Cholesterol signaling in single cells: lessons from STAR and sm-FISH

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Abstract

Cholesterol is an important regulator of cell signaling, both through direct impacts on cell membranes and through oxy-metabolites that activate specific receptors (steroids, hydroxy-cholesterols, bile acids). Cholesterol moves slowly through and between cell membranes with the assistance of specific binding proteins and transfer processes. The prototype cholesterol regulator is the Steroidogenesis Acute Regulatory (STAR), which moves cholesterol into mitochondria, where steroid synthesis is initiated by cytochrome P450 11A1 in multiple endocrine cell types. CYP27A1 generates hydroxyl cholesterol metabolites that activate LXR nuclear receptors to control cholesterol homeostatic and transport mechanisms. LXR regulation of cholesterol transport and storage as cholesterol ester droplets is shared by both steroid-producing cells and macrophage. This cholesterol signaling which is crucial to brain neuron regulation by astrocytes and microglial macrophage, is mediated by ApoE and is sensitive to disruption by β-amyloid plaques.

sm-FISH delivers appreciable insights into signaling in single cells, by resolving single RNA molecules as mRNA and by quantifying pre-mRNA at gene loci. sm-FISH has been applied to problems in physiology, embryo development and cancer biology, where single cell features have critical impacts. sm-FISH identifies novel features of STAR transcription in adrenal and testis cells, including asymmetric expression at individual gene loci, delayed splicing and 1:1 association of mRNA with mitochondria. This may represent a functional unit for the translation-dependent cholesterol transfer directed by STAR, which integrates into mitochondrial fusion dynamics. Similar cholesterol dynamics repeat with different players in the cycling of cholesterol between astrocytes and neurons in the brain, which may be abnormal in neurodegenerative diseases.

Introduction

Cholesterol is both a key heterogeneously distributed component of cell membranes and a substrate for the synthesis of steroids and other biologically active derivatives. The transfer of cholesterol between and within cells has a significant effect on their activity but is typically a local change that benefits from analysis at the single cell level. Here, we focus on the Steroidogenesis Acute Regulator (STAR) as an intracellular organizer of such processes. We aim to supplement many excellent reviews of cholesterol trafficking and STAR with a perspective based on the importance single cell activities. These processes are shared by steroid-producing cells and macrophage,
particularly as the latter function in the brain as microglia to process the remarkably high levels of cholesterol. We review how direct imaging of gene expression in single cells mainly as exemplified for STAR, can be measured by a new imaging approach, sm-FISH, and thereby provide insight into local cholesterol signaling.

**Discovering cholesterol networks**

A new direction in steroid synthesis emerged from the finding that ACTH stimulation of adrenal cholesterol metabolism was rapidly stopped by protein synthesis inhibitors, such as cycloheximide (CHX) (Davis & Garren 1968), even though the metabolism was mediated by a specific stable mitochondrial, cytochrome P450ccc/CYP11A1 (Simpson & Boyd 1967, Jefcoate & Boyd 1971). This CYP11A1 formed a potent complex with cholesterol that was quantified by unique optical and magnetic resonance spectra (Jefcoate et al. 1973). Quantitation of this complex was used to characterize a pool of reactive cholesterol in the inner mitochondrial membrane (IMM). In cultured primary bovine adrenal cells, ACTH stimulation of cholesterol access to this cytochrome P450 was stopped by CHX, while causing cholesterol accumulation in the outer mitochondrial membrane (OMM) (DiBartolomeis & Jefcoate 1984). This restraint was overcome by hydroxyl cholesterol derivatives that reached the CYP11A1, without the need of this translation-coupled factor. This led to a search for a gene that delivered a protein that generated a translation-coupled cholesterol transfer from OMM to IMM that could be by-passed by 25-hydroxycholesterol (Jefcoate et al. 1973). Ten years later, STAR was discovered (Clark et al. 1994, Manna et al. 2009). The effects on steroid synthesis were demonstrated by the results of deletion in mice (Ishii et al. 2002).

Over a further 20 years, a family of diverse STARD cholesterol exchange proteins has been characterized for cholesterol mobilization (Letourneau et al. 2015). Cholesterol is restricted to cell membranes and even to one side of a bilayer and directly exerts local effects by producing islands of low fluidity. These changes affect the distribution of membrane proteins, notably signaling proteins. These local effects are enhanced by the transfer of caveolin, which has the effect of co-localizing proteins that engage in signaling crosstalk. Cholesterol only moves between cell membranes through direct membrane contacts or more typically transfer to and from cholesterol-binding proteins such as STAR and the family members STARD3 and STARD4.

The STAR cholesterol partnership functions as the core signaling component for steroid signaling (Manna et al. 2009). Much of this work on steroid synthesis has been carried out with mouse Y-1 and MA10 cell lines that derive from respectively adrenal and testis Leydig tumors. These lines exhibit similar CAMP-induced levels of STAR expression and reproduce fundamental adrenal/testis differences. Thus, Y-1 adrenal cells show a low basal STAR expression with a rapid steroidogenic response to CAMP analogs that peaks within 15 min, whereas MA10 Leydig cells have minimal basal STAR expression that only appears with steroid synthesis after about 30 min. These cells have low expression of some contributors to the equivalent primary cells; notably, CYP11B1 in Y-1 cells and CYP17 in MA10 cells. We describe here how sm-FISH distinguishes these lines but also emphasize their shared features.

Hydroxyl cholesterol and carboxy-cholesterol ( bile acids) derivatives expand this cholesterol network through, respectively, LXR and FXR receptors (Evans & Mangelsdorf 2014). Cholesterol also signals by linkage to hedgehog proteins, which are critical mediators of development, notably for limbs, cranial structures and the nervous system (Luchetti et al. 2016). Cholesterol additionally controls signaling by generating functionally distinct membrane domains, which can be imaged by high-resolution microscopy (Maekawa et al. 2016).

Cholesterol trafficking has been separately studied in macrophage (Rong et al. 2013). Phagocytic macrophage and steroid-producing cells are notably distinguished from the fat-accumulating cells of adipose, muscle and liver in that cholesterol esters accumulate rather than triglycerides (Khor et al. 2014). Regulation of cholesterol trafficking and activity is provided by the LXR receptors and their activation by hydroxycholesterol derivatives. Here STAR plays an essential role by mediating cholesterol transfer to intra-mitochondrial CYP27A1. STAR transfection into macrophage decreases secretion of inflammatory factors (Graham 2015), whereas deletion introduces the expression of inflammatory macrophage markers in adrenal cortex cells (Ishii et al. 2012).

With aging populations, neurodegenerative diseases, notably Alzheimer’s disease, have become a significant focus of research. All evidence points toward cholesterol signaling as a major contributor, most notably through abnormalities in neural circuits for the activities of Apo E, oxidative stress and various hydroxycholesterol derivatives (Czuba et al. 2017). Cholesterol is ten times more concentrated in the brain than in any other organ, in ways that are functionally selective. Cholesterol trafficking...
networks, involving neurons, astrocytes, microglia and the blood–brain barrier, provide a new understanding. Notably, loss of the cholesterol trafficking mediator, NPC1, causes both early neurodegenerative disease, while increasing oxidative stress and innate immune responses in macrophage (Zhang et al. 2008, Platt et al. 2016). A variety of lipophilic amines that inhibit NPC-mediated cholesterol export from endosomes cause both oxidative stress and increased β-amyloid deposition in cultured astrocytes (Yang et al. 2017).

Introducing STAR and other cholesterol mediators

STAR was discovered as a cAMP-induced 37 kDa mitochondrial protein in MA10 testis cells that is expressed in nearly all steroid-producing cells. STAR transfection reconstituted cholesterol transfer and steroidogenesis in non-steroidogenic COS1 cells (Manna et al. 2009). This same protein had previously been shown to be stimulated by ACTH in adrenal cells, and to be highly sensitive to CHX, in line with the steroidogenic response (Pon et al. 1986). We now know that this CHX sensitivity derives from the unique mitochondrial import and inactivation of p37 STAR, directed by the N-terminal segment. Import of STAR into the IMM is sensitive to CHX due to the need for continuous re-synthesis to maintain activity (Artemenko et al. 2001, Bose et al. 2002, Sasaki et al. 2008). The reactive pool of IMM cholesterol is rapidly metabolized by CYP11A1 to pregnenolone through three successive oxygenase cycles, supported by electron donation from the ferredoxin, adrenodoxin and NADPH flavoprotein reductase (Hanukoglu & Jefcoate 1980). A shuttle mechanism for ADX has been confirmed by the crystal structures of the CYP11A1–adrenodoxin and adrenodoxin–NADPH reductase complexes (Strushkevich et al. 2011). In the steroidogenic mitochondria, electron microscopy and protein crosslinking positions HSD3B2, which converts pregnenolone to progesterone, on the outside of the IMM with dependence on OMM TOM22. CYP11A1 is located on the inside of the IMM (Rajapaksha et al. 2016).

