2**) and fluorescence was localized in the Golgi complex by 10 min (**Fig. 3e**). After 15 min, granule-like post-Golgi carriers appeared and plasma membrane staining became visible whereas signal at the Golgi decreased (**Fig. 3b** and **Supplementary Video 2**), disappearing almost completely after 30 min (**Fig. 3f**). Intensity measurements suggest that TNFα proteins crossed the Golgi in 10–15 min on average (**Fig. 3e**).

Trafficking of E-cadherin was slightly different. After a rapid diffusion in the ER, SBP-EGFP-E-cadherin progressively accumulated in the Golgi apparatus until 20 min after biotin addition (**Fig. 3c**,**f** and **Supplementary Video 3**). Then we observed many tubular structures emanating from the Golgi (**Fig. 3c** and **Supplementary Video 3**) and, in contrast to TNFα-SBP-EGFP, no granule-like post-Golgi transport intermediates were visible. Fluorescence of SBP-EGFP-E-cadherin in the Golgi gradually decreased with time without reaching a plateau before the end of the acquisition (**Fig. 3f**). Our observations of intra-Golgi cargo segregation are in agreement with studies carried out using either temperature block or single-cell analysis approaches.

We also monitored the trafficking of VSVGwt tagged at its cytoplasmic domain. VSVGwt-SBP-EGFP reached the Golgi apparatus within 10 min after the addition of biotin and exited from the Golgi after 20 min, forming tubes and vesicles (**Supplementary Video 4**). This recapitulates previous observations using the thermosensitive VSVG[13,14,26]. We observed a similar acquisition

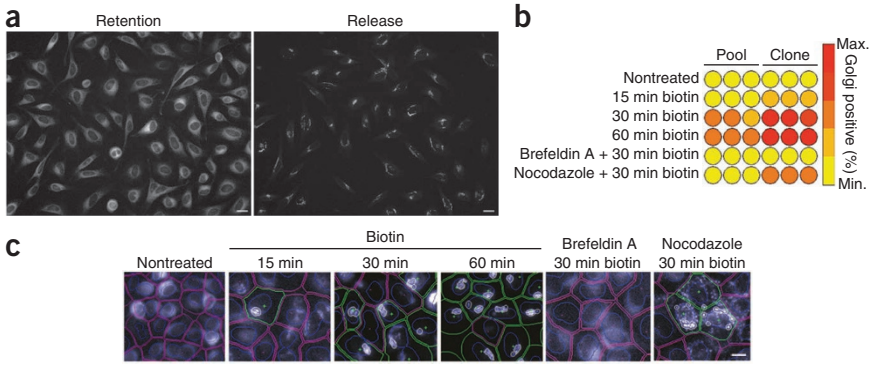

Figure 5 | Potential of the RUSH system for automated screening. (**a**) The micrographs show HeLa cells stably expressing Str-KDEL as a hook and ManII-SBP-EGFP as a reporter, either treated (30 min) or not with biotin. Images were acquired in 96-well View plate using an automated microscope (Incell 2000, GE Healthcare) with a 20× air objective. Scale bar, 20 µm. (**b**) Cells as in a were treated with biotin for indicated times in presence or absence of brefeldin A for 15 min or nocodazole for 1 h. Segmentation was performed using the software InCell Analyzer Workstation. The image shows a schematic color-coded representation (from yellow to red) of the relative percentage of cells with Golgi-localized fluorescence ('Golgi positive') as detected by the algorithm in automated fashion. Cells used in this experiments were either a population of stably expressing cells (pool) or a selected clone (clone). (**c**) The images show segmentation of the micrographs used to obtain the data in **b**. Nuclei are outlined in blue; cells are delineated in magenta or green. Green cells were classified as positive for Golgi transport, magenta cells were classified as negative. Scale bar, 10 µm.

of Golgi-specific endoglycosidase-H–resistant glycosylation by VSVG proteins synchronized using VSVGtsO45 or the RUSH approach (**Supplementary Fig. 2**). Because RUSH allows tight synchronization of the reporter, we could image exocytosis at the plasma membrane without prior accumulation of cargo in the *trans*-Golgi network by a 20 °C temperature block (**Supplementary Fig. 7** and **Supplementary Video 5**).