Human mutations, which prevent STAR activity, are mostly localized to the cholesterol-binding region (Stocco 2002). The complexity of cholesterol import is shown by microscopy images, which show the extensive cytoplasmic cholesterol ester accumulations in steroid-producing cells when STAR is disrupted (Clark et al. 1994). Deletion of the N-terminal domain causes similar accumulations, but with appreciable tissue selectivity in the loss of steroid synthesis (Sasaki et al. 2008). Fifteen genes share homology with STAR, five of which have detailed structures. Two others function with cholesterol (STARD3 and STARD4) (Letourneau et al. 2015). However, only STAR has the N-terminal sequence. Significantly, STAR is active in mesenchymal progenitor cells (Anuka et al. 2013) and macrophage (Graham 2015) without associated steroid synthesis.

Cholesterol affects cell signaling through a network in which local micro-domains function as regulatory foci. STAR, related STARD family members, and other cholesterol-sensing proteins provide the organization for this system through particular functions in different cell locations (Fig. 1 and Table 1). The unique activity in cell membranes derives from the formation of cholesterol islands of low fluidity that additionally recruit cholesterol sensor proteins like STAR.

Figure 1

STAR effects on cholesterol dynamics in steroid-producing cells are substantially shared by macrophage/microglia. Cholesterol (CH) enters cells via LDL/LDLR and HDL/SRB1 complexes. LDLR delivers CH esters to the endosome network (E). SRB1 delivers CH esters to lipid droplets (LD) via vimentin microfilaments (MF). CH also transfers from HDL into the plasma membrane. CH becomes available to STAR from the NPC1/NPC2 combination in late endosomes and through hormone-sensitive lipase (HSL) from LD. Late endosomes also transfer CH to the endoplasmic reticulum (ER) through bridging activity of STARD3. Extended arrows indicate CH transfer steps. In brain microglia, CH processing in the endosomes overlaps with the uptake of β-amyloid from plaque accumulations, which is mediated by the receptor TREM2 and also by ApoE. CH is sensed in the ER by the SCAP, which tethers the CH biosynthesis stimulant, SREBP2. When CH is low, SREBP2 is released to Golgi (Go) where cleavage provides the SRE transcription factor, which transfers to the nucleus. STAR transfers CH into mitochondria (OMM to IMM) for conversion by CYP11A1 and HSD3B2 to progesterone (PROG). A minor IMM conversion of CH to hydroxyl cholesterols (HO-CH) by CYP27A1 generates an activator of LXR, which induces ATP-dependent CH export pumps, ABCA1 and ABCG1. STARD4 mediates this export and other CH redistribution steps. These transporters associate with, respectively, LDLR and SRB1, to transfer CH back to LDL and HDL, respectively.
A different type of sensor protein, caveolin, was identified as a source of plasma membrane indentations, now called caveolae (Sala-Vila et al. 2016). Caveolin acts differently by forming multimers when bound by cholesterol. These structures recruit cell–cell adhesion proteins, receptors and other contributors to intercellular and intracellular signaling networks (Jansen et al. 2008, Sowa 2012). Caveolin also functions at mitochondrial/endoplasmic fusion domains (MAM sites) that partner with STAR in cooperation with the sigma receptor (Su et al. 2016) and another cholesterol-binding protein, TSPO (Fan et al. 2015).

The transporter Nieman Pick Type C1 (NPC1) plays a core role in the endosome network, which imports cholesterol through LDL receptors (Prasad et al. 2015). NPC1 not only switches cholesterol from the inside to the outside of endosomes but also nucleates a complex with SLC38A9, which when bound by cholesterol recruits and activates the key energy regulator, TORC1 (Eid et al. 2017). The dual function STARD3 forms a bridge between late endosome and endoplasmic reticulum (ER) (Raiborg et al. 2015, Wilhelm et al. 2017). The cholesterol-binding domain that is highly homologous to the equivalent STAR domain binds to ER, while a Rho-Gap domain binds to the endosomes. Another cholesterol-binding protein, ORP5, which also

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<td>SF1/NR5a1 transcription factor</td>
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<td>Cyp11a1 IMM CYP</td>
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<td><strong>Distinctive activities</strong></td>
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<td>Abcg1: Plasma membrane CH export pump (to ApoA and HDL)</td>
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<td>ApoE: Serum protein that transport CH and esters. Enters endosomes</td>
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<td>ApoA-HDL: CH exported via Abcg1 pump to ApoA, a component of HDL</td>
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<td>ApoE-LDL: ApoE binds receptor and also Caveolin: Core protein of CH-rich caveolae signaling domains</td>
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<td>LDLR: LDL receptor; binds ApoE and mediates CH-ester endocytosis</td>
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<td>SRB1: Scavenger class B receptor – binds ApoA mediates CH/ester uptake</td>
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<td><strong>Endosomes</strong></td>
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<td>NPC1: CH-binding endosome exporter</td>
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<td>NPC2: NPC1 co-activator</td>
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<td>SLC38a9: CH sensor that binds NPC1 and also TORC complex</td>
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<td>STARD3: Rho-GAP domain links endosome and ER; C-terminal CH binding</td>
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<td>STARD4: STAR-related lipid transfer (START) domain containing 4</td>
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<td><strong>Endoplasmic reticulum (ER)/Golgi (Go)</strong></td>
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<td>Scap: CH-binding sensor sequesters SREBP2; prevents Golgi cleavage</td>
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<td>Cyp11a1: Cholesterol side chain cleavage</td>
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<td>Cyp27a1: CH side chain hydroxylase at 25- and 27-positions</td>
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<td>STAR: N-terminal OMM targeting; C-terminal CH binding</td>
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<td>TSPO/PBR: OMM protein, CH-binding domain; enriched at OMM/ER contacts</td>
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<td>Affects membrane channels</td>
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<td>TOM22: Translocase of outer mitochondrial membrane</td>
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<td><strong>Lipid droplets (stores cholesterol esters)</strong></td>
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<td>HSL: Hormone-sensitive lipase – liberates CH from esters</td>
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<tr>
<td>ACAT: CH – acyl CoA acyl transferase</td>
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<td>Perilipins: Peripheral structural proteins</td>
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<td><strong>Nucleus</strong></td>
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<td>LXRa: Nuclear receptors activated by side chain 25- or 27-hydroxy-CH</td>
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<td>LXRb</td>
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<td><strong>Cytoplasm</strong></td>
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<td>Orp5: CH/oxysterol activated proteins/bind phosphoinositides</td>
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<td>STARD4: CH transfer protein</td>
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targets phosphoinositides (Olkkonen & Li 2013), transfers the cholesterol between these organelles and to STAR at the mitochondrion. NPC1 and STARD3 mark different micro-domains of late endosomes (van der Kant et al. 2013). STARD4, which corresponds to STAR without the N-terminal domain, mediates more general inter-membrane cholesterol transfer, including export through the plasma membrane (Iaea et al. 2017). This export functions in caveolae through ATP-dependent pumps that deliver cholesterol to LDL or HDL (ABC1A, ABCG1). Import is mediated at these same sites by, respectively, the LDL receptor and the SRB1 (Fig. 1).

**Cholesterol signaling processes**

Cholesterol levels are subject to tight homeostasis involving these import/export processes and synthesis. Cholesterol binding to the sensor, SCAP, located in the ER controls the activation of the central synthesis stimulant, SREBP2, which regulates all genes in the cholesterol synthesis pathway. When bound by cholesterol, Scap tethers SREBP forms to the ER, thus preventing their protease activation in the Golgi (Shimizu-Albergine et al. 2016). The mediation of cholesterol exchange between multiple organelles by STARD4 ensures that the cholesterol readout by SCAP is broadly representative (Iaea et al. 2017). STARD4 is coupled to the cholesterol homeostasis neonatal liver (Maguire et al. 2017).

Cholesterol directs signaling through conversion to numerous signaling derivatives, including hydroxyl cholesterols (LXR activation), steroids, vitamin D and bile acids (FXR activation), each with their specific receptors. Multiple forms of Cytochrome P450 contribute to the formation of these derivatives. Thus, hydroxyl cholesterol products are formed by mitochondrial CYP27A1, which functions in many cell types, and by the ER enzyme, CYP46A1 (hydroxylcholesterol derivatives) (Mast et al. 2017). In diverse cells, including hepatocytes and macrophage, STAR provides cholesterol access to CYP27A1, thereby producing 25- and 27-hydroxycholesterol, which are potent LXR activators. These nuclear receptors stimulate a network consisting of STAR, cholesterol export pumps and SREBP regulators of cholesterol and fatty acid synthesis (Cummins et al. 2006, Jefcoate 2006).

The tight control over cholesterol micro-domains is coordinated with activities of the hedgehog family of developmental regulators (Sonic/SHH, Desert/DHH and Indian/HHH). These signaling molecules commonly function in mesenchymal cells during development. They contribute to limb and skeletal morphogenesis during embryogenesis (Luchetti et al. 2016). Donor cells release Hh forms to activate response cells that through binding to Patch (PTCH) receptors, thereby activating a plasma membrane partner smoothened (Smo) and one of three Gli transcription factors. The PTCH receptor represses the transmembrane protein Smo until activated by Hh ligands, which then stimulates signaling to activate Gli transcription factors. Cholesterol modifies this signaling through linkage to the C-terminal amino acid, while C-16 palmitic acid links to the N-terminus. These modifications do not change the Hh affinity for PTCH receptors, but rather increase the stability and range of Hh transfer. These signaling processes play key roles in the development of steroidogenic tissues where STAR is also an early participant. Disruptions of cholesterol synthesis cause serious deficiencies in Hh signaling. An interesting caveat is that hydroxyl cholesterols activate Smo, notably 20S-hydroxycholesterol (Nachtergaele et al. 2012). Hydroxyl cholesterols activate multiple response proteins (Guillemot-Legris et al. 2016).

These developmental processes function on a single cell basis, which can now be measured by sm-FISH through the expression of functional marker RNA molecules.

**Cholesterol dynamics of steroid-producing cells extends to microglia astrocytes and neurodegeneration**

STAR and other cholesterol network players recognized from endocrine cells function together in astrocytes and microglia, the resident neural macrophage (Sierra et al. 2003, Benmessahel et al. 2004). In the brain, single cell biochemistry is critical to specificity. Cholesterol circulation between the blood–brain barrier, astrocytes, microglia and neurons plays important functional roles. Notably, storage of cholesterol acyl esters by phagocytic macrophage and astrocytes delivers cholesterol is a key neuron repair mechanism (Khor et al. 2014).

ApoE complexes with cholesterol, the esters and other lipids to form LDL (Fryer et al. 2005). The lipoprotein interacts with LDLR by binding ApoE with subsequent uptake through endosomes. ApoE functions in the cell but is either recycled or degraded (Schmukler et al. 2018). Cholesterol is released from esters by an acid hydrolase in the late endosomes, which merge with lysosomes. Thus cholesterol is transferred across the vesicle membrane by NPC1 and a partnering protein, NPC2 (Maxfield et al. 2016). This cholesterol has broad effects on cell metabolism through NPC1-mediated autophagy (Eid et al. 2017). STARD3 and STARD4...
transfer cholesterol to, respectively, the ER or to other cell membranes. HDL, on the other hand, interacts with the SRB1 receptor through the APOA components (forms 1 or 2) with the transfer of mostly esters to lipid droplets (Maiga et al. 2014).

In the brain, ApoE also complexes β-amyloid (β-A) plaques that are additionally recognized for clearance by phagocytosis into microglia through TREM2, a surface receptor. This complex also generates signals through a partnering protein DAP12 that effects microglia proliferation around the plaques. This βA is also transferred from plaques by TREM2 into endosomes for eventual degradation in lysosomes. TREM2 exhibits extensive sequence variation in humans with functional deficiencies predictive of ND (Jay et al. 2017). Aging mice and mouse neurodegenerative disease (ND) and demyelination models each link TREM2 and APOE (Krasemann et al. 2017). Functional deficiencies are effectively seen by single cell imaging (Wang et al. 2016, Krasemann et al. 2017). The morphology of microglia that associate with βA plaques changes with defective Trem2 variants (Condello et al. 2018). These microscopy studies are opening up future possibilities for linking Trem2 variation to single cell changes in cholesterol-associated gene expression.

Loss-of-function mutation in NPC1 causes early onset ND. The disease in NPC−/− mice is appreciably reversed by sequestering cholesterol in vivo with hydroxypropyl-cyclodextrin (Tamura & Yui 2018). Restoration of NPC, specifically to microglia or astrocytes, each partially modifies the ND indicating dual contributions (Fan et al. 2015, Prasad et al. 2016, Marshall et al. 2018). STAR, STARD3, STARD4 and other cholesterol-binding proteins, such as the sigma receptor in the ER and TSP0 in mitochondria, provide overlapping cholesterol signaling in both steroidogenesis and these microglial functions (Borthwick et al. 2010) (Table 1).

Another predictor for ND in humans is the extent of enhanced expression of APOE4 relative to APOE3, which can involve threefold changes (Schmukler et al. 2018). The single R/C112 amino acid change in ApoE4 diminishes the import and cycling of both cholesterol and βA. APOE4 also differs in the interactions between βA and TREM2, potentially favoring an inflammatory response over phagocytosis (Condello et al. 2018). This substitution preferentially forms monomers over the normal tetramer (Mahley 2016, Chetty et al. 2017). Disruption of the overlapping trafficking of cholesterol and βA through the endosomes to lysosomes may also contribute to ND by disrupting cholesterol signaling in ways seen with NPC1 disruption. STAR-dependent cholesterol dynamics also function in microglia and astrocytes, notably by controlling LXR activity (Graham 2015). The substantial positive effects of LXR and PPARγ activators on ND models also support a central role of cholesterol dynamics in the microglial–astrocyte–neuron circuitry (Skerritt et al. 2015). Increases in the cholesterol exporter, ABCA1, which is induced by LXR, decreases βA deposits, whereas reduced expression increases deposition and causes aberrant macrophage functions (Sene et al. 2013, Mahley 2016). STAR induction in microglia is a notable response to brain injury (Garcia-Ovejero et al. 2005), possibly by increasing LXR activity through transference of cholesterol to mitochondrial CYP27A1. Cholesterol ester hydrolysis also delivers arachidonic acid, which activates PPARγ following conversion to 15-HETE by CYP1B1 (Lefevre et al. 2015).

Single cell transcriptomics: sm-FISH complements sc-RNAseq: spatiotemporal and high-resolution advances stimulated by STAR

Single cell heterogeneity is a significant part of these cholesterol-dependent functions. Single cell evaluation of the transcriptome delivered by sc-RNAseq in comparison to the genome sequence (Junker & van Oudenaarden 2014, Saliba et al. 2014) can now be further dissected at the level of individual cell epigenetic controls (Clark et al. 2017). The spatial relationship of individual cells identified by their distinctive expression profiles can be characterized by sm-FISH (Raj & van Oudenaarden 2008). Cholesterol trafficking and the gatekeeper function of STAR have provided unexpected features that, as we will discuss, may expand beyond steroidogenic processes (Lee et al. 2016a).

sc-RNAseq provides a readout of cell diversity within a population based on more highly expressed sequences (Junker & van Oudenaarden 2014, Saliba et al. 2014). The single cells are isolated and separated by flow methods, thus providing a relationship between cell surface markers and the internal gene expression. The positioning of a cell within a tissue is approximately fixed by initial laser capture dissection (Kolodziejczyk et al. 2015). The many thousand sequences obtained from a single cell, measure diversity and provide the opportunity to identify rare cells along with insight into their function and origin. Such analyses have advanced understanding of developmental processes from stem cells (Kumar et al. 2017), while also characterizing the diversity of cells arising from IPS tissue regenerations (Phillips et al. 2017).
Adaptation of clustering algorithms developed for macro-RNA-seq combined with the statistical power of large cell numbers provides a unique perspective on intercell relationships and developmental transitions that are blurred in macro-RNA-seq. Single cell analyses improve the precision of pathway associations, including raising important questions concerning whether differences are stochastic or arise from differences in regulation (Kolodziejczyk et al. 2015). Biological variation in single cells inevitably appear from local signaling (endocrine, paracrine, adhesion) and cell or clock cycles. Much of this variability is predictable from prior macrostudies and can be avoided in the selection of cell type markers. Thus, STAR exhibits very diverse expression including from Clock signaling (Son et al. 2008). The counterpoint between stochastic and regulated processes is fundamental to expression uncertainty at the single cell level.