Cargo partitioning in the Golgi and post-Golgi stages

We analyzed the simultaneous trafficking of two pairs of cargos: the plasma membrane protein TNFα (tagged with EGFP) either with ManII or with E-cadherin (tagged with mCherry). We transiently expressed the pairs of cargo in HeLa cells, in each case associated with the same hook, Ii-streptavidin.

The traffic kinetics upon reporter expression were consistent with our observations after transfection of a single plasmid for each reporter (**Figs. 3** and **4**). Before the addition of biotin, both reporters localized in the ER. After release, TNFα-SBP-EGFP was first to leave the ER. The localization in ER exit sites and in the Golgi were clearly visible, whereas ManII-SBP-mCherry or SBP-mCherry-E-cadherin were still localized in the ER (**Fig. 4a–c**, **Supplementary Videos 6** and **7** and **Fig. 4h–j**), highlighting differences in the kinetics of ER export. At 15 min, TNFα and ManII were largely localized in the Golgi (**Fig. 4d**,**e** and **Supplementary Video 6**). TNFα then started to segregate inside the Golgi complex until large granule-like structures were pulled out en bloc from the Golgi to traffic toward the plasma membrane, suggesting intra-Golgi partitioning (**Fig. 4f**,**g** and **Supplementary Video 6**).

Simultaneous imaging of TNFα and E-cadherin demonstrated that TNFα-SBP-EGFP exited first from the Golgi mainly as large granule-like structures (**Supplementary Video 7**). More rarely we observed faintly fluorescent tubes on which the granule structures moved. After 25 min, we frequently observed SBP-mCherry-E-cadherin in tubes emanating from the Golgi but saw no large vesicular post-Golgi carriers. After 30 min of release, we observed long tubules continuously bearing SBP-mCherry-E-cadherin and decorated at their tips with large vesicular-like structures containing only TNFα-SBP-EGFP (**Fig. 4** and **Supplementary Video 7**). These tubes were also faintly positive for TNFα-SBP-EGFP. These data suggested a sequential intra- and post-Golgi segregation between two plasma membrane cargos, as has been previously reported (for example, ref. 27). The RUSH system should allow a systematic analysis of partitioning in diverse plasma membrane cargo under physiological conditions.

The RUSH system is usable in automated screens

A strength of the RUSH system is its potential applicability to large-scale systematic screens of protein traffic. In a proof-of-concept experiment, we tested whether the system could be used in the future for automated high-content screening. We generated stable cell lines expressing streptavidin-KDEL as a hook and ManII-SBP-EGFP as a reporter (**Fig. 5a** and **Supplementary Fig. 8**), and imaged them in 96-well plates using an InCell 2000 microscope. We used granularity-based algorithms to analyze the localization of ManII-SBP-EGFP in fixed cells at various time points after reporter release with biotin, in the presence or absence of drugs (nocodazole and brefeldin A) that perturb secretory traffic (**Fig. 5b**,**c**). The algorithms efficiently detected normal and perturbed trafficking. We note that clonal cell lines allowed better discrimination of traffic than a heterogeneous pool of stable clones, although the latter can still be used. We conclude that the localization of a RUSH reporter, at least for the robust effects monitored here, can be efficiently analyzed using automated imaging systems.

DISCUSSION

The RUSH system allows synchronization of secretory cargo trafficking at physiological temperature. As with any tagging system, the position of the tag must be chosen carefully. We relied on prior knowledge from GFP tagging and observed that the addition of the SBP tag did not seem to modify the trafficking of the chosen cargos. However, addition of SBP to a new reporter will need careful validation.

Retention of reporters in the ER did not appear to be toxic. We did not observe morphological change of the ER by electron microscopy analysis or induction of an ER stress response protein (**Supplementary Fig. 2a**). Furthermore, the ER export of the cargo protein VSVG-tsO45 occurred normally in cells that retain a RUSH reporter in the ER (**Supplementary Fig. 2b**). Finally, we could select several stable cell lines, suggesting that steady-state reporter retention was not toxic (**Fig. 5** and **Supplementary Fig. 7**).

One or multiple proteins can be observed at the same time using the RUSH system, enabling the study of cargo partitioning

and differential regulation of trafficking. The mechanisms of intra-Golgi trafficking and Golgi maintenance are still under debate[28,29]. It will be interesting to use the RUSH system to address systematically this question for a large set of cargos. For example, when considering the kinetics of Golgi exit displayed by E-cadherin and TNFα, it is tempting to propose that the former follows a proposed rapid partitioning model[30] whereas the latter displays the dynamics expected according to the cisternae maturation model. Because the RUSH system allows the synchronization and quantification of a vectorial flow of very diverse cargos using an identical setup, it could lead to a more precise view of the diversity of Golgi-intersecting pathways.