The core sc-RNAseq method, which is highly adaptable to needs, requires single cell isolation, separation and lysis followed by RNA isolation. Most manipulation occurs in reverse transcription (RT), in which primer barcoding is used to link transcripts to cell identity (Fu et al. 2014). In the subsequent PCR amplification the use of specific primers provides accurate quantitation when linked to fluorescent primers (Universal Molecular Indexing/UMI). Fundamental limitations include single cell recovery, which depends on size and shape. The RT step usually targets only the mRNA and is complicated by the large excess of non-coding RNA and ribosomal RNA. Usually mRNA recovery is only about 10 percent although with special efforts this has been improved to about 40 percent (Junker & van Oudenaarden 2014). An advance in sensitivity and spatial location has been provided by the FISSEQ procedure, which combines random in situ PCR to generate clusters of cDNA (Lee 2017).

Single molecule fluorescence in situ hybridization of mRNA (sm-FISH) has been developed to complement sc-RNAseq (Kwon 2013, Tsanov et al. 2016, Halpern et al. 2017) (Table 2). sm-FISH is based on the high affinity binding of multiple tandem fluorescent 20mers (Raj & van Oudenaarden 2008, Kwon 2013). sm-FISH provides precise 3D positioning of individual cells based on mRNA markers identified by sc-RNAseq. At high resolution, mRNA particles provide a quantitative digital readout of single cell expression. The labile expression at the gene loci including primary/spliced ratios (p-RNA/sp-RNA) provides a near-instant digital probe of transcript, splicing and further processing. We have developed such single cell analyses for STAR through comparison of 20–30 adjacent single cells to parallel macro-PCR analyses (Lee & Jefcoate 2017).

To multiplex mRNA species in order to identify a cell type or a regulatory network the probe design employs combinatorial features (Moffitt & Zhuang 2016, Tsanov et al. 2016). Such multiplexing is achieved by dividing the 40 oligomers into several sets marked by different dyes banded in various combinations. The FISH probe is also constructed in two parts; an oligomer recognition segment and a readout segment comprising either distinguishing fluorescent tags or a barcode. This approach is used in the MERFISH protocol (Moffitt & Zhuang 2016).

We have developed a high-resolution sm-FISH that resolves primary and spliced transcripts (p-RNA, sp-RNA) at STAR gene loci (Lee et al. 2016b). p-RNA and sp-RNA were co-localized with locus DNA that was detected after removal of all RNA. STAR mRNA is seen at specific locations within the cytoplasm by using the sp-RNA oligomer set. The mRNA molecules have been counted as single particles using image quantitation software.

sm-FISH has been expanded to image mRNA in live cells initially through large 3′UTR RNA inserts. The first strategy used 24 repeat RNA loops that capture fluorescent bacterial coat proteins as the RNA appears. This fluorescence is sufficient to track a single molecule (Tutucci et al. 2018), but the bulk inserts can potentially perturb the process. A recent alternative approach detects the new mRNA with a single-stranded oligonucleotide molecular beacon (MB) (Chen et al. 2017) comprising a fluorophore flanked by a quencher. The MB becomes fluorescent after hybridization to complementary mRNA sequence. This method needs only 8 repeat probe structures comprising 400 nucleotides. The MB inserts do not affect the target mRNA distribution when determined by sm-FISH in fixed cells. The MB reporter insert delivers live cell video images that track complex stochastic intracellular movement of single mRNA molecules.

**STAR as a challenge for single cell biochemistry and sm-FISH**

This single cell biochemistry has greatly accelerated by progress in high-resolution fluorescent microscopy (Shashkova & Leake 2017). sm-FISH highlights RNA and, therefore, is complementary to electron microscopy, which locates single proteins with organelle membranes, notably in recent studies of STAR (Prasad et al. 2015, Rajapaksha et al. 2016). STAR has shown remarkable characteristics that allow adaptation to diverse functions. The roles of STAR and cholesterol signaling range beyond...
### Table 2 Integration of sc-RNAseq and sm-FISH in single cell functional analyses.

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**Notes:**
- FISSEQ: in situ RT-PCR
- Multiplex detection methods
- Quantitation of copy numbers
- Live cell probe design
- Application development
- In situ CRISPR editing

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**References:**
- C R Jefcoate and J Lee. Cholesterol dynamics and StAR imaged by sm-FISH. *Journal of Molecular Endocrinology*. 2018. [DOI: 10.1530/JME-17-0281](https://doi.org/10.1530/JME-17-0281)
steroid synthesis to multi-potential progenitor cells in heart repair (Anuka et al. 2013) and neural networks (Sierra et al. 2003, Czuba et al. 2017). Hedgehog signaling highlights local cholesterol-sensitive signaling, which has been examined by sm-FISH (Adolphe et al. 2017). This cholesterol-controlled hedgehog signaling directs the development of the mesenchymal cells, which differentiate into STAR-regulated steroid-producing cells. sm-FISH and single cell biochemistry will improve the analysis of localized steroid synthesis, notably the distinction between de novo synthesis from cholesterol, where STAR is a likely participant, and the metabolic diversion of systemic steroids.

The scope of sm-FISH and single cell characterization

What advantages does sm-FISH provide?

sm-FISH is based on the much greater response and binding specificity of 20-base fluorescent oligomers, as compared to the traditional 300–800 base FISH probe. We have used the synergy between oligomers to separately probe primary STAR RNA transcripts and their spliced products at the gene loci, together with single mRNA molecules in the cytoplasm. The intensity of fluorescence at loci is proportional to the number of transcripts, whereas the number of cytoplasmic mRNA particles determines this single cell expression level. The levels of each RNA species exhibit a wide range. However, when summed for 20–30 adjacent cells mean copy numbers per cell match numbers obtained by PCR from equivalent macro-cultures (Lee et al. 2016a).

The examination of transcription at the level of individual gene loci in single cells reveals random ‘bursts’ of activation of labile reporters. These kinetics led to a stochastic model for activation of transcription (Suter et al. 2011). In this burst model, an activator protein associates randomly with the transcription initiation complex, stimulates POL2 to generate some transcripts and then dissociates. The association step depends on the nuclear concentration of the activator, whereas the dissociation is concentration independent. Such stochastic time courses have been analyzed over a few hours in individual cultured cells by using labile reporters (Nicolas et al. 2017). This simple model surprisingly provides a good fit for several more complex systems (Bahar Halpern et al. 2015b, Halpern et al. 2017). We find that this model extends well to the single cell stimulation of STAR in cultured adrenal and testis cells.

Application of sm-FISH to single cell characterization in development

Single cell analyses have made vital contributions to questions relating to the spatiotemporal organization of cells during in vivo development. Major contributions by sm-FISH are found in studies on Drosophila (Trecz et al. 2017), C. elegans development (Bolkova & Lanctot 2016), IPS cell analyses (Klemm et al. 2014), stem cell fate (Toth et al. 2017), mouse skin differentiation (Adolphe et al. 2017) and human breast tumor variance (Annaratone et al. 2017).

In colonic crypts, sm-FISH shows that a small number of individual stem cells precisely commit to forming absorptive and secretory cells in a precise ratio in each crypt (Toth et al. 2017). In skin, spatiotemporal development of hair follicles is mediated by Sonic hedgehog (SHH) signaling (Adolphe et al. 2017). sm-FISH mRNA probe sets for Patch1 and the signal mediator, Gli1, provide spatiotemporal markers for SHH-induced changes. Cells that expressed mRNA for, respectively, Patch1 and Gli1 identified sites of peak SHH activity at the base of hair follicles and local gradients of signaling activity.

SHH signaling also plays a central role in adrenal cortex development from progenitor cells (Finco et al. 2015) that are located between the glomerulosa and fasciculata zones. Cells expressing Gli forms differentiate to cells expressing SF1. This core steroidogenic transcription factor then induces STAR, which typically marks an early stage in the steroidogenic development. STAR expression can precede CYP11A1 and other steroid-producing genes, suggesting a role in cholesterol distribution and signaling as a prelude to steroidogenesis (Budefeld et al. 2009). While lineage mapping of these processes has been extensive, complementary sm-FISH analyses provide insight into the individual signaling in different cell locations.

Application of sm-FISH to cell heterogeneity in tumors

sm-FISH analysis of intratumor signaling provides a powerful cancer diagnostic tool. Thus, E\textalpha\ receptors and HER2 transcripts have been analyzed in single cells of normal and archival breast cancer samples (Annaratone et al. 2017). These analyses exposed considerable local variability in the expression levels of E\textalpha\ and HER2. The crosstalk between HER2 and E\textalpha\ provides a critical guide to choices of endocrine therapy. Examination by sm-FISH at the single cell level revealed extensive variability in the spatial distribution of these key activity markers, even
within breast cancers previously diagnosed to be of the same type. The quantitative sm-FISH digital readout of ERα and HER2 provides a major improvement over more traditional histology methods.

sm-FISH analyses could also be used to identify heterogeneity at the single cell level, based on markers of steroidogenesis. Cushing’s syndrome (London et al. 2015) tumors show gene mutations in the cAMP stimulation pathway that increase protein kinase A activity (PRKACA (+), PRKARIA (−) and GNAS (+)), which potentially increase STAR mRNA expression (Zhou et al. 2016). Tumors that produce aldosterone and hypertension (Nishimoto et al. 2015) appear as clusters of single cells that express CYP11B2. A study of 91 adrenal tumors identified driver genes with mutations or epigenetic changes that were assessed through a set of 25 steroidogenic genes, including STAR (Zheng et al. 2016). Again, sm-FISH is readily adapted to the characterization of pathway diversity at the single cell level.

Normal human prostate stromal fibroblasts and tumor cell lines often express STAR, but not CYP17A1, which is needed to produce testosterone. In normal human prostate stroma (Lewis et al. 2014), hyperplasia (BPH) and tumors, the expression of STAR, CYP11A1 and CYP17A1 is variable and uncorrelated. Occasional donors have remarkably high STAR, but little CYP11A1 or CYP17A1. These findings raise important questions concerning whether STAR is functioning in a cholesterol signaling/transport mode seen in heart stroma (Anuka et al. 2013) or as a mediator of steroidogenesis, which requires CYP11A1 expression. sm-FISH analysis brings the added capability of determining the relative active expression in each cell.

**Niche effects in physiology**

In many respects, sm-FISH provides new molecular tools to study physiology; for example, how the location of cells within a tissue ‘niche’ affects their activity. Critical functional niche modulators include proximity to blood flow, lymphatic ducts or nerve terminals. Oxygen, nutrient delivery, access to immune cells and sympathetic neuronal stimuli are particularly critical. This is particularly relevant to steroidogenic cells in the adrenal, testis and ovary, where there are several different cell types, each associated with the complex organization of blood flow and innervation.

Spatiotemporal signaling is pre-eminant in the liver, which has a gradation of oxygen ranging from moderate in the outer region, which receives blood from both the hepatic artery and the portal vein, to low around the central vein (Bahar Halpern et al. 2015b, Halpern et al. 2017). Gene expression and their associated functions are highly polarized; for glucose homeostasis, gluconeogenesis is dominant in the outer zone, for glycolysis, in the inner zone. Most P450 cytochromes are localized close to the central vein. The intervening hepatocytes are engaged with the capillary structure of the sinusoids and their access to the bile duct. Additional cell types contribute signaling, including the endothelial, Kupffer resident macrophage and stellate cells, which store retinoids. This oxygen gradient is complicated by changes in metabolite concentration.

sm-FISH has been used to measure the distribution of single mRNA molecules from multiple genes in intact livers. These analyses have been matched to sc-RNAseq data (Bahar Halpern et al. 2015b, Halpern et al. 2017). Half of the expressed genes exhibit zonal patterning with preferences for one or other extreme or, occasionally, for the middle. The sm-FISH gene expression matched expression from the single cell sequencing. The broad distribution of mRNA content for each gene in their preferred region fits a 2-step burst transcription model. The separation of mRNA between nuclei and cytoplasm show that some mRNA is preferentially retained in the nucleus (Bahar Halpern et al. 2015a). As expected, expression patterns under fasting conditions shift drastically within an hour of feeding. The zonal preferences of markers reflect the overlap of the metabolic gradient and of specific regulatory factors. For example, growth hormone mostly impacts liver sinusoid zones close to the hepatic vein.

The steroidogenic organs show these features of local regulation. Thus, the adrenal gland has three cortex zones, each with different hormonal regulation, steroid synthesis and connections to sympathetic neuronal stimuli provided by the central medulla. The cells have high mitochondrial content and are particularly sensitive to changes in blood flow, which provides oxygen, nutrients and the endocrine signals for aldosterone (glomerulosa/angiotensin) and cortisol (fasciculate/ACTH). The central medulla receives innervation from the sympathetic nervous system and releases catecholamines and other factors to locally affect zonal cortex responses (Ehrhart-Bornstein & Bornstein 2008, Huang et al. 2012, Steenblock et al. 2017). The gland is subject to complex diurnal control by hypothalamic Clock factors, which affect both ACTH and the medulla releases (Fahrenkrug et al. 2012). The testis provides different opportunities. The interstitial regions of each comprise a small number of Leydig cells (15–20 cells in a section) that receive both systemic and local stimuli.
These cells can potentially be characterized individually. We have developed a systematic approach to examining STAR RNA expression, as a marker of such niche effects.

**Regulation of STAR transcription and mRNA regulation**

The stimulation of STAR transcription in MA10 and Y-1 cells by PKA (Manna et al. 2009, Hatano et al. 2016) substantially matches the respective ACTH and LH activations of the adrenal and testis. We have used sm-FISH as a means to compare the mechanisms at the level of single cells. A separate stimulation process activated in these cells by phorbol esters provides a reference for spatiotemporal comparison. This process that is mediated by protein kinase C and MEK/ERK kinase activation (Manna et al. 2009) increases STAR expression similarly but without the STAR phosphorylation, which is needed for cholesterol transfer and metabolism.

The PKA activation, which is typically stimulated by cAMP analogs, depends on specific complexes within a 300bp proximal promoter. The key activators are SF1, CREB and GATA4 and API1, with the further recruitment of various co-activators (Manna et al. 2009, Hatano et al. 2016) (Fig. 2). PKA not only directly phosphorylates CREB and GATA4 but also directs activation through de-sumoylation of SF1 (Yang et al. 2009) and nuclear translocation of CRTC2, an essential co-activator of CREB. CRTC2 recruits the histone acetyltransferase CBP. A second factor, MEF2, associates in a similar fashion with GATA4 (Daems et al. 2015). The second phase of PKA-initiated transcription and translation is mediated by induction of the additional transcription factors, C/EBPβ and NR4A1, which also bind to elements in the proximal promoter (Abedou et al. 2016). The result of this second set of contributors is a surge in transcription after about 75 min in both MA10 and Y-1 cells (Fig. 3A).

The participation of CRTC2 is repressed by a pair of SIK kinases that act in concert (Takemori & Okamoto 2008, Lee et al. 2015, 2016b). SIK1 and SIK2 are each blocked by PKA phosphorylation of equivalent serines (SIK1 S577 and SIK2 S587). SIK2 has high basal expression in the cytoplasm that restrains CRTC2 through dual phosphorylation and sequestration by 14-3-3 (Sonntag et al. 2017). This process is reversed by PKA, with the nuclear transfer of CRTC2 to pS133-CREB on the STAR promoter. SIK1 has a low basal expression, which is mostly nuclear but is extensively induced by PKA within 60 min. However, PKA maintains this form in an inactive state that is mainly outside the nucleus. The S577A-SIK PKA-resistant mutant is completely localized to nuclear speckles that are shared by imported CRTC2. We envisage regulatory inhibition by SIK1 during the decline in cAMP that occurs after the peak of ACTH pulses, which are delivered *in vivo* (Liu et al. 2013). The dominant role of SIK regulation of CRTC2 in STAR regulation is
demonstrated by drugs, such as staurosporine, which potently inhibits SIK forms. In the absence of any PKA activity or CREB phosphorylation, near maximum STAR expression is achieved (Takemori & Okamoto 2008, Lee et al. 2016b).

STAR was among the first genes to be profiled for changes in the association of methylated and acetylated H3 histones during transcription. Increases (H3K4Me2) and decreases (H3K9Me2) in the specific promoter and transcribed regions, which are quantified by chromatin immune-precipitation (ChIP) during PKA stimulation, complement the changes in transcription factor activity (Hiroi et al. 2004).