Because it is based on simple addition of an available small molecule and works at physiological temperatures, the RUSH system is amenable to large-scale screens. This could improve our understanding of the fundamental mechanisms of intracellular trafficking and pave the way to comparative, quantitative and systematic study of multiple trafficking pathways. It could enable new therapeutic strategies by helping to identify regulatory factors responsible for the transport of proteins essential for disease development (for example, receptors, growth factors and adhesion molecules) or to screen for inhibitory molecules.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemethods/.

Note: Supplementary information is available on the Nature Methods website.

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AUTHOR CONTRIBUTIONS

G.B. carried out most of the experiments, designed and set up some of the approaches, analyzed the data, prepared the figures and wrote the manuscript. S.D. was involved in most of the molecular biology experiments and carried out the biochemical experiments. N.G. was involved in the molecular biology experiments and in selection of the stable cell lines. H.d.F. carried out the total internal reflection fluorescence experiment. A.L. contributed to automated screening. L.L. established the stable cell lines. V.M. was involved at the very beginning of the project. F.J. was involved at the very beginning of the project. G.R. did the electron microscopy experiments, prepared the corresponding figure and corrected the manuscript. F.P. designed and supported the study, directed the work, analyzed the data and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturemethods/.

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ONLINE METHODS

Cells and transfection. HeLa cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen) supplemented with 10% of FCS (Biowest), 1 mM sodium pyruvate and 100 µg/ml penicillin and streptomycin (Invitrogen) (complete medium) at 37 °C and 5% of CO_2. HeLa cells were transfected using calcium phosphate as described previously[31].

Plasmids. The IRES vector is based on the pIRESneo3 (Clontech-Takara). The hook was inserted using the multicloning site of the vector. We purchased the core streptavidin as a synthetic gene from Mr. Gene. To insert the reporter, we modified the vector by replacing the *Neo* cassette by a multicloning site containing the 8-base cutter recognition sites AscI, SfiI and PacI. A patent application has been submitted to cover commercial application of the RUSH, but no restriction will be applied to the academic use of the system. The plasmids will be made available upon request to the corresponding author. If the number of requests is too high, reference plasmids will be added to a public repository such as Addgene.

Immunofluorescence and antibodies. For cell surface immunostaining, coverslips were transferred in ice-cold PBS. Mouse antibody to GFP (anti-GFP, clones 7.1 and 13.1 (1:1,000); Roche) was incubated in ice-cold PBS, cells were washed in PBS, fixed with 2% paraformaldehyde, and free aldehydes were quenched with 50 mM NH_4Cl. For other immunostaining, after fixation and aldehydes quenching, cells were permeabilized with 0.05% saponin in PBS with 2 mg/ml bovine serum albumin. Coverslips were mounted in Mowiol (Calbiochem) supplemented with DAPI to stain DNA. A monoclonal antibody to streptavidin (clone S10D4; 1:500) was purchased from Santa Cruz. The anti-giantin (1:100) was previously described[32]. The anti-Ii (1:100) was a gift of P. Benaroch (Institut Curie). The antibody to the ectoplasmically exposed VSVG (anti-VG; 1:500) had been obtained from T. Kreis (University of Geneva). The rabbit Sec24 antibody (1:100) was a gift from D. Stephens (University of Bristol). Fluorescent secondary antibodies were purchased from Jackson ImmunoResearch. Anti-BiP (C50B12; 1:1,000) was purchased from Cell Signaling Technology and the anti-actin (AC-10; 1:5,000) from Sigma. The polyclonal anti-GFP (1:10,000) and monoclonal anti-HA tag (12CA5; 1:2,000) used for immunoblot and immunofluorescence were obtained from the Proteins and Antibodies Laboratory (Institut Curie). The anti-GFP (IgG fraction) used for electron microscopy staining was purchased from Invitrogen.