Figure 3
Uncoupled and coupled transcription of STAR in MA10 cells. (A) sm-FISH profiles for Br-cAMP stimulation of p-RNA and sp-RNA at loci, and mRNA in the cytoplasm (×4). (B) The kinetic model for dual transcription of STAR at separate loci: early uncoupled (first 60 min) and delayed high activity coupled (after 75 min). (C) Diagram of 40-oligomer sets used to detect different transcripts formed at STAR loci: primary (p-RNA), spliced or mRNA (sp-RNA). A third set 40 oligomers (3′EU) extended across the end of the 3′ UTR.

The STAR 3′UTR plays a vital role in the regulation of the translation that often determines STAR activity. This exon 7 sequence includes multiple regulatory sites in the extended 3.5 kb form. Such sites are absent in the 1.6 kb form, which differs through polyadenylation at an alternative earlier 3′UTR polyadenylation site (Ariyoshi et al. 1998). This extended 3′UTR sequence includes specific AU-rich elements that bind the PKA inducible protein, TIS11b/ZNF36l1 (Fig. 2). Also, the mitochondrial targeting protein, AKAP1, interacts with this extended 3′UTR through an adjacent 10 base pyrimidine sequence (UCUUCCUUCCU) (Duan et al. 2009, Grozdanov & Stocco 2012, Lee et al. 2017). sm-FISH images of MA10 cells after stimulation show that one STAR mRNA molecule is associated with most mitochondria that ring the nuclear envelope. These mitochondria are each marked by STAR protein that accumulates in the matrix in an inactive location (Lee et al. 2016b) (see below). TIS11B functions to enhance turnover of the mRNA by recruiting ribonucleases to the poly A tail. The degradation is completely prevented by transcription inhibitors, possibly indicative of partnership with miRNA species. Significantly, AKAP1 has been shown to inhibit translation, probably through recruitment of AGO1 and miRNAs.

A frequent scenario is that shortening of the 3′UTR to remove inhibitory elements, such as those controlled by TIS11B and AKAP1, increases translation (Mayr & Bartel 2009). In MA10 cells, STAR 3.5 kb mRNA is 10 times less efficient than the 1.6 kb mRNA in generating the protein, through effects on both mRNA stability and translation (Duan & Jefcoate 2007). STAR translation and cholesterol metabolism each decreased when TIS11B was suppressed (Duan et al. 2009). TIS11B may, therefore, be co-stimulated with STAR 3.5 kb mRNA to facilitate this cycle (Fig. 2).

PCR analysis of STAR transcription: uncoupling of splicing from transcription is linked to delayed elongation

When analyzed with q-PCR probes for both primary and spliced transcripts, Br-cAMP stimulation of STAR transcription in MA10 cells reveals several unusual features. Although there is an immediate increase in transcription, there is a significant delay (20 min) between the appearances of primary (p-RNA) and spliced transcripts or mRNA (sp-RNA/mRNA) (Fig. 3A). There is also a pause in elongation that occurs at the end of the final intron 6. Elongation, therefore, needs to proceed beyond this intron 6/exon 7 before splicing removal of the six introns can occur. p-RNA and sp-RNA increase...
to similar steady-state levels that characterize this uncoupled transcription process. This is very unusual since transcription typically proceeds with removal of introns as soon as they form (Bentley 2014). However, a surge in sp-RNA without accompanying p-RNA suggests a delayed, but efficient coupled transcription, which also has an appreciably increased overall rate. Initially, p-RNA remains unchanged but declines at a later stage as splicing becomes more engaged. A model for the dual transcription of STAR, as coupled and uncoupled processes, is shown in Fig. 3B.

In Northern blots of STAR expression (Duan et al. 2009), about 75 percent of the mRNA is detected as a 3.5 kb form, while the remaining 25 percent is mostly a 1.6 kb form. Surprisingly, PCR analyses in the initial 60 min failed to catch either spliced or unspliced forms, with products corresponding to the short 3'UTR of this 1.6 kb form. This absence suggests formation from a later processing step.

**sm-FISH analysis of STAR transcription in Y-1 and MA10 cells**

**Resolution of primary and spliced transcripts at STAR loci**

This dual mechanism for STAR expression has been evaluated by using the sm-FISH approach. The design of oligomer sets that distinguish STAR p-RNA and sp-RNA is shown in Fig. 3C. Successful resolution of p-RNA and sp-RNA at STAR loci led us initially to call this design high-resolution FISH (HR-FISH) (Lee et al. 2015, 2016b). The exponential dependence on the number of head-to-tail oligomers has been used in our STAR probe design to distinguish primary from spliced RNA at gene loci. Exons are distinguished in sp-RNA by taking advantage of their short length, which is typically sub-optimal for oligomer synergy: a typical exon of 200 bases is spanned sub-optimally by only ten 20mers. Combination across adjacent exons in the sp-RNA, with additional inter-exon bridging oligomers, expands the network to optimal numbers. A further RNA probe set was created to probe the 3' end of the primary and spliced transcripts (3'EU). This probe set binds 3.5 kb mRNA, but not 1.6 kb mRNA.

After digestion of RNA and with strong denaturation conditions, the p-RNA probe sets also hybridized gene DNA at about half of the STAR and CYP11A1 loci. This appreciable locus selectivity may reflect more open chromatin structures and less DNA methylation. This detection efficiency is similar when the STAR locus is assessed with either p-RNA or 3'EU probes but is weaker with a distal STAR promoter probe set (Lee & Jefcoate 2017).

The selectivity of p-RNA and sp-RNA probes was established during the early stimulation of MA10 cells by Br-cAMP (Lee et al. 2015, 2016b). There are no RNA transcripts in basal loci. Primary transcripts appear first at single loci in increasing numbers of cells without detection of spliced transcripts, which only appear after about 30 min as more loci become transcriptionally active. Evidently, the sp-RNA oligomer sets do not recognize the primary transcripts. Most loci show both p-RNA and sp-RNA after 60 min, with a range of 20–80 transcripts. These p-RNA and sp-RNA accumulations at the loci have intensities that are proportional to the number of transcripts. mRNA in the cytoplasm is scarcely detectable until the surge of stimulation (75–120 min), which matches the increase in sp-RNA detected by PCR during this period.

**Asymmetric activation, elongation and splicing**

The striking new feature of STAR transcription revealed by the sm-FISH images is the asymmetry of the activation of the two loci and the way in which this connected to delayed splicing. These differences between loci extend to asynchrony in the individual cells. Quantitation of these images for 20–30 adjacent cells matches the time courses indicated by the q-PCR analyses. Based on the PCR analyses, a delay in elongation appears to be a limiting step in splicing. Epigenetic changes involving H3K marks (Hiroi et al. 2004) may apply asymmetrically to the two loci in MA10 cells and include an additional exon 7 pause site that is not seen in Y-1 cells.

The simple two-step bursting model for mRNA generation in single cells is consistent with the observed broad distribution curve for mRNA copy numbers per cell. This model is equivalent to the coupled transcription that produces most of the mRNA. The broad distribution of intensities for STAR p-RNA, sp-RNA and mRNA across a range of about 4-fold fits a set of stochastic steps.

At high resolution (Nikon NSIM), p-RNA and sp-RNA accumulations resolve at individual loci, but with variable separations (100–300 nm) (Fig. 4A). These sp-RNA accumulations, however, did not separate from 3'EU RNA oligomers, which target the end of the 3'UTR (Lee et al. 2015, 2016a). We propose that the spatial separation corresponds to two pause sites: an accumulation of multiple p-RNA, each attached to POL2 at the end of intron 6, and a second accumulation, including both
spliced sp-RNA and 3′EU sequences at the end of exon 7. This second accumulation may reflect the delay in polyadenylation and ribonucleoprotein (RNP) packaging of mRNA that limits export to the cytoplasm. This model is shown in Fig. 5.

Following Br-cAMP stimulation, CRTC2 transfers to the nucleus within 5 min. Single cell immunohistochemistry (IHC) images show that CRTC2 only slowly associates with key splicing factor, SC35, which is also captured at many active STAR loci that are identified by sp-RNA (Fig. 4B). CRTC2 may play a role in the slow engagement of splicing complexes at STAR loci during uncoupled STAR transcription. sm-FISH analyses show that the PKA/SIK/CRTC2 crosstalk is central to this transcription. Thus, transfection of S577A SIK1–GFP removes all STAR mRNA expression in cells marked by the GFP fluorescence (Lee et al. 2017). The expression of TIS11B–GFP is similarly effective at removing STAR mRNA (Fig. 2 upper right), presumably in this case through enhanced turnover of STAR 3.5 kb mRNA.