Live-cell imaging and image quantification. HeLa cells were seeded onto 25-mm-diameter glass coverslips, 1 d before transfection. Twenty hours after transfection with the indicated RUSH plasmids, coverslips were transferred into L-shape tubing Chamlide (Live Cell Instrument), filled with pre-warmed carbonate independent Leibovitz's medium (Invitrogen). At time 0, medium was removed and D-biotin (Sigma-Aldrich) at 40 µM final was introduced in the chamber using the tubing. Time-lapse acquisitions were done at 37 °C in a thermostat-controlled chamber using an Eclipse 80i microscope (Nikon) equipped with spinning disk confocal head (Perkin) and a CoolSnapHQ2 camera (Roper Scientific). Images were acquired using a 63× objective and MetaMorph software (Molecular Device). Integrated intensity was measured at each time point in a region of interest (ROI) comprising the Golgi complex. The position of the Golgi complex was obtained using later time point and used for the entire time-lapse quantification. Integrated intensity of an identical size ROI corresponding to background was measured and subtracted from the values of the integrated intensity of the Golgi for each time point. These values were then normalized to the maximum value. These quantifications were made using the MetaMorph software, and we used Prism 4 (GraphPad Software) for drawing the plots.

For live-cell total internal reflection fluorescence (TIRF) microscopy, cells were seeded and release was performed as described above. Acquisition was performed through a 100× TIRF objective installed on an Eclipse TE2000 inverted microscope (Nikon) equipped with a QuantEM electron-multiplying charge-coupled device (EMCCD) camera (Roper Scientific).

Immunoprecipitation. HeLa cells expressing Ii-streptavidin-HA and ST-SBP-EGFP or ManII-SBP-EGFP were detached with 0.05% trypsin-EDTA (Invitrogen) and resuspended in complete medium. Biotin was added in the cell suspension and at indicated times an aliquot of cells was collected. Cells were pelleted by short centrifugation at 4 °C, washed in ice-cold PBS and centrifuged again. The cell pellets were then lysed in TNE buffer (10 mM Tris-HCl, 150 mM NaCl and 5 mM EDTA) containing 1% of NP-40 and protease cocktail inhibitor (Sigma-Aldrich) 20 min on ice. The lysates were precleared by centrifugation at 17,700*g* 15 min at 4 °C, the protein content of supernatants was measured using BioRad protein assay and BSA standards. We incubated 340 µg of total protein of each sample with 50 µl of protein A coated Dynabeads (Invitrogen) and 600 µl of anti-GFP scFv-hFc containing supernatant (clone G3a)[33], for 3 h, with rotation. After three washes in TNE containing 0.1% NP-40, immunoprecipitated proteins were incubated with Laemmli buffer containing β-mercaptoethanol, boiled and analyzed by SDS-PAGE using Nupage 4–12% Bis-Tris acrylamide gels (Invitrogen) for western blotting detection with anti-HA and polyclonal anti-GFP followed by incubation with secondary antibodies coupled to horseradish peroxidase (Jackson ImmunoResearch).

Electron microscopy. For immunoelectron microscopy, cells were fixed with a mixture of 2% PFA and 0.2% glutaraldehyde in 0.1 M phosphate buffer and processed for ultracryomicrotomy and immunogold labeling[34]. Ultrathin cryosections were single immunogold–labeled with antibodies and protein A coupled to 10-nm gold particles. Sections were observed under a CM120 electron microscope (FEI company), equipped with a KeenView camera (Soft Imaging System).

Generation of stably expressing cells. HeLa and RPE-1 cells stably expressing Str-KDEL as a hook and either ManII-SBP-EGFP or ManII-SBP-mCherry as a reporter (in a pCDH1 vector) were transduced using lentiviral particles produced in HEK293T cells. We then enriched GFP-positive cells by cell sorting.

High-content assay. HeLa cells stably expressing ManII-SBP-GFP with Str-KDEL as a hook were seeded at 15,000 cells per well in a plastic bottom high content screening plate (View Plate, Packard). The day after, biotin was added for 0 min, 15 min, 30 min or 60 min at 37 °C and cells were then fixed using paraformaldehyde

3% in PBS. In some wells, nocodazole or brefeldin A was added for 60 min or 15 min, respectively, before biotin addition. In this case a single point of release (30 min) was chosen. Cells were then imaged using an InCell 2000 microscope (GE Healthcare) and images were analyzed using the InCell Analyzer to quantify the fluorescence recovered in the Golgi and the overall shape of Golgi elements.

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