When transcription surges to deliver detectable mRNA, most loci retain p-RNA and sp-RNA corresponding to uncoupled transcription. Additional loci show only sp-RNA. Each fluorescent particle represents a single mRNA molecule. This mRNA is initially seen at mitochondria located at the nuclear periphery and is translated to STAR protein, which accumulates within these mitochondria. The turnover of mRNA leaves a ratio of one mRNA per mitochondria (Lee et al. 2015, 2017). Although increasing with mRNA, STAR protein is appreciable after 60 min, when mRNA is
scarcely detectable (Lee & Jefcoate 2017). STAR cholesterol metabolism is nearly maximum at this time. This absence of detectable mRNA is consistent with slow generation from the uncoupled transcription/splicing process and rapid mRNA turnover.

The mean positioning of STAR loci in this XY plane shifts from close to the nuclear envelope prior to stimulation to a mean displacement of about 2 microns after 60 min of stimulation (Fig. 4E). This expansion is within the span that could be provided by an unwinding of the promoter during activation of the gene. A full 3D resolution of STAR expression in MA10 cells shows loci confined to an XY plane near the midline of the nucleus, a positioning shared by CYP11A1. The mRNA is located below these loci (Fig. 4F). The specificity of this this nuclear spatial positioning points to an additional level of regulation of STAR and CYP11A1 loci, perhaps linked to a shared transcription factor such as SF1.

**Distinctive features of basal expression of STAR in Y-1 cells**

The basal expression of STAR mRNA, which is detected in Y-1 cells, but not MA10 cells, sustain maximum cholesterol metabolism, which is reached after 10 min of stimulation by Br-cAMP (Artemenko et al. 2001). This short treatment does not have an effect on the mRNA level but is sufficient to phosphorylate STAR. This basal mRNA level is scarcely detectable in MA10 cells, which generate cholesterol metabolism much more slowly. In Y-1 cells, this basal expression represents 10–20 percent of the maximum level. The induced STAR mRNA is not a limiting factor in the cholesterol transfer activity. This basal expression exhibits a diurnal fluctuation, mediated by the CLOCK/BMAL dimer through a distal Ebox (Nakao et al. 2007, Son et al. 2008).

Again, the features provided by sm-FISH match q-PCR analyses, but reveal single cell heterogeneity with highly variable levels of p-RNA and sp-RNA in the loci (Lee & Jefcoate 2017). STAR mRNA is detectable in the cytoplasm of only about 20 percent of the cells, which may, therefore, mediate the rapid PKA-induced steroidogenesis (Artemenko et al. 2001). The respective levels of STAR p-RNA and sp-RNA at the loci, and of mRNA in the cytoplasm appear unrelated. Since locus transcripts turnover much faster, STAR expression in single cells may correspond to various activation stages of either the Clock cycle (Son et al. 2008) or a stochastic burst (Nicolas et al. 2017).

Remarkably, the p-RNA expression in many Y-1 loci corresponds to a single transcript, possibly representing a pro-active state that speeds the response to ACTH stimulation. These loci become more active within 15 min, but with no net increase in cytoplasmic mRNA for 60 min. Overall, Y-1 cells present as an additive combination of heterogeneous basal expression, with a superimposed multistep response similar to that in MA10 cells.

**STAR-mediated cholesterol transfer and mitochondrial fusion/fission cycles**

The sm-FISH analyses of STAR mRNA and protein provide substantial evidence for cholesterol transfer that is linked to a single mRNA at each active mitochondrion. The 400 cholesterol molecules transferred for each new p37 STAR (Artemenko et al. 2001), and the activation of this protein by cholesterol (Rajapaksha et al. 2013) suggest a receptor-like process. EM imaging and associated protein crosslinking show an increasingly complex picture (Fan et al. 2015, Issop et al. 2015, Paz et al. 2016, Prasad et al. 2016). The N-terminal sequence is not a limiting factor in model reconstitutions with isolated mitochondria (Kraemer et al. 2017) or COS1 cells (Bose et al. 2002). However, inhibition of IMM metalloproteases selectively blocks cholesterol metabolism in Y-1 cells at an intermediate stage, without affecting the maximum CYP11A1 activity for hydroxyl cholesterol derivatives, which bypass STAR (Artemenko et al. 2001). The conserved N-terminal cleavage sites (Lee et al. 2016b) appear to play a regulatory role. Notably, N-terminal deletion produces tissue-selective suppression effects in mice (Sasaki et al. 2008).

The N-terminal domain connects the translation of STAR mRNA to the trafficking from late endosomes and lipid droplets that occurs in other cells (Rong et al. 2013, Khor et al. 2014) and thereby enhances the specialized Cyp11a1-mediated cholesterol metabolism of steroidogenic cells (Fig. 1). Cholesterol-rich domains can be imaged in live MA10 cells with fluorescent derivatives of the bacterial toxin Perfringolysin O, and after fixation by fluorescent–filipin cholesterol complexes in fixed cells (Maekawa et al. 2016, Venugopal et al. 2016, He et al. 2017).

Serum lipoproteins play a critical role in steroid synthesis (Fig. 1). The LDL/APOE complex at LDLR delivers cholesterol to STAR via late endosomes and NPC1 in competition with transfer to the ER. The HDL/APOA complex directs an equivalent process through the SRB1 surface protein, which delivers cholesterol esters to lipid droplets via the vimentin-microfilament network (Rong et al. 2013, Khor et al. 2014). Cholesterol reaches STAR via the HSL/ACAT equilibration on lipid droplets. However,
a reverse export to APOE/LDL and APOA/HDL directed by STARD4 through ABCA1 and ABCG1, can exceed the metabolism of cholesterol in the mitochondria during hormonal stimulation. In cultured MA10 and Y-1 cells, the replacement of serum and lipoprotein content with defined media redistributes cholesterol to the plasma membrane as a source of steroids (He et al. 2017).

A central feature of STAR activity is an association with the continuous fusion and fission of mitochondria (Castillo et al. 2015), which is also associated with key cellular processes such as ATP generation, apoptosis and autophagy (Murley & Nunnari 2016, Pernas & Scorrano 2016). This cycling contributes a mechanism for cells to adapt to external changes in oxygen and nutrients, much as characterized by sm-FISH in liver (Bahar Halpern et al. 2015b, Halpern et al. 2017). The cycles are rapid with times in the 5–20 min range, close to the translation/import time for p37 STAR in adrenal cells (Artemenko et al. 2001). In this mitochondrial cycle, DNA and protein are exchanged, including the accumulated matrix STAR.

This fusion process is mediated by a set of GTPases (MFN1, MFN2 and OPA1) and their associated enhancer GEFs (Murley & Nunnari 2016, Pernas & Scorrano 2016) (Fig. 6). MFN1 and MFN2 function as dimers at the OMM to initiate fusion, while Opa1 controls the morphology of the IMM including the cristae (Rampelt et al. 2017) and mediates the IMM exchange process. A fourth GTPase DRP1 then completes the cycle by directing the fission to two separate mitochondria. DRP1 is a cytoplasmic protein that is inactive when phosphorylated by PKA or Ca/CaM kinase but is recruited to the OMM after de-phosphorylation by the phosphatase calcineurin. Disruption of these GTPases leads to neurodegenerative disease (Mast et al. 2017).

STAR may redirect this cycle such that cholesterol redistributes from OMM to IMM (Fig. 6). MFN2 is both elevated by Br-cAMP and is a major contributor to STAR stimulation of cholesterol metabolism (Castillo et al. 2015, Paz et al. 2016, Sala-Vila et al. 2016). Mfn2 stimulates the generation of MAM ER/OMM contacts, which enhance the binding of DRP1. Interestingly, STAR and DRP1 proteins show opposite regulation by kinases. Cholesterol may play a central role in these contacts, as evidenced by the enrichment of caveolin at these sites along with the sigma receptor, TSPO and STAR (Castillo et al. 2015, Issop et al. 2015). Critical effects of membrane potential and ERK-mediated STAR phosphorylation add to this process (Duarte et al. 2014). A series of complexes involving interactions between p37 and p32 STAR forms and both TOM22 and VDAC2 in the OMM has been proposed (Prasad et al. 2015, 2016). How phospho-STAR mediates OMM-IMM cholesterol transfer requires an understanding of how the transfer is restrained as membranes redistribute during a fusion/fission cycle in its absence.

Reconstitution of mitochondria with recombinant STAR and lipid droplet components exhibits STAR-mediated stimulation of cholesterol metabolism but without consideration of mitochondrial fusion (Kraemer
et al. 2017). This fusion process can also be reconstituted in vitro, potentially adding to such modeling (Schauss et al. 2010). Ca and GTP have long been implicated in the cholesterol transfer process in isolated mitochondria (Kowluru et al. 1995).

LXR-regulated transcription mediated by STAR and mitochondrial CYP27A1 also contributes to steriodogenesis (Cummins et al. 2006, Jefcoate 2006, Manna et al. 2013). Cholesterol in the plasma membrane is a source of cholesterol for steriodogenesis (Venugopal et al. 2016), but this pool arises from serum LDL and HDL or from ER synthesis. This transfer is directed to STAR and mitochondria through the endosome network, as evidenced by the inhibition of steroid synthesis by U18666A, a selective inhibitor of NPC1 (Lu et al. 2015, Venugopal et al. 2016). The availability of cholesterol in the plasma membrane is readily manipulated by β-methyl cyclodextrin, which sequesters cholesterol (Venugopal et al. 2016).

**Application to in situ CRISPR CAS9 editing of STAR**

Based on these features of cholesterol movement and signaling, we expected STAR to exert diverse effects beyond stimulation of steroid synthesis (Ishii et al. 2002, Ikonen 2008, Manna et al. 2013, Castellano et al. 2017, Kraemer et al. 2017). We have applied a dual guide RNA/CAS9 CRISPR strategy to Y-1 and MA10 cells to delete 1.8 kb of STAR sequence from the proximal promoter, exon 1 and intron 1 (Lee & Jefcoate 2017). sm-FISH was used to show suppression in single CRISPR(+) cells of Br-cAMP stimulation of STAR mRNA, in parallel with deletion of targeted DNA from intron 1. The STAR DNA deletion in the total cell population, determined by PCR, was 50 percent efficient and mostly occurred between 12 h and 24 h after transfection.

In this 12-h period, STAR-deleted cells adapted to the loss of activity by exhibiting a large increase in cholesterol ester droplets in both endosomes and lipid droplets. This Br-cAMP-sensitive response mimics the change seen in adrenals, testes and ovaries of STAR−/− mice (Ishii et al. 2002) and in humans with loss-of-function STAR mutations (Stocco 2002). Similar lipid accumulations are seen when STAR is low, through the removal of SF1 (Hatano et al. 2016) or NPC activity is compromised (Kennedy et al. 2012, Lu et al. 2015). This immediate change implicates STAR in cholesterol trafficking, not only from lipid droplets but also from endosomes that is mediated by NPC1 and STARD3 (Ikonen 2008, Kennedy et al. 2012). This method provides the means to screen for the effects of different editing sites in the STAR gene, including the BMAL-E-box element and exon 7 sites that determine mRNA processing, turnover and translation (Fig. 2).

**sm-FISH and in vivo signaling to STAR**

The sm-FISH methodology, when applied to frozen sections of mouse testis interstitial Leydig cells, reproduces many features of STAR expression that is seen in MA10 cells (Jorgensen and Lee, unpublished). Thus, expression of STAR p-RNA and sp-RNA are each detectable at loci, in variable proportions and intensities. sp-RNA also identifies cytoplasmic particles of STAR mRNA. Like MA10 cells, basal expression of STAR is very low compared to CYP11A1. Stimulation with HCG introduces p-RNA and sp-RNA at the STAR loci within 60 min, and mRNA in most cells within 4 h. A 3D de-convolution resolves the STAR and CYP11A1 expression in the stimulated Leydig cells and shows their different distributions (Fig. 7). The images look similar to those of MA10 cells but have less definition due to the absence of a defined adherence surface.

The interstitial regions of the testis provide only about 15 Leydig cells in each section that can each be characterized (Hatano et al. 2016). Initial studies indicate heterogeneity of expression comparable to that seen in MA10 and Y-1 cells in vitro. *In vivo*, of course, we expect additional heterogeneity derived from local physiological features, including blood vessels and nerve endings, combined with effects of age and hormonal stimulation. The intensity of STAR p-RNA and sp-RNA at loci mark active expression and probe locus asymmetry and splicing activity. The heterogeneity of STAR mRNA expression is seen when STAR is low, through the removal of SF1 (Hatano et al. 2016) or NPC activity is compromised (Kennedy et al. 2012, Lu et al. 2015). This immediate change implicates STAR in cholesterol trafficking, not only from lipid droplets but also from endosomes that is mediated by NPC1 and STARD3 (Ikonen 2008, Kennedy et al. 2012). This method provides the means to screen for the effects of different editing sites in the STAR gene, including the BMAL-E-box element and exon 7 sites that determine mRNA processing, turnover and translation (Fig. 2).

![Figure 7](image_url)

**Figure 7**

Distribution of STAR and CYP11A1 mRNA in the interstitial zone of mouse testis during stimulation by HCG. Frozen testis sections were probed with oligomers that specifically recognize STAR and CYP11A1 mRNA. 1 µm sections are reconstructed by Olympus IX 80 software into a projection through the 3D assemblage. Each cell is in a single interstitial region. The same sections are probed with STAR (left) and CYP11A1 (right) oligomer sets. DAPI blue marks the nuclei. The merge shows separate distributions in cells with the similar expression. STAR mRNA closely rings the nuclei.
in relation to other steroidogenic genes (CYP11A1, HSD3B2, CYP17A1 and SRBI) provides another sm-FISH characteristic for each cell. The relationship of STAR mRNA expression to mitochondrial dynamics opens new avenues for sm-FISH, notably in relation to the presence of MFN2 as a regulator.

**Highlights and future directions**

Steroidogenic cells, astrocytes and the neuronal macrophage (microglia) share complex local cholesterol trafficking and signaling effects, which show remarkable single cell diversity. STAR and NPC1 function as gatekeepers for such cholesterol trafficking.

sc-RNAseq effectively identifies different cell types including rare stem cells or individual neurons and partnering astrocytes and microglia. The relationships between tissue cell types including pathway controls are effectively characterized by hierarchical clustering. Spatiotemporal relationships emerge from the integration of sc-RNAseq with sm-FISH, which delivers 3D positioning of specifically marked cells. The separate selection of low variation gene markers for cell type and markers of pathway stimulation responses is critical.

Analysis of STAR expression by sm-FISH reveals an underlying mechanism heterogeneity that is unattainable to standard macro RNA methods. The multimeric oligomers used for sm-FISH provide a digitized single molecule readout of locus primary and spliced RNA transcripts. The sensitivity and simplicity of these methods can reveal similar features in other regulatory genes. Novel findings include a remarkably diverse basal expression (adrenal cortex cells) that becomes synchronized after stimulation. For testis Leydig cells, fully suppressed loci show an asymmetric stimulation indicative of locus differences in epigenetic control. Each stimulation reveals concerted delays in elongation, splicing and polyadenylation that restrict mRNA appearance in the cytoplasm until each step accelerates after 60 min. The initially formed 3.5kb STAR mRNA after transfer to mitochondria by association with AKAP1 and TIS11B then becomes shortened to 1.6kb via 3’UTR processing thus enhancing translation and dissociation from mitochondria. A single STAR mRNA associates with most mitochondria proximal to the nuclear envelope. Adrenal cortex cells superimpose a similar sequence of changes on the appreciable basal STAR expression.

sm-FISH analyses can similarly probe other contributors to cholesterol trafficking including NPC1 or contributors to mitochondrial cycling such as MFN2 to test their coordinated expression in single cells. sc-RNAseq could identify other contributing players. These methods work equally well in the cultured cell line models, in adrenal or testis tissue sections thus allowing direct comparison.

Coordination of sc-RNAseq to spatiotemporal sm-FISH approaches could be linked to identified ND features thereby testing how closely aberrations in cholesterol trafficking overlap differences in β-amloid plaques or other ND risk factors.

sm-FISH is effective for the rapid implementation of in situ CRISPR gene editing. Rapid deletion of a key gene (as shown for STAR) can be applied efficiently and directly to cultured cell lines like MA10 or Y-1 without the disruptive effects of isolating clonal lines.

**Declaration of interest**
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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**Author contribution statement**
Colin Jefcoate wrote the text and designed the figures. Directed all the research from this laboratory that is described in the review (see ‘The scope of sm-FISH and single cell characterization’ to ‘Application to in situ CRISPR Cat9 editing of STAR’ sections). Jinwoo Lee: Developed the sm-FISH methodology used for this work and carried out all the experiments from this laboratory described in ‘The scope of sm-FISH and single cell characterization’ to ‘Application to in situ CRISPR Cat9 editing of STAR’ sections.

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Cholesterol dynamics and STAR imaged by sm-FISH